Community structure of gut microbes in *Busseola fusca* (Lepidoptera: Noctuidae)

M Snyman

21155852

Dissertation submitted in fulfilment of the requirements for the degree *Magister Scientiae* in Environmental Sciences at the Potchefstroom Campus of the North-West University

Supervisor: Prof CC Bezuidenhout

Co-supervisor: Dr S Claassens

Assistant Supervisor: Prof J van den Berg

May 2015

NORTH-WEST UNIVERSITY
YUNIBESITI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT ®

It all starts here ™

ABSTRACT

Bt-maize is engineered to express insecticidal toxins derived from the bacterium *Bacillus thuringiensis* and has been shown to be very effective against pests like *Busseola fusca*. However, resistance of this pest against Bt-maize has developed and spread throughout South Africa. This study was inspired by the lack of knowledge over the microorganisms associated with the gut of these insects as they play a vital role in insect growth and development. Microbial-derived enzymes may have a role during an insect's adaption in different environmental conditions and to new diets. Previous studies suggest (1) that gut bacteria are required for *B. thuringiensis*-induced mortality in most Lepidoptera species and (2) that the toxicity of *B. thuringiensis* depends on microbial community interactions within the gut. The aim of this study was to determine the microbial diversity present in the midgut of *B. fusca* larvae occurring in maize. *Busseola fusca* larvae were collected from 30 sites throughout South Africa and dissected to collect their midgut contents. Serial dilutions were made of the contents and spread plated onto nutrient agar after which morphotypes were identified. One-hundred and five morphotypes were identified; DNA were extracted from the selected morphotypes and subjected to PCR analysis followed by secquencing. Sequencing results revealed the dominance of *Enterococcus* spp., specifically *Enterococcus casseliflavus* and *Enterococcus gallinarum*, *Klebsiella* spp., espesially *Klebsiella pneumoniae* and *Klebsiella oxytoca* and *Bacillus* spp. such as *.B. thuringiensis* and *B. subtilis*. Other organisms isolated, included *Achromobacter* spp., *Brevudimonas* spp., *Caulobacter* spp., *Enterobacter* spp., *Halomonas* spp., *Ochrobactrum* spp., *Pantoea* spp., *Pseudomonas* spp., *Serratia* spp., *Stenotrophomonas* spp., *Arthrobacter* spp., *Brevibacterium* spp., *Leucobacter* spp., *Microbacterium* spp., *Planomicrobium* spp. and *Staphylococcus* spp. The microbial diversity of larvae collected at the respective sampling sites were determined with the Shannon diversity index. The data were compared to several factors regarding the sampling sites. No significant differences were observed between the microbial diversities isolated at the respective sites. This may imply that the microbial community within *B. fusca* larvae are relative consistent throughout the maize production area. It is important to understand the distribution and structure of gut microbial communities within insects and whether the gut community is influenced by the geographical distribution of the insects. A better understanding of the distribution of the insects and community structure of their gut microbiota may aid in the development of better insect control strategies.

Keywords: *Busseola fusca*, microbial community, gut microbes, PCR, resistance, symbionts.

PREFACE

The experimental work done and discussed in this dissertation for the degree Master of Science in Environmental Sciences was carried out in the School of Biological Sciences, North-West University, Potchefstroom Campus, South Africa. This study was conducted fulltime during the period of January 2013 - November 2014, under the supervision of Prof. Carlos Bezuidenhout, Dr. Sarina Claassens and Prof. Johnnie van den Berg.

The research done and presented in this dissertation signifies original work undertaken by the author and has not been submitted for degree purposes to any other university. Appropriate acknowledgements in the text have been made where the use of work conducted by other researchers have been included.

References were done according to: NWU. 2014. NWU Referencing Guide. Potchefstroom: Library Services of North- West University, Potchefstroom Campus.

Maxi Snyman
November 2014

ACKNOWLEDGEMENTS

I would like to express my appreciation to the following persons at the North-West University, Potchefstroom Campus for their contributions towards the successful completion of this study.

My supervisors Prof Carlos Bezuidenhout, Dr. Sarina Claassens and Prof. Johnnie Van den Berg for giving me this exceptional opportunity and for all of their support and guidance throughout this period.
Dr. Jaco Bezuidenhout for assisting with the statistical aspects, and Dr. Arvind Gupta with the phylogenetic analysis of this study.
Megan van Staden for the time spent sampling and assistance in the laboratory.
Stephan Barnard for constructing the map that illustrates the respective sampling sites.

Furthermore I would also like to thank my family and friends for their encouragement and support during my studies:

Simone Booyens and my fellow colleagues
My parents, Kenny and Marinda Snyman for supporting me since the beginning of my studies.
My lifelong friends, Marizaan de Jong and Carla Pohl for their friendship and emotional support.
Johan Smit, for the love and inspiration that kept me from giving up.

Without you none of this would have been possible.

TABLE OF CONTENTS

ABSTRACT ..... **i**

PREFACE ..... **ii**

ACKNOWLEDGEMENTS ..... **iii**

TABLE OF CONTENTS ..... **iv**

LIST OF ABBREVIATIONS ..... **vii**

LIST OF FIGURES ..... **ix**

LIST OF TABLES ..... **xii**

CHAPTER 1 ..... **1**

INTRODUCTION **1**

1.1. General introduction and problem statement **1**

1.2. Research aim and objectives **3**

CHAPTER 2 ..... **4**

LITERATURE STUDY **4**

2.1. The importance of maize **4**

2.2. *Busseola fusca* **4**

2.2.1 Distribution 4

2.2.2. Behaviour and life-cycle 6

2.2.3. Pest status 8

2.2.4. Management and control 9

2.3. Genetically modified crops **11**

2.3.1. What are genetically modified crops? 11

2.3.2. Development of genetically modified crops 12

2.4. Bt Maize **13**

2.4.1. *Bacillus thuringiensis* 14

2.4.2. Mechanism of Bt 16

2.4.3. Advantages and disadvantages of Bt crops 18

2.5. Resistance development of *Busseola fusca* to Bt maize **21**

2.6. Associations between insects and gut microbes 23
2.6.1. Structure and physical conditions of the insect gut 23
2.6.2. Function of gut microbes 26
2.6.3. Lepidopteran gut community structure 29
2.6.4. Effect of geographical distribution of insects on gut microbe content 32
2.7. Methods to identify gut community structure 33
2.8. Approaches to evaluate microbial community structure 35

CHAPTER 3 37
MATERIALS AND METHODS 37
3.1. Sample collection 37
3.2. Sample preparation 38
3.3. Isolation of bacteria 39
3.4. Gram stain 40
3.5. DNA isolation 41
3.6. DNA amplification 41
3.7. Agarose gel electrophoresis 42
3.8. First clean-up 42
3.9. Sequencing 42
3.10. Statistical and data analyses 43

CHAPTER 4 44
RESULTS 44
4.1. Bacterial morphotypes 44
4.2. Identification of bacterial morphotypes 49
4.2.1. Gram staining and DNA isolation 49
4.2.2. DNA amplification 50
4.2.3. 16S rRNA gene sequencing analysis 51
4.3. Phylogenetic analysis 55
4.4. Species distribution 62
4.5. Geographical differences 64
4.6. Summary of results 66

CHAPTER 5 .... **68**

DISCUSSION **68**

5.1. Introduction **68**

5.2. Bacterial morphotypes **68**

5.3. Identification of bacterial morphotypes **71**

5.3.1. Gram staining, DNA isolation and amplification 71

5.3.2. 16S rRNA gene sequencing analysis 72

5.4. Phylogenetic analysis **73**

5.5. Potential function of gut microbes **75**

5.5.1. Enhancing nutrition 81

5.5.1.1. Aid in digestion 81

5.5.1.2. Improving quality of nutrient poor diets 85

5.5.1.3. Detoxification 86

5.5.2. Affect efficiency as disease vectors 89

5.5.3. Govern mating and reproductive systems within insects 89

5.5.4.2. Immune homeostasis 92

5.5.4.3. Development and fitness 92

5.5.5. Insect pathogens 92

5.6. Species distribution **95**

5.6.1. Geographical differences 96

CHAPTER 6 .... **99**

CONCLUSION AND RECOMMENDATIONS **99**

6.1. Conclusion **99**

6.1.1. Identification of the microbial community structure within *Busseola fusca* larvae 99

6.1.2. Comparison of microbial diversity at geographically separate sites 100

6.2. Recommendations **101**

REFERENCES **103**

APPENDIX 1 .... **138**

APPENDIX 2 .... **143**

APPENDIX 3 .... **149**

APPENDIX 4 .... **151**

APPENDIX 5 .... **153**

LIST OF ABBREVIATIONS

a.s.l.	above sea level
AHL	*N-Acyl*homoserine lactones
ANOVA	analysis of variance
BLAST	Basic local alignment search tool
BLASTn	Basic local alignment search tool for nucleotide
cAMP	cyclic adenosine monophosphate
CCA	Canonical correspondence analysis
CFUs	colony forming units
CMC agar	carboxymethyl-cellulose agar
CR	colony resistance
Cry	crystal proteins
Cyt	cytolytic proteins
CY	cypermethrin
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
E value	expected value
EDTA	thylenediamine-tetra-acetic acid
FAO	food and agriculture organization
FAOSTAT	food and agricultural commodities production
GM	genetically modified
GMO	genetically modified organism
H	flagellar antigen
HT	herbicide tolerant
IPM	integrated pest management
IRM	insect resistant management
LAB	lactic acid bacteria
LB agar	Luria-Bertoni broth agar
MAMPs	microbe-associated molecular patterns
MEGA	molecular evolutionary genetics analysis
MT	morphotypes
NCBI	National Centre for Biotechnology Information
OTUs	operational taxonomic units
PAHs	polycyclic aromatic hydrocarbons
PCR	polymerase chain reaction

PG	*polygalacturonase* enzyme
pH	the co-logarithm of the activity of dissolve hydrogen ions (H+)
PNP	p-Nitrophenol
QS	quorum sensing
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
TAE solution	Tris-acetate-EDTA buffer
Tris buffer	Tris (hydroxymethyl) aminomethane buffer
TSA	tryptic soy agar
Tukey’s HSD	Tukey’s honest significant difference

LIST OF FIGURES

Figure 2.1: Distribution of *Busseola fusca* in Africa (Harris and Nwanze, 1992). **5**

Figure 2.2 A-B: (A) Leaf damage caused by young larvae that leads to (B) death of the growing points in the plants. **6**

Figure 2.3 A-C: (A) Larvae tunneling in stems of maize plants. (B-C) Ear damage caused by larvae. **7**

Figure 2.4: Illustration of GM crops growth from 1996 to 2013 (Matthews, 2014). **12**

Figure 2.5: Scanning electron micrograph of Bt spores (a) and Bt crystals (b) (Xue *et al*., 2008). **15**

Figure 2.6: Transmission electron micrograph illustrating the parasporal body of *B. thuringiensis* subsp. *israelensis* together with the entailed crystal proteins (Federici *et al*., 2010). **15**

Figure 2.7: Illustration of the two modes of action of Cry toxins causing larval death. Step 1: Ingestion of Bt protein. Step 2: Activation of protoxins. Step 3: Toxins bind to receptors. Pore formation mechanism- Step 4: Proteolytic cleavage. Step 5: Toxins bind to secondary receptors. Step 6: Pore formation in the lumen membrane. Signal transduction mechanisms- Step 4a: Protein binds to cadherin. Step 5a: Cell death occurs (Bravo and Soberón, 2008). **18**

Figure 2.8: Overview of an insect gut structure (Engel and Moran, 2013). **24**

Figure 3.1: Sampling points at which *Busseola fusca* larvae were collected in South Africa. **37**

Figure 3.2 A-D: Aseptic dissections of *Busseola fusca* larvae. **38**

Figure 3.3: Illustration of the dilution series made from the supernatant. **39**

Figure 3.4 A: Illustrates differences in the surface appearance and edges of the colonies. Both colonies a and b has a smooth surface appearance and colony c has a granular appearance. Colony a have round edges, while colonies b and c is considered irregular. ……………………………………………………………………………...........………………......**40**

Figure 3.4 B: Elevation of the colonies and colour variances are demonstrated in the figure. ……………………………………………………………………………………………......................**40**

Figure 4.1: The various morphotypes that occured at each site respectively. Morphotype 2 (MT2) was mostly present in high percentages and occured at 28 of the 30 sites, while several other morphotypes were isolated in low numbers only at one site such as MT21, MT24, MT26, MT43 and MT50. ……………………………………………………………………………………………......................**45**

Figure 4.2: Agarose gel image of isolated DNA from pure cultures of the selected morphotypes. A 1 Kb molecular weight marker (O'GeneRuler, Fermentas Life Science, US) was loaded into lane 1. ……………………………………………………………………………...........………………......**49**

Figure 4.3: Image of a 1 % (w/v) agarose gel illustrating the amplified products of nine pure cultures (lanes 1 to 9) from the selected morphotypes. The first two lanes contain the 1 Kb and 100 bp molecular weight markers (O'GeneRuler, Fermentas Life Science, US), respectively, and lane C is the no template control. ……………………………………………………………………………...........………………......**50**

Figure 4.4: Distribution of genera within the respective phyla. ……………………………………………………………………………...........………………......**52**

Figure 4.5: Phylum Proteobacteria with percentage representation of different species isolated from *Busseola fusca* larvae. ……………………………………………………………………………...........………………......**54**

Figure 4.6: Phylum Firmicutes with percentage representation of different species isolated from *Busseola fusca* larvae. ……………………………………………………………………………...........………………......**54**

Figure 4.7: Phylum Actinobacteria with percentage representation of different species isolated from *Busseola fusca* larvae. ……………………………………………………………………………...........………………......**55**

Figure 4.8: Species distribution for the isolates that were selected for phylogenetic analysis. ……………………………………………………………………………………………......................**58**

Figure 4.9 A-B: Compares the main families identified from (A) the selected sequences (≥ 97 %) and, (B) the total number of isolated sequences (≥ 74 %). The composition and structure of the major families is similar in both cases, however, inconsistency occured in 6 % of the structure (*Brevibacteriaceae* and *Pseudomonadaceae*), which is expected since microbial communities are dynamic.
……………………………………………………………………………………..............…….......**59**
Figure 4.10: Neighbor-Joining tree constructed from partial 16S rRNA gene sequences collected from *Busseola fusca* larvae. Obtained Bootstrap confidence values (1000 replicates) are given at the branch point. Entries include the selected bacterial groups (Table 4.3) together with species names and accession numbers obtained from the GenBank database.
……………………………………………………………………………………..............…….......**61**
Figure 4.11: Canonical Correspondence Analysis illustrating the species composition at the different sites in relation to the direct distance between the respective sites. The sampling sites are represented by numbers 1-30 (refer to Table 4.2). The eigenvalues for the first two axes were 0.221 (x-axis) and 0.173 (y-axis), respectively.
……………………………………………………………………………………..............…….......**64**
Figure 5.1: Summary of the potential roles the isolated gut microbes may facilitate in the lifestyle of *B. fusca* larvae. [Information was compiled from: (1) Anand *et al.*, 2009; (2) Bora *et al.*, 1994; (3) Broderick *et al.*, 2006; (4) Busse *et al.*, 2012; (5) Busse, 2012; (6) Cappellozza *et al.*, 2011; (7) Chen *et al.*, 2011; (8) Dai *et al.*, 2005; (9) Dillon and Dillon, 2004; (10) Dillon *et al.*, 2002; (11) Engel and Moran, 2013; (12) Fedhila *et al.*, 2010; (13) Gullan and Cranston, 2010; (14) Inglis *et al.*, 2000; (15) Kallimanis *et al.*, 2007; (16) Li *et al.*, 2011; (17) Li *et al.*, 2014; (18) Liu *et al.*, 2007; (19) Liu *et al.*, 2011; (20) Liu *et al.*, 2014; (21) Logan and De Vos, 2011; (22) Lundgren *et al.*, 2007; (23) Mano and Morisaki, 2008; (24) Morohoshi *et al.*, 2012; (25) Özkan-Çakici *et al.*, 2014; (26) Rhee *et al.*, 2014; (27) Shida *et al.*, 1995; (28) Suen *et al.*, 2010; (29) Suribabu *et al.*, 2014; (30) Suzuki and Hamada, 2012; (31) Tang and You, 2012; (32) Wang *et al.*, 2010; (33) Wiese *et al.*, 2013; (34) Yadav *et al.*, 2012].
……………………………………………………………………………………..............………......…**79**

LIST OF TABLES

Table 2.1: Comparison of gut microbes in laboratory reared and field-collected bollworms (*Helicoverpa armigera*) described by Xiang and co-workers (2006). The abundance in which these groups were isolated is also presented. ……………………………………………………………………………………………………...................**31**

Table 4.1: Details regarding the various sampling sites as well as the Shannon diversity index calculated at each site …...………………………………………………………………………………...........………..**47**

Table 4.2: Comparison between the mean Shannon diversity values and the type of maize, production area and the type of production system, respectively (Means ± standard error, superscript characters denote statistical significant differences, p = 0.05, Tukey's Unequal HSD). ……………………………………………………………………………………….............................**48**

Table 4.3: Sequencing results and percentage of similarity (BLASTn, NCBI) as well as the assigned complex names and the number of isolates it entails. …………………………………………………………………………….............……………......**56**

Table 4.4: Comparison between the mean Shannon diversity values and the type of maize, production area and the type of production system, respectively (Means ± standard error, superscript characters denote statistical significant differences, p = 0.05, Tukey's Unequal HSD).

…………………………………………………………………………….............………………......**63**

Table 4.5: Species unique to one sampling site.

....**63**

Table 5.1: Acid producing bacteria isolated from the midgut of *Busseola fusca* as well as the sources from which acid is produced.
…………………………………………………………………………...........………….............**78**

CHAPTER 1
INTRODUCTION

1.1. General introduction and problem statement

Maize is one of the most important food crops in Africa (Kfir *et al*., 2002). Lepidopteran stem borers are the most important pests attacking maize in Africa and are responsible for major yield losses (Kfir *et al*., 2002; George *et al*., 2011). In Africa 21 stem borer species have been described as economically important pests on cereal crops. From these species seven belong to the family Noctuidae, two to the family Pyralidae, and 12 are from the family Crambidae. In South Africa the most important species are *Busseola fusca* (Lepidoptera: Noctuidae) and *Chilo partellus* (Lepidoptera: Crambidae) (Kfir *et al*., 2002). *Busseola fusca* larvae tunnel inside the maize stems, which make it difficult to control this pest by using pesticides (Calatayud *et al*., 2007; Calatayud *et al*., 2014). Tunnelling also weakens the stems and cause interference with the translocation of nutrients and metabolites in the maize plant. This results in the malformation of grains, which has a substantial influence on food production (Fandohan *et al*., 2003). Early control measures consisted of cultural control and the application of insecticides to the whorls of plants when symptoms of infestation were observed (Van Rensburg and Flett, 2008).

Through genetic engineering insect resistant maize cultivars were developed to control agricultural pests. These maize plants express insecticidal toxins derived from the bacterium *Bacillus thuringiensis*, which is known as Bt maize (Schnepf and Whiteley, 1981; Höfte and Whiteley, 1989; Federici, 1998; Van den Berg *et al*., 2013). Since Bt crops were commercialised in 1996, its use increased rapidly worldwide (James, 2014). The increase in Bt cultivation resulted in an increase in toxin exposure to insects, which added to selection pressure for resistance evolution (Oppert, 1999).

The first case of field resistance to Bt maize in *B. fusca* was reported at the Vaalharts irrigation scheme in 2006 (Van Rensburg, 2007). Several mechanisms for resistance to develop have been proposed. This includes changes to the toxin binding sites (Oppert, 1999; Ma *et al*., 2005), quick replacement of cells affected by Bt toxins (Martínez-Ramírez *et al*., 1999; Ma *et al*., 2005) and variations in the pH of the gut lumen (Oppert, 1999). Another mechanism suggests a shift in the microbial content in the midgut of *B. fusca* larvae (Broderick *et al*., 2006).

Numerous symbiotic relationships exist between insects and bacteria (Engel and Moran, 2013). These associations are known to (1) enhance nutrition, (2) develop and maintain the immune system, (3) affect efficiency as disease vectors and (4) govern mating and reproductive systems within insects (Dillon *et al*., 2002; Engel and Moran, 2013; Tagliavia *et al.,* 2014). The dynamics between lepidopteran insects and their gut microbes is not well comprehended (Broderick *et al*., 2009). However, several authors have identified gut microbes associated with different Lepidoptera species in an attempt to better understand this interaction (Broderick *et al*., 2004; Xiang *et al*., 2006; Anand *et al*., 2010; Belda *et al*., 2011; Priya *et al*., 2012). From literature it is strongly suggested that gut microbes have an essential role within the nutrition of these insects. Microbial-derived enzymes may aid in the digestion of refractory or toxic food components such as lignin or allelochemicals. Alterations in insect diet may cause changes within the microbial community it harbours as well as in the digestive enzymes that these bacteria produce. As a result gut microbes may aid in the adaption of insects to new diets and environments by facilitating nutrition (Anand *et al*., 2010).

For Bt toxins to activate, an extreme alkaline environment is required. Therefore, the specificity of Bt to Lepidoptera is mainly as a result of the high alkalinity within the gut. After the toxins are activated, pores will form in the gut membrane that allows bacteria in the gut to enter the haemolymph, causing septicemia that result in larval death (Broderick *et al*., 2006; Broderick *et al*., 2009; Rajagopal, 2009). Broderick and co-workers (2006) concluded that gut bacteria are required for *B. thuringiensis*-induced mortality in most lepidopteran species and that the toxicity of *B. thuringiensis* depends on the interactions between the gut microbes (Broderick *et al*., 2006).

Microorganisms are able to alter their environment in different ways to facilitate survival and adaption. For instance, most species belonging to Lactobacillales produces lactic acid from carbohydrates. Acid production can cause the pH within the midgut to decrease (Dillon and Dillon, 2004; Cappellozza *et al*., 2011), which may prevent Bt toxins to activate (Broderick *et al*., 2006; Broderick *et al*., 2009).

Microbial communities are dynamic and can regularly experience alterations in their composition and structure. This results from changes in nutrient availability, environmental characteristics, and proximity to other organisms (Broderick *et al*., 2004; Robinson *et al*., 2010; Priya *et al*., 2012). Several authors observed a higher microbial diversity within field-collected larvae than in laboratory-reared larvae. It was suggested that the microbial community might be influenced as a result of variations within the environment and diet that larvae encounter (Mead *et al*., 1988; Xiang *et al*., 2006; Belda *et al*., 2011; Priya *et al*., 2012; Hammer *et al*., 2014).

Information regarding the distribution and species composition of microbial communities associated with insects may provide a better understanding of the interactions between these organisms. This may also aid in determining the potential roles that gut microbes may have within the lifestyle and survival of insects, which will in return aid in the development of better insect control strategies (Rajagopal, 2009).

Apart from two preliminary studies conducted at the North-West University no information is available on the microbial community associated with *B. fusca* larvae (Brink *et al*., 2011; Snyman *et al*., 2012, Unpublished data). It is important to understand the diversity and geograpical distribution of gut microbes associated with this insect pest to determine if it may have a role in its survival, and possibly in resistance development to Bt maize.

1.2. Research aim and objectives

The aim of this study was to further our knowledge on the microbial community structure within the midgut of *B. fusca* larvae.

The specific objectives of the study were:

i) to characterise the microbial community in the midgut of *B. fusca* larvae by using culture dependent methods.

ii) to collect *B. fusca* larvae from Bt-maize and non Bt-maize under different farming practises (irrigated and dry land) in the maize producing region of South Africa.

iii) to compare the microbial diversity of larvae collected at the different sampling sites to determine whether differences occur between geographically separated sites.

CHAPTER 2
LITERATURE STUDY

2.1. The importance of maize

Maize (*Zea mays*) is the major staple food crop in Africa (Kfir *et al*., 2002). It is grown in temperate, sub-tropical and tropical regions where rainfall or irrigation is adequate (Adeyemo, 1984). Maize is a key crop in securing food availability (Mboya *et al*., 2011), since it has a higher nutritious value than sorghum and wheat. It contains more carbohydrates and is a source of phosphorus, calcium, iron, thiamine, niacin, protein, vitamin A and fat (Adeyemo, 1984).

Food security is defined as "a state reached when all people at all times have access to adequate amounts of safe and nutritious food to meet their dietary needs and food preferences for an active and healthy life" (FAO, 1996; Mannion and Morse, 2013). There are three pillars that determine food security: (1) the availability, which relates to the production of food, (2) access to food, which includes the distribution and processing, (3) as well as the appropriate use of food. Genetically modified (GM) food crops may provide an opportunity to increase and secure food availability by addressing any limitations inherent within crops (Mannion and Morse, 2013).

Maize production is limited by several abiotic (drought, soil fertility and/or mineral toxicity) and biotic (arthropods, nematodes, diseases, weeds, rodents and / or birds) factors. Of the various insect pests attacking maize, lepidopteran species are considered among the most injurious (Kfir *et al*., 2002). Seventeen Pyralidae and Noctuidae stem borer species have been reported as pests in Africa (Gressel *et al*., 2004). *Chilo partellus* (Lepidoptera: Crambidae) and *Busseola fusca* (Lepidoptera: Noctuidae) are considered as the most important species attacking maize in South Africa (Van den Berg *et al*., 2013).

2.2. *Busseola fusca*

2.2.1 Distribution

Busseola fusca is indigenous to sub-Saharan Africa and responsible for major maize and sorghum losses (Kfir, 2002; Sezonlin *et al*., 2006; Calatayud *et al*., 2014). Stem borer population densities vary greatly among different regions as well as different seasons, within

each region. This is due to climatic, abiotic and biotic factors such as human activity, natural enemies, rainfall and host plant availability (Chabi-Olaye *et al*., 2005; Ong'amo *et al*., 2006; Sezonlin *et al*., 2006).

In West Africa *B. fusca* is more dominant in the Northern dry savannah areas than in the Southern humid parts (Harris and Nwanze, 1992; Ndemah *et al*., 2001; Calatayud *et al*., 2014). Although this species occurs throughout East and central Africa, (Cardwell *et al*., 1997; Ndemah *et al*., 2001), it is only predominant in areas above 1500 m a.s.l. (Ong'amo *et al*., 2006; Sezonlin *et al*., 2006; Calatayud *et al*., 2014). In Southern Africa, *B. fusca* occurs throughout the maize producing region of South Africa (Krüger *et al*., 2008) and at altitudes of up to 2131 m a.s.l. in Lesotho (Ebenebe *et al*., 1999; Catalayud *et al*., 2014). The distribution of *B. fusca* is illustrated in Figure 2.1.

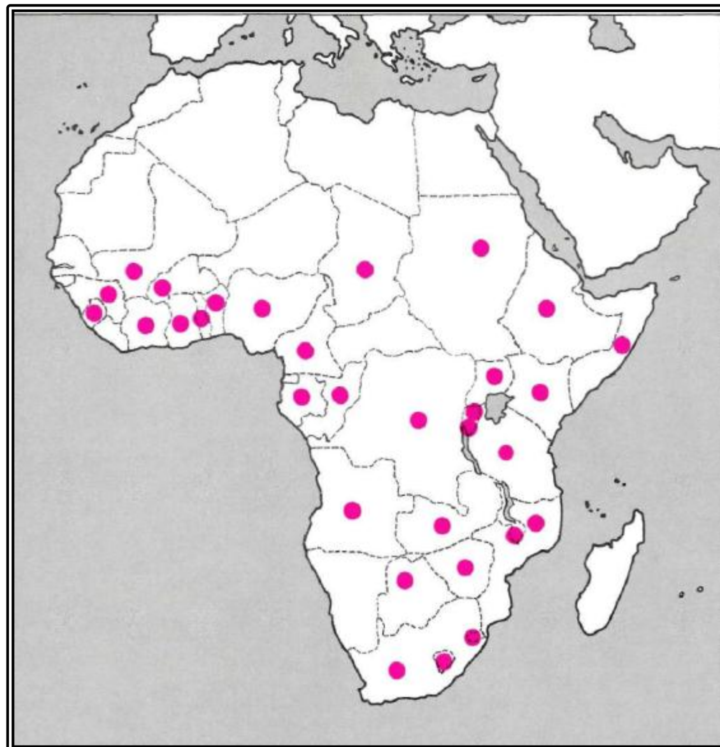

Figure 2.1: Distribution of *Busseola fusca* in Africa (Harris and Nwanze, 1992).

2.2.2. Behaviour and life-cycle

Busseola fusca was first described and named by Fuller in 1901 (Harris and Nwanze, 1992). Larvae first feed on young leaves (Figure 2.2 A) before they start tunnelling into the stems, which leads to the destruction of growing points in young plants. This occurrence is referred to as "dead hearts" (Figure 2.2 B; Harris and Nwanze, 1992). Larvae tunnel in the stems (Figure 2.3 A), which causes weakening and interferences with the translocation of nutrients and metabolites within the plant. This results in malformation of the grains, stem breakage, plant stunting, lodging and direct damage to ears. The weakened stems are prone to breakage and plants are mostly underdeveloped (Kfir *et al*., 2002). Maize ears are directly damaged by tunnelling larvae (Figure 2.3 B-C), which lead to substantial crop losses (Harris and Nwanze, 1992). Additionally, the increased activity of stem borers causes secondary infections through fungi such as *Fusarium* spp. (Fandohan *et al*., 2003).

Figure 2.2 A-B: (A) Leaf damage caused by young larvae that leads to (B) death of the growing points in the plants.

A

B

C

Figure 2.3 A-C: (A) Larvae tunnelling in stems of maize plants. (B-C) Ear damage caused by larvae.

Moths have a wingspan of 20 - 40 mm, and have lighter coloured forewings than hind wings. They emerge from their pupae late in the afternoon, and are active during the night. On the night of emergence, female moths release pheromones in order to attract males to mate with. Within the following 3 - 4 nights, females lay their eggs under the inner surfaces of leaf sheaths. Each female lays about 200 eggs (Harris and Nwanze, 1992; Calatayud *et al.*, 2014). A week later eggs hatch and larvae initially migrate to the whorls of maize plants. After entering the whorls they start feeding on the leaves and soon afterwards start to bore into the stems where they will feed for 3 - 5 weeks. A fully grown *B. fusca* larvae is about 40 mm long with a dark brown head, a yellowish-brown prothorax and a creamy white body. Their feeding causes tunnels to form within the stems and ears. They pupate within these tunnels after constructing emergence windows for adult moths. Pupae are about 25 mm long and are a yellow brown colour. Female pupae are somewhat bigger than the male pupae. Within 9 - 14 days adults emerge. The life cycle is completed within 7 - 8 weeks during optimal conditions. During the off-season larvae undergo a diapause period of approximately six months, which only ends with the start of the next cropping season (Harris and Nwanze, 1992; Calatayud *et al.*, 2014).

2.2.3. Pest status

Busseola fusca is considered to be the most important lepidopteran pest of maize and sorghum in Africa (Calatayud *et al.*, 2014). The pest status of *B. fusca* varies between different regions and agroecological zones (Ndemah *et al.*, 2001; Sezonlin *et al.*, 2006; Calatayud *et al.*, 2014). In East Africa it is considered as one of the most important pests (Kfir *et al.*, 2002; Sezonlin *et al.*, 2006), and is responsible for an average loss of 14 % in Kenya's maize production (Groote, 2002; Calatayud *et al.*, 2014). The humid forest areas in Cameroon (central Africa) experience crop losses of around 40 % (Cardwell *et al.*, 1997; Chabi-Olaye *et al.*, 2005) while in West Africa, *B. fusca* has a low economic impact on maize (Sezonlin *et al.*, 2006; Calatayud *et al.*, 2014). In South Africa *B. fusca* is also considered as the most important insect pest of maize (Kfir, 1995; Van Rensburg and Flett, 2008). During the early part of the 1900s annual losses of 10 % were caused by this pest (Mally, 1920 cited in Van Rensburg and Flett, 2008). South African maize production increased from less than one million tons in 1910 (Van Rensburg and Flett, 2008) to 11.8 million tons in 2012 (FAOSTAT, 2013). This increase in production raised the economic pest status of this pest significantly (Van Rensburg and Flett, 2008). Due to fluctuations in population sizes each year, the pest status of *B. fusca* is unpredictable (Kruger *et al.*, 2009).

2.2.4. Management and control

In order to control maize stem borers an integrated pest management (IPM) programme should be adapted to local conditions and resources (Harris and Nwanze, 1992). Such IPM programmes consist of four pillars (chemical, biological, and cultural control as well as host plant resistance) in which multiple methods are coordinated to optimise pest control (Ehler, 2006; Calatayud *et al.*, 2014).

(A) Chemical control

The first reports of successful chemical control of stem borers were from South Africa during the 1920s, in which maize crops were treated with hycol solution, sheep-dip and botanical insecticides such as Derrisol®, Pulvex®, Kymac®, DDT and carbofuran (Harris and Nwanze, 1992). Today, a large variety of insecticides are available to control economically important pests (Singh *et al.*, 2007; Slabbert and Van den Berg, 2009).

Stem borer control is challenging because of their cryptic feeding habitat inside the plant whorls. In order to control maize stem borers, insecticides have to be applied into the whorls of plants (Slabbert and Van den Berg, 2009). However, the use of insecticides is only effective as a short term solution. If it is used over long periods, farmers may be faced with problems such as resistance development, negative effects on non-target species and other harmful impacts of insecticides (Van den Berg *et al.*, 1998). Chemical control is expensive and inadequate when used on its own (Kfir, 1995; Van den Berg *et al.*, 1998; Van Rensburg, 1999).

(B) Biological control

Biological control agents involve living organisms to suppress pest populations such as predators, parasitoids, parasites, nematodes and pathogens (Thomas and Waage, 1996; Kfir *et al.*, 2002). Parasitic wasps are able to detect larvae through volatile semiochemicals produced by plants when larvae feed on it (Hassanali *et al.*, 2008). Stem borer larvae are mainly parasitised by parasitoids from either Hymenoptera or Diptera (Kfir *et al.*, 2002). Examples of such parasitoids include *Cotesia sesamiae* (Hymenoptera: Braconidae), *Goniozus* sp. (Hymenoptera: Bethylidae), *Syzeuctus* sp. (Hymenoptera: Ichneumonidae), *Enicospilus* sp. (Hymenoptera: Ichneumonidae), *Pediobius furvus* (Hymenoptera: Eulophidae), *Sturmiopsis parasitica* (Diptera: Tachinidae) and *Descampsina sesamiae* (Diptera: Tachinidae) (Gounou and Schulthess, 2006; Hassanali *et al.*, 2008).

To reduce infestation levels below the economic injury level, biological control needs to be integrated into an IPM strategy (Van den Berg *et al.*, 1998). Numerous authors have questioned the effectiveness of parasitoids as biological control agents (Kfir, 1995; Chabi-Olaye *et al.*, 2001; Kfir *et al.*, 2002; Gressel *et al.*, 2004; Van Rensburg and Flett, 2008). Successful establishments of newly introduced control agents are very scarce (Hufbauer, 2002). *Cotesia flavipes* (Hymenoptera: Braconidae) is one of the few parasitoids that were successfully introduced for managing *C. partellus* in East Africa (Kfir *et al.*, 2002).

Several studies also concluded that natural enemies, nematodes or pathogens are unable to regulate population numbers effectively (Kfir *et al.*, 2002). However, one bacterium, *Bacillus thuringiensis*, showed potential as a biological control agent, since it is toxic to several insect pests, but not to humans and other animals (Van Rensburg, 1999).

(C) Cultural control

Cultural control involves traditional methods that alter the environment, making it unfavourable for pests. It is an important part of IPM programmes and is considered as the first line of defence. In Africa, it is the most economic and relevant method of control for resource poor farmers (Van den Berg *et al.*, 1998; Kfir *et al.*, 2002). Although these practices are labour-intensive, it does not require expensive equipment and generally has little to no negative effects on the environment (Van den Berg *et al.*, 1998).

Effective cultural control methods against *B. fusca* includes (1) Destroying crop residues, to prevent populations carrying over from one growing season to another (Kfir *et al.*, 2002). (2) Tillage, by burying maize debris 5 - 15 cm in the soil, kills pupae and limits successful emergence of moths from the soil (Harris and Nwanze, 1992; Kfir *et al.*, 2002). (3) Intercropping maize with a crop that is not a host of *B. fusca* is an effective method to reduce infestations. Since females are unable to lay their eggs on pearl millet, intercropping maize, sorghum and pearl millet will result in a decrease in population numbers (Adesiyun, 1983 cited in Harris and Nwanze, 1992; Kfir *et al.*, 2002). Similarly, Gounou and Schulthess (2006) reported lower infestation levels of stem borers in maize / rice intercrops compared to monocropped maize. (4) The “push-pull” tactic involves planting highly susceptible trap plants (napier grass, *Pennisetum purpureum*, and sudan grass, *Sorghum sudanensis*) to attract stem borers along with repellent plants (molasses grass, *Melinis minutiflora*, silverleaf, *Desmodium uncinatum* and greenleaf, *Desmodium intortum*) to limit ovipositing (Khan *et al.*, 2000; Khan *et al.*, 2008). Cultural control may be effective in suppressing population numbers, but it is not the solution for complete pest control, especially for large scale commercial farmers.

(D) Host-plant resistance

Host-plant resistance was successfully used against the European corn borer, *Ostrinia nubilalis* (Lepidoptera: Crambidae) in North America (Reddy, 1985 cited in Harris and Nwanze, 1992). Efforts to develop insect resistant maize cultivars against *B. fusca* were unsuccessful due to a lack of effective screening methods. Most of the studies were conducted in locations where several stem borer species were present on the same crop, making it difficult for resistance selection. In South Africa attempts to inoculate plants with larvae reared on artificial diets failed due to poor survival of first-instars (Harris and Nwanze, 1992).

Due to failed attempts to breed resistance in cereal crops, alternative solutions were developed to control stem borers. With rDNA technology plant resistance to *B. fusca* was achieved. Genes encoding for toxins, derived from the bacterium *B. thuringiensis*, were inserted into the maize genome. Although biological plant resistance is inherited, Mendelian inheritance also applies to this transgenic resistance, called Bt crops (Van Rensburg, 1999). From the late 1990s the popularity of genetically modified crops increased drastically (James, 2014).

2.3. Genetically modified crops

2.3.1. What are genetically modified crops?

A genetically modified organism (GMO) refers to both animals and plants in which the genetic material has been altered through genetic engineering (Anklam *et al.*, 2002). Genetic engineering is when genes are transferred between unrelated organisms making it possible to break species barriers, which are not achievable through traditional plant breeding (Sharma, 2006). Plant breeding is time consuming and expensive compared to genetic engineering (Mannion and Morse, 2013). This biotechnology tool is crucial in pest management programmes, since it is a key factor to obtain desirable traits to improve agricultural practices (Sharma, 2006).

2.3.2. Development of genetically modified crops

The first genetically modified crop released was the Flavr Savr tomato in 1994 in America (Krieger *et al.*, 2008; Mannion and Morse, 2013). With genetic engineering the *polygalacturonase* (PG) enzyme was supressed, delaying fruit softening after harvesting. Therefore, the Flavr Savr tomato had a longer shelf life (Bagwan *et al.*, 2010). Production of the Flavr Savr tomato stopped in 1999 due to limited success and anti-GM groups. In 1996 staple crops such as maize, canola, soybean and cotton were engineered to express herbicide tolerance and insect resistance (Mannion and Morse, 2013).

In spite of all the debates about the potential risks and benefits of genetically modified (GM) foods, its development grew rapidly. In 1996 GM crops were commercialised and 1.7 million hectares were grown globally. Nearly two decades later in 2013, a total of 175.2 million hectares were grown worldwide (James, 2014). Figure 2.4 shows the fast growing adaptation of this technology from 1996 onwards.

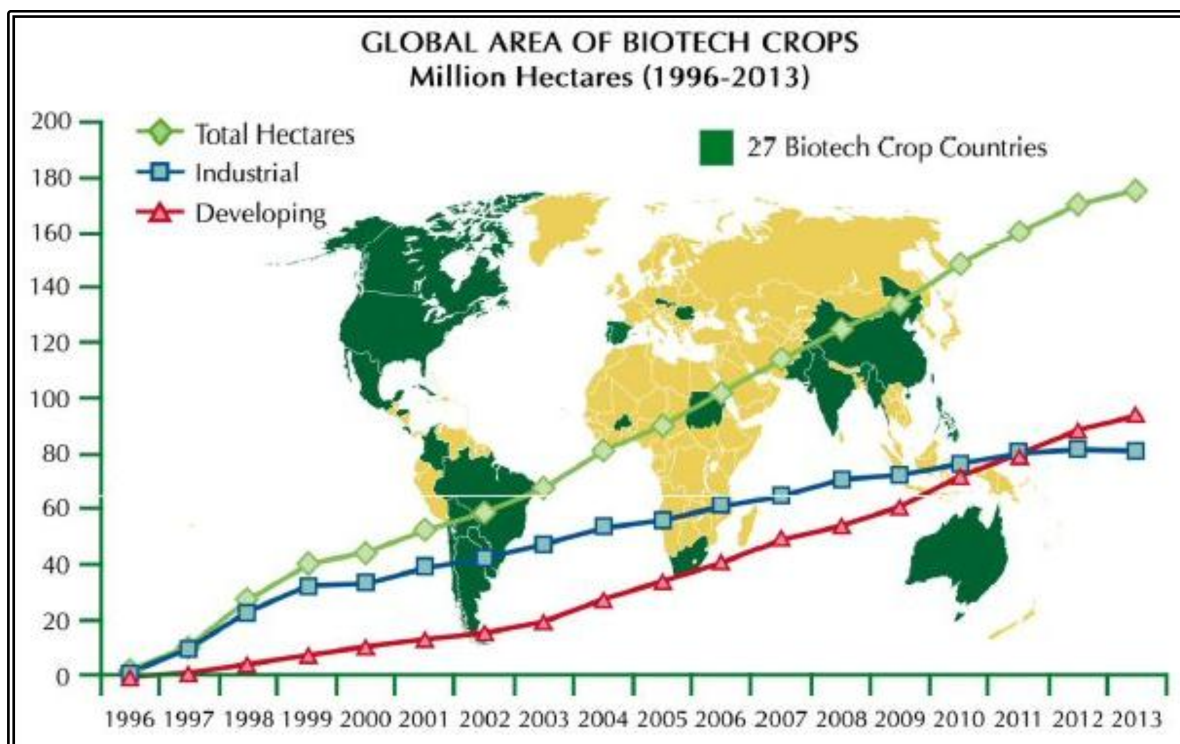

Figure 2.4: Illustration of GM crops growth from 1996 to 2013 (Matthews, 2014).

2.4. Bt Maize

One of the most widely cultivated GM crops is insect resistant maize (Bt maize), which is engineered to express insecticidal toxins derived from the bacterium *B. thuringiensis* (Schnepf and Whiteley, 1981; Höfte and Whiteley, 1989; Federici, 1998; Van den Berg *et al.*, 2013). Bt crystal proteins (called Cry proteins) display a high degree of specificity towards agricultural insect pests (Bagwan *et al.*, 2010; Cheeke *et al.*, 2012). The first Bt crystal protein was cloned by Schnepf and Whiteley in 1981, which led to the development of Bt plants in the mid 1980s (Federici, 1998). After the ingestion of Bt plant material, Cry proteins are activated into protoxins that bind to specific midgut epithelial receptors. This leads to pore formation in the digestive tract, which results in larval death. The type and amount of protoxins that is produced, determines the specificity towards a certain insect order (De Maagd *et al.*, 2001; Pigott and Ellar, 2007; Bravo *et al.*, 2011).

In 1996 Bt maize was commercialised in the USA. The main objective for its development was to control two stem borer species, *O. nubilalis* and *Diatraea grandiosella* (Lepidoptera: Crambidae) in North America (Van den Berg *et al.*, 2013). In 1998 seven million hectares of transgenic maize (Bt maize) were planted in the USA (Van Rensburg, 1999). Originally, Bt plants expressed single toxins in order to kill target pests. The first maize varieties contained the Cry1Ab gene and cotton expressed the Cry1Ac gene. Newer varieties of transgenic crops were quickly developed, and in 2009 Tabashnik and co-workers reported 18 different combinations of 11 Bt toxins registered in the USA (Tabashnik *et al.*, 2009).

In 1997 Bt cotton was approved in South Africa for commercial planting. The second GM crop approved for commercial planting was Bt yellow maize, which contained a Cry1Ab gene. It was first planted during the 1998 / 1999 cropping season to control two lepidopteran pests, *B. fusca* and *C. partellus* (Van Wyk *et al.*, 2009; Kruger *et al.*, 2010; Van den Berg *et al.*, 2013). Bt white maize was first introduced in South Africa in 2001, and commercialised during the 2002 / 2003 season (Van den Berg *et al.*, 2013). South Africa is the leader in cultivating genetically engineered crops in Africa, and one of the five leading countries in the world. The other four is China, India, Brazil and Argentina. Together these five countries grew 47 % (82.7 million hectares) of the global GM crops in 2013 (James, 2014).

Insect pests are not the only threat farmers are faced with when growing crops, other threats such as weeds are also responsible for yield losses. Unwanted weeds are controlled by herbicides, although it can also cause harm to crops in some cases. These chemicals bind to

specific target sites (proteins and enzymes) within plants and in this manner disrupt natural plant functions (Prather *et al.*, 2000).

The first genetically engineered herbicide resistant crops were grown in the United Kingdom in 1998. Maize and canola varieties were made resistant to glufosinate-ammonium and beet to glyphosate (Firbank *et al.*, 2003). Tolerance to the herbicide glyphosate enables glyphosate to kill all weeds without damaging crops. Since its cultivation, herbicide tolerance has been the dominant trait in GM crops (James, 2012). When crops are engineered to express herbicide tolerance together with insecticidal properties or a combination of other traits, it is referred to as stacked-gene crops.

2.4.1. *Bacillus thuringiensis*

Bacillus thuringiensis is a spore-forming, Gram-positive, motile bacterium commonly found in natural environments (Ferré *et al.*, 2008) such as soil, water, plant surfaces, grain dust and insects (Federici, 1998). This bacterium grows as a vegetative cell in the presence of sufficient nutrients and reproduces by binary fission. When food sources are inadequate, a dormant spore forms (Knowles, 1994). During this sporulation the bacterium synthesises crystalline inclusions (Figure 2.5). This structure is made up of protoxin subunits called delta-endotoxins (δ-endotoxins). Two types of proteins are found based on their host specificity, namely Cry (crystal) and Cyt (cytolytic) proteins (Federici *et al.*, 2010). The δ-endotoxins are accumulated into a parasporal body (Figure 2.6) and are responsible for the Cry proteins specific toxicity (Federici, 1998; Pigott and Ellar, 2007; Ben-Dov, 2014).

Genes encoding for 150 Cry and 12 Cyt proteins have been sequenced and cloned (Federici *et al.,* 2010). Thousands of *B. thuringiensis* insecticidal proteins have been isolated and characterised since it was first cloned (Schnepf and Whiteley, 1981), revealing the extreme diversity of these proteins (McLinden *et al.*, 1985; De Maagd *et al.*, 2001; Pigott and Ellar, 2007; Federici *et al.*, 2010; Ben-Dov, 2014; Deng *et al.*, 2014). Cry proteins primarily target insects belonging to Lepidoptera (moths and butterflies), Diptera (flies and mosquitoes), Coleoptera (beetles) (Federici, 1998; Broderick *et al.*, 2006; Pigott and Ellar, 2007) and a few are toxic to nematodes (Bravo and Soberón, 2008). Cyt proteins are less toxic and only active against mosquito (Diptera: Culicidae) and black fly (Diptera: Simuliidae) larvae (Federici *et al.*, 2010).

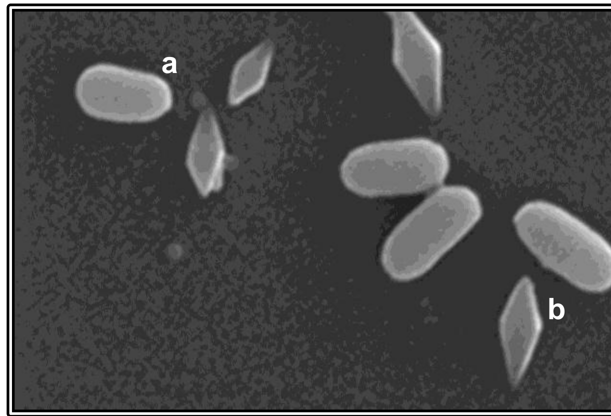

Figure 2.5: Scanning electron micrograph of Bt spores (a) and Bt crystals (b) (Xue *et al*., 2008).

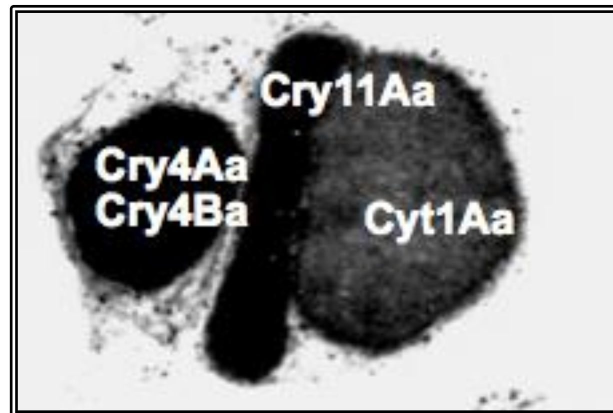

Figure 2.6: Transmission electron micrograph illustrating the parasporal body of *B. thuringiensis* subsp. *israelensis* together with the entailed crystal proteins (Federici *et al.*, 2010).

Bacillus thuringiensis are divided into several subspecies based on the antigenic properties of the flagellar (H) antigen. These are given an H antigen serovariety number and a sub-specific name. The four subspecies generally used for their insecticidal properties are: (1) *Bacillus thuringiensis* subsp. *kurstaki* (H 3a3b3c) that targets Lepidoptera. It produces Cry1Aa, Cry1Ab (used for Bt maize), Cry1Ac (used in Bt cotton) and Cry2Aa endotoxins. (2) *Bacillus thuringiensis* subsp. *aizawai* (H 7) is effective against Lepidoptera, with the major endotoxin proteins being Cry1Aa, Cry1Ab, Cry1Ca and Cry1Da (Federici *et al*., 2010). (3) *Bacillus thuringiensis* subsp. *israelensis* (H 14) is used against Diptera such as mosquitoes and blackfly larvae. It produces Cry4Aa, Cry4Ab, Cry11Aa1 (Oppert, 1999; Federici *et al*., 2010) and Cyt1Aa toxins (Ben-Dov, 2014). (4) *Bacillus thuringiensis* subsp. *morrisoni* (H 8a8b) controls

several coleopteran pests and encodes for Cry3Aa and Cry3Bb endotoxins (Federici *et al.*, 2010).

In a review article by Ramírez-Lepe and Ramírez-Suero (2012) the discovery of *B. thuringiensis* as a biological control agent is described. This bacterium was first isolated from diseased silkworm, *Bombyx mori* (Lepidoptera: Bombycidae) larvae in 1901. It was described by Iwabushi as *Bacillus sotto*. In 1915 Ernst Berliner isolated the bacterium from *Anagasta kuehniella* (Lepidoptera: Pyralidae) in Thuringia, Germany. He officially described it as *Bacillus thuringiensis* as it is known today. The toxicity of Bt to Lepidoptera species was established by Edward Steinhaus. The research showed that Bt had potential in controlling the alfalfa caterpillar, *Colias eurytheme* (Lepidoptera: Pieridae). After this breakthrough, many studies on Bt followed. It was then discovered that Bt produces a parasporal body that is responsible for larval death (Ramírez-Lepe and Ramírez-Suero, 2012).

2.4.2. Mechanism of Bt

A multi-step process is undergone in which the midgut cells of the insect larvae are erupted by 3D-Cry toxins, in order to kill the host insect. Two different mechanisms of action for these toxins have been proposed, with one relying on signal transduction and the other on pore formation (Bravo and Soberón, 2008). The first three steps are identical in both mechanisms and from step four onward differences occur. These mechanisms are shown and compared in Figure 2.7.

(A) Pore formation and signal transduction mechanisms

Step 1: Ingestion of Bt Cry proteins
After ingestion of a Bt protein, the crystalline inclusions are solubilised in the highly alkaline insect midgut into smaller inactive protoxins (Bravo *et al.*, 2007).

Step 2: Activation by midgut proteases
The inactive protoxins are cleaved by midgut proteases, giving rise to 60-70 kDa 3D-Cry toxins (Bravo *et al.*, 2007; Bravo and Soberón, 2008). Cry toxins are activated through the removal of an N-terminal peptide and half of the remaining protein from the C-terminus (Bravo *et al.*, 2007).

Step 3: Binding to primary receptor cadherin
Microvilli within the midgut cells have a cadherin receptor that binds to the activated toxin (Bravo and Soberón, 2008; Gómez *et al*., 2014).

(B1) Pore formation mechanism

Step 4: Interaction with cadherin helps with additional protein breakdown (proteolytic cleavage), which results in the oligomerisation of the toxin (Bravo and Soberón, 2008).

Step 5: Aminopeptidase (or alkaline phosphatase) acts as anchors for proteins in the membrane. The toxin oligomer binds to these anchor proteins that can be considered as the secondary receptors (Bravo and Soberón, 2008).

Step 6: The toxin oligomer inserts into the lumen membrane, which leads to pore formation in the microvilli. This subsequently causes cell lysis that disrupts the midgut epithelium of the larvae. Microbes within the midgut are now able to enter the haemocoel where spores can germinate and reproduce, leading to severe septicaemia and larval death (Broderick *et al*., 2006; Bravo *et al*., 2007; Bravo and Soberón, 2008).

(B2) Signal transduction mechanism

Step 4a: When cadherin binds to the Cry proteins it activates a pathway that mediates the activation of the G-protein (Bravo and Soberón, 2008).

Step 5a: Activation of the G-protein triggers adenylyl cyclase. The levels of cyclic adenosine monophosphate (cAMP) increases, which then activates protein kinase A. This leads to oncotic cell death (Bravo and Soberón, 2008).

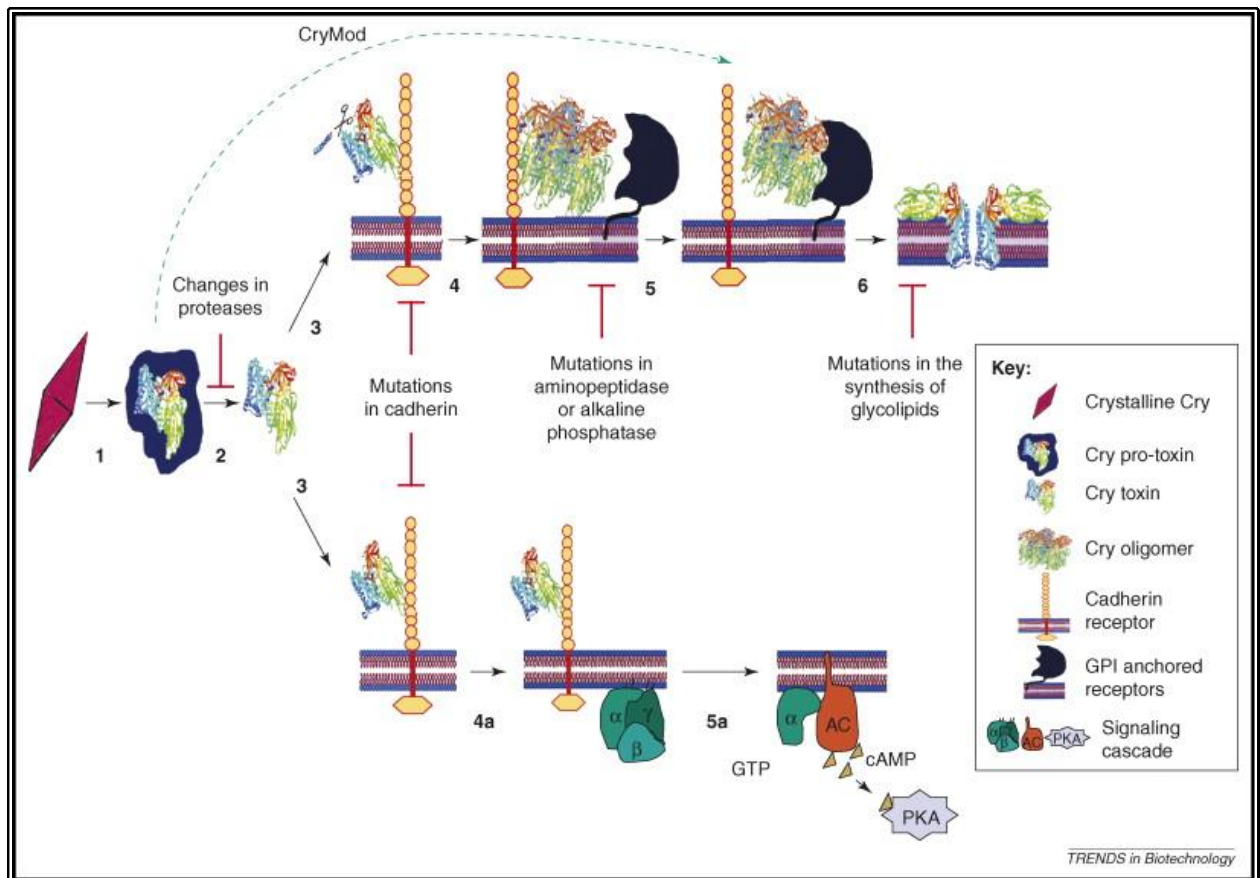

Figure 2.7: Illustration of the two modes of action of Cry toxins causing larval death. Step 1: Ingestion of Bt protein. Step 2: Activation of protoxins. Step 3: Toxins bind to receptors. Pore formation mechanism- Step 4: Proteolytic cleavage. Step 5: Toxins bind to secondary receptors. Step 6: Pore formation in the lumen membrane. Signal transduction mechanisms- Step 4a: Protein binds to cadherin. Step 5a: Cell death occurs (Bravo and Soberón, 2008).

2.4.3. Advantages and disadvantages of Bt crops

Debates regarding GM crops have been on going since it was first commercialised. Claims concerning the advantages and disadvantages of GM crops are based on laboratory and field experiments as well as the history of GM crops, but some are only speculative. These can be considered in four categories: agronomic, environmental, economic and social issues (Mannion and Morse, 2013).

Bt crops have a positive effect on agronomic aspects such as weed, insect and disease control. Bt crops are engineered to reduce the competition that competitors (weeds, viruses, fungi, insects) have on production, which leads to an increase in yield per unit area (Mannion and Morse, 2013). Crops with improved water use efficiency and drought tolerance were developed to overcome factors such as water shortages, rising temperatures and changes in rainfall patterns, which are responsible for significant reductions in seed and biomass yields each year (Cominelli and Tonelli, 2010). These crops present the possibility to expand cropping systems into remaining natural ecosystems (Mannion and Morse, 2013). Although more crops will aid in food security it can also be considered a cause of concern for natural habitats.

From an environmental view it is beneficial since the cultivation of Bt crops reduces the use of conventional insecticides. However, secondary pests such as aphids are still present in these crops, which still require the use of insecticides to control these non-target pests (Cannon, 2000). The threat to beneficial non-target organisms is minimised with the reduced insecticide usage. This also leads to less water contamination by these chemicals. The cultivation of herbicide tolerant (HT) maize and soybean requires reduced tillage practices, which reduces soil erosion and nutrient loss (Mannion and Morse, 2013).

For farmers the cultivation of Bt crops holds economic benefits such as: (1) Reduced input costs (Kumar, 2003), because of a reduction in insecticide usage and the need for scouting (Cannon, 2000). (2) Season long protection against insect pests despite weather conditions (Kumar, 2003), since the toxin is expressed throughout the whole plant it cannot wash off or become inactive like insecticides (Ferré *et al.*, 2008). (3) Effective control of burrowing insects (Kumar, 2003), which cannot be controlled successfully through insecticides. (4) Another essential trait of these crops is that it only affects insects that feed on it (Kumar, 2003).

The increasing cultivation of GM crops raised social issues concerning human health such as: (1) Allergies caused by GM proteins however, this theory also applies to hybrids obtained through conventional plant breeding. (2) Adverse effects on the development of rats as well as mortalities caused by ingestion of GM crops (Mannion and Morse, 2013). On the other hand, the reduced environmental pollution has positive effects on human health (Romeis *et al.*, 2006; Bravo *et al.*, 2011).

The potential effects that Bt toxins may have on non-target organisms, especially biological control agents, is another concern associated with the cultivation of Bt crops. Biological control agents include parasitoids and predators. Parasitoids are easily affected by changes in host quality because of the close relationships with their hosts (Romeis *et al.*, 2006; Yu *et al.*, 2011).

In studies using Bt susceptible hosts, adverse effects were observed on parasitoid survival, development, weight, longevity and reproduction (Liu *et al*., 2005b; Romeis *et al*., 2006; Chen *et al*., 2008; Yu *et al*., 2011). These effects are primarily caused by a decrease in host quality after ingestion of Bt toxins, rather than direct toxicity (Romeis *et al*., 2006; Chen *et al*., 2008; Yu *et al*., 2011). Another contributing factor to parasitoid mortality is that not all susceptible hosts are able to survive long enough on Bt plants for parasitoids to complete their development (Schuler *et al*., 2003). However, parasitoids are able to develop in Bt resistant hosts without any adverse effects (Schuler *et al*., 2003; Romeis *et al*., 2006). Although several studies reported adverse effects of Bt on parasitoids most of the authors concluded that no significant effects occurred (Liu *et al*., 2005b; Chen *et al*., 2008; Yu *et al*., 2011). Bt may also have negative effects on predators such as green lacewings, *Chrysoperla carnea* (Neuroptera: Chrysopidae). Hilbeck and co-workers (1999) reported the first adverse prey-mediated effects of Bt on *C. carnea* larvae. Immature larvae were reared on prey fed with Bt and non-Bt diets respectively. High mortality rates were observed in *C. carnea* larvae reared on prey that fed on diets containing Bt (Hilbeck *et al*., 1999).

Non-target organisms also include butterflies, pollinators and aquatic and soil organisms. Studies investigating the potential effects that Bt may have on soil organisms such as Collembola (Heckmann *et al*., 2006; Bai *et al.,* 2010), earthworms (Vercesi *et al*., 2006; Krogh *et al*., 2007), snails (De Vaufleury *et al*., 2007), nematodes (Saxena and Stotzky, 2001) and protozoa (Saxena and Stotzky, 2001) generally reported little to no adverse effects (Carpenter, 2011; Yu *et al*., 2011). Rosi-Marshall and co-workers (2007) reported a decrease in growth rates in the aquatic organism, *Lepidostoma liba* (Trichoptera: Lepidostomatidae) (caddisflies) after the ingestion of Bt maize byproducts (such as detritus and pollen) expressing Cry1Ab proteins. However, due to the apparent lack of necessary background information and poor experimental design the results were doubtful, and no conclusions of adverse effects were drawn from the study. A similar study by Jensen and colleagues (2010) reported no negative effects of Bt on caddisflies (Jensen *et al*., 2010).

Pollinators such as honeybees (Hymenoptera: Apidae) have vital roles within terrestrial ecosystems. Therefore several studies have been done to determine whether Bt may have adverse effects on these insects (Bailey *et al*., 2005; Liu *et al*., 2005a; Rose *et al*., 2007). These studies observed no negative effects of Bt pollen on the longevity, behavior, development of hypopharyngeal glands, superoxide dimutase activity and the gut microbial communities of honey bees (Yu *et al*., 2011). Studies evaluating the effects of Bt on non-target lepidopteran species such as the European swallowtail, *Papilio machaon* (Lepidoptera: Papilionidae) (Lang and Vojtech, 2006), monarch butterfly, *Danaus plexippus* (Lepidoptera:

Nymphalidae) (Prasifka *et al.*, 2007), peacock butterfly, *Inachis io* (Lepidoptera: Nymphalidae) (Perry *et al.*, 2010) and the red admiral, *Vanessa atalanta* (Lepidoptera: Nymphalidae) (Perry *et al.*, 2010) observed adverse effects on mortality, development, body weight, and larval behaviour. During these studies larvae were artificially exposed to high levels of Bt toxins (Lang and Vojtech, 2006; Prasifka *et al.*, 2007; Perry *et al.*, 2010; Yu *et al.*, 2011).

The main risk of cultivating Bt crops is the development of resistance in target pests (Hernández-Martínez *et al.*, 2010; Bravo *et al.*, 2011). The ability of insects to develop resistance to Bt products has been described for several insect species. Only a few lepidopteran species have evolved field resistance to Bt so far (Hernández-Martínez *et al.*, 2010). However, studies involving artificial selection under laboratory conditions have reported the possibility for resistance developing in many other insect species (Ferré and Van Rie, 2002), such as the Indianmeal moth, *Plodia interpunctella* (Lepidoptera: Pyralidae) (McGaughey, 1985) and the tobacco budworm, *Heliothis virescens* (Lepidoptera: Noctuidae) (Gould *et al.*, 1992).

2.5. Resistance development of *Busseola fusca* to Bt maize

The term "resistance" can refer to either field-evolved or laboratory selected resistance. Tabashnik *et al.* (2009) explain the difference between the two concepts as follows: (1) Field-evolved resistance - "A genetically based decrease in the susceptibility of a population to a toxin caused by exposure of the population to the toxin in the field". (2) Laboratory selected resistance - "Exposure to a toxin in the laboratory that causes a heritable decrease in susceptibility of a laboratory strain" (Tabashnik *et al.*, 2009).

Before Bt crops were commercially grown, scientists predicted that resistance would evolve within target insect pests (Tabashnik *et al.*, 2003). The possibility of resistance developing in the Indianmeal moth (*P. interpunctella*) to Bt was reported by McGaughey in 1985. *Plodia interpunctella* is an important lepidopteran pest of stored grain products. McGaughey (1985) collected *P. interpunctella* larvae from Bt-treated and Bt-untreated grain storage facilities respectively. Larvae were reared in the laboratory and their susceptibility to Bt formulations was determined. It was found that larvae collected from treated bins showed more resistance than the larvae collected from the untreated bins. It was also found that the larvae developed resistance after only a few generations were exposed to Bt. This indicated the possibility of field evolved resistance (McGaughey, 1985). Laboratory experiments on other target species have also shown the potential to develop resistance to Bt toxins (Meihls *et al.*, 2008). Akhurst

and co-workers (2003) reared *Helicoverpa armigera* (Lepidoptera: Noctuidae) larvae on an artificial diet containing the Cry1Ac toxin. They found that after several generations larvae showed resistance to Bt spray formulations containing the Cry1Ac toxin alone, but were susceptible to formulas containing multiple Cry toxins. Toxin binding assays showed that high affinity binding sites that were detected in susceptible larvae were absent in resistant larvae (Akhurst *et al.*, 2003).

Insect resistant management (IRM) programmes have been proposed in order to control further resistance development. This includes mechanisms such as the high-dose / refuge strategy and pyramided maize hybrids (Tabashnik *et al.*, 2009; Bravo *et al.*, 2011; Hellmich and Hellmich, 2012). These strategies were mainly developed to reduce selection pressure on target pests and should therefore, be a main concern for farmers (Van den Berg *et al.*, 2013).

The success of Bt crops led to an increase in the use of these crops, which resulted in an escalated toxin exposure to insects, thus adding to the selection pressure for resistance evolution (Oppert, 1999). Resistance development in agricultural insect pests threatens the success of Bt crops (Liu and Tabashnik, 1997; Wang *et al.*, 2007). So far, incidents of field evolved resistance have only been documented in a few lepidopteran species. The African stem borer, *B. fusca* to Cry1Ab (Van Rensburg, 2007; Kruger *et al.*, 2011), the fall armyworm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae) to Cry1F (Storer *et al.*, 2010) and the diamond back moth, *Plutella xylostella* (Lepidoptera: Plutellidae) (Shelton *et al.*, 2002) as well as the cabbage looper, *Trichoplusia ni* (Lepidoptera: Noctuidae) (Janmaat and Myers, 2003; Wang *et al.*, 2007) have developed resistance against Bt sprays used in greenhouses and open fields (Janmaat and Myers, 2003).

Plutella xylostella, a major pest of vegetables, is one of the most challenging pests to control because of its ability to develop resistance against extensively used insecticides (Zhao *et al.*, 2006). It was the first to develop resistance to DDT, most synthetic insecticides and Bt sprays (Talekar ans Shelton, 1993; Ferré and Van Rie, 2002; Van Rensburg, 2007). Most studies found that resistance of *P. xylostella* to *B. thuringiensis* subsp. *kurstaki* were unstable (Tabashnik *et al.*, 1994), inherited as an autosomal recessive or partly recessive trait (Liu and Tabashnik, 1997), and mainly controlled by one or a few loci (Tang *et al.*, 1996).

Several mechanisms for resistance development against Bt have been proposed. This includes: (1) Alterations to the toxin binding receptors in the midgut membrane will reduce the amount of toxins able to bind to the midgut (Oppert, 1999; Ma *et al.*, 2005; Bravo *et al.*, 2011). (2) Cells in the midgut that are affected by Bt toxins are quickly replaced (Martínez-Ramírez *et*

al., 1999; Ma *et al.*, 2005). (3) Changes occur in the crystal protein solubilisation and / or activation reactions such as variations in physicochemical conditions (pH) and proteases in the gut lumen (Oppert, 1999; Bravo *et al.*, 2011). (4) And sequestering the toxin through esterases (Gunning *et al.*, 2005).

Busseola fusca was the first pest to develop resistance against Bt maize expressing the Cry1Ab gene (Van den Berg *et al.*, 2013). The first report of field resistance was in 2006 at the Vaalharts irrigation scheme in South Arica (Van Rensburg, 2007). In 1994 specific Bt events were tested to control the South African stem borer complex, and *B. fusca* already showed more tolerance than the spotted stem borer, *C. partellus* (Van Rensburg, 2007).

2.6. Associations between insects and gut microbes

Insects are the most abundant and diverse animal group globally in a number of species, ecological habitats and in biomass (Basset *et al.*, 2012). The countless relationships insects have with beneficial microorganisms played a large part in their diversification and evolutionary success. Associations with bacteria are known to (1) upgrade nutrient-poor diets, (2) aid in the digestion of recalcitrant food components, (3) protect from predators, parasites, and pathogens, (4) contribute to inter- and intraspecific communication, (5) affect efficiency as disease vectors, (6) and govern mating and reproductive systems (Dillon *et al.*, 2002; Azambuja *et al.*, 2004; Broderick *et al.*, 2004; Rajagopal, 2009; Gullan and Cranston, 2010; Engel and Moran, 2013; Gimonneau *et al.*, 2014; Tagliavia *et al.,* 2014).. Microbial communities are especially prominent in the digestive tracts of insects where they facilitate the various lifestyles of their hosts (Engel and Moran, 2013; Powell *et al.*, 2014).

For agricultural and ecological assessments the contribution of gut microorganisms to insect function is highly relevant. Several insect species provide beneficial laboratory models to better understand the microbial community and their interactions with hosts. The impact both agricultural insect pests and pollinators have on crop plants are influenced by the microorganisms associated with them (Engel and Moran, 2013).

2.6.1. Structure and physical conditions of the insect gut

The basic structure of an insect gut consists of three regions: the foregut, the midgut and the hindgut. In some cases the foregut or hindgut is divided into different functional parts. The foregut may have a separate diverticula (crop) for temporary food storage and the hindgut separate sections such as fermentation chambers and a distinct rectum for holding faeces

before defecation (Engel and Moran, 2013). The midgut arises from endodermal cells and is the primary site for digestion and adsorption in many insects. In many cases the midgut epithelial cells secretes a peritrophic membrane that divides it into an ecto- and endoperitrophic space. Usually microorganisms are restricted to the endoperitrophic space to prevent them from making direct contact with the epithelium (Engel and Moran, 2013). The peritrophic membrane consists of small pores that block most microorganisms, but allows small molecules to cross. Enzymes must cross the peritrophic membrane in order to reach the food bolus and digested food molecules have to cross the membrane in the opposite direction to reach the absorptive epithelium (Shao *et al.*, 2001; Engel and Moran, 2013). The physical properties (thickness) of the peritrophic membrane are maintained by chitinase, an enzyme produced by several of the gut microbes. In return, this chitinous sheath provides chitin for the bacteria, which they utilise as carbon and nitrogen sources (Indiragandhi *et al.*, 2007).

The peritrophic membrane provides a barrier that protects the epithelium from (1) mechanical damage by food particles, (2) exposure to large toxin molecules present in food, (3) and pathogenic invasion. The peritrophic membrane further concentrates food and digestive enzymes (Shao *et al.*, 2001). Figure 2.8. is a illustration of the gut structure in insects.

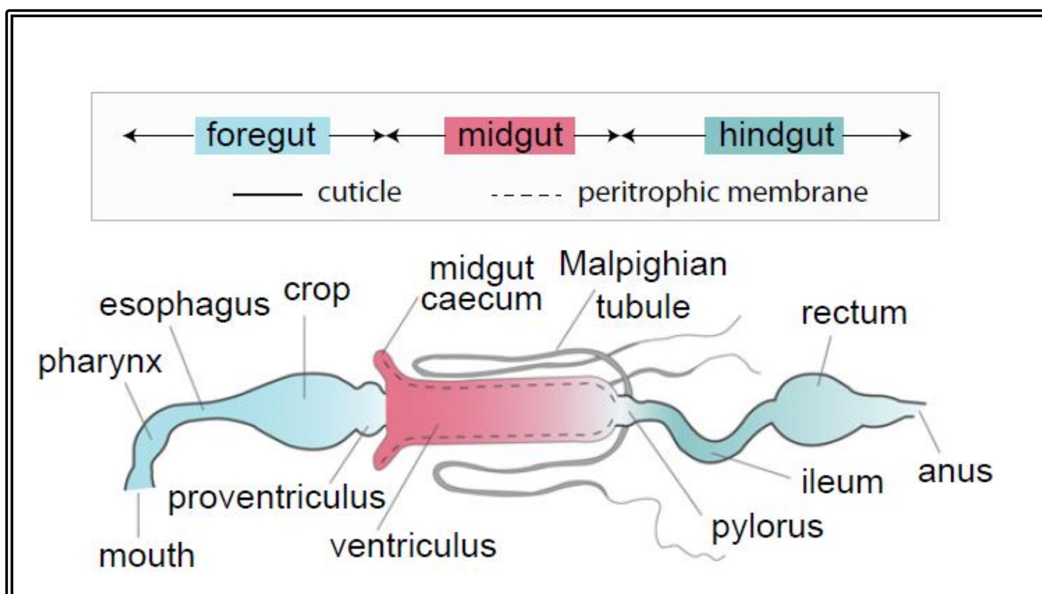

Figure 2.8: Overview of an insect gut structure (Engel and Moran, 2013).

Although the digestive tract of different insect species is similar, a variety of modifications occur between species as a result of their adaptions to different feeding methods and habitats. Many of these changes have evolved for hosting microorganisms in specific compartments of the gut (Engel and Moran, 2013).

Microbial colonisation depends on the physicochemical conditions in the lumen of the different gut compartments. These conditions such as pH and oxygen availability can fluctuate between the various sections in the gut. Microbial metabolism of the gut microbes actively shapes the conditions within the different gut compartments. The pH in the lumen often differs from the pH of the haemolymph, which is normally 7 (Engel and Moran, 2013). Alkalinity within gut regions is associated with the dietary preferences of the host insect (Lemke *et al.*, 2003). Lepidopteran larvae have an extremely alkaline midgut with a pH of up to 12 (Harrison, 2001; Engel and Moran, 2013). Their diet includes tannin-rich leaves which correlate the pH within their guts; this lowers the binding of dietary proteins, improves nutrient availability and also excludes most bacteria (Engel and Moran, 2013).

The different compartments within termite guts display extreme pH gradients, which can vary between five and 12. Microbial colonisation is not completely prevented by the extreme alkalinity of some compartments within the gut. Instead it supports the growth of specialised alkaline-tolerant symbiotic bacteria such as Firmicutes, Planctomycetes and *Clostridium* (Köhler *et al.*, 2012; Engel and Moran, 2013).

Oxygen availability within insect guts can range from anaerobic to aerobic. Larger insects, insects with bigger gut compartments and vigorous gut communities generally have anaerobic conditions within their guts (Engel and Moran, 2013). Johnson and Barbehenn (2000) reported relatively low oxygen levels within the gut of *Helicoverpa zea* (Lepidoptera: Noctuidae) larvae. The oxygen levels remained low even after they were fed with antibiotics to eliminate most of the microbes within their guts. It was suggested that the low oxygen levels within *H.* zea may not be a result of the microbial activity within the gut, but rather the insects natural chemical processes. However, nine other Lepidoptera species were also used during this study, in which they reported higher oxygen levels within the foregut than in the midgut. Oxygen may enter the gut while the larva feeds and is then depleted as the food moves posteriorly. Therefore, the gut microbes may reduce the oxygen levels during oxidation processes (Johnson and Barbehenn, 2000).

2.6.2. Function of gut microbes

Microbial communities within insect guts may include bacteria, archaea, protists (single cell eukaryotes) and fungi (Brennan *et al*., 2004; Engel and Moran, 2013). Microbes may assist in defining insect metabolic traits. Microbial groups participate in carbon metabolism, nitrogen recycling, methano- and acetogenesis (Brennan *et al*., 2004). Symbiotic associations between microbes and insects can be divided into primary and secondary symbionts. Primary symbionts exist in specialised cells namely bacteriocytes or bacteriotomes. This is the most common type of symbiosis and is usually beneficial (or often obligated) for their host (Moran and Baumann, 2000; Vega and Dowd, 2005; Lundgren *et al*., 2007). Secondary symbionts are extracellular and live within the gut or other tissues of the host (Lundgren *et al*., 2007).

(A) Bacteria

Bacterial species represent most or all of the microorganisms found within insect guts (Engel and Moran, 2013). Gut microbial communities greatly differ between insect species in terms of their size, composition, functions and where it is located within the gut. It is estimated that honey bees, *Apis mellifera* (Hymenoptera: Apidae) (Martinson *et al*., 2012), bloodsucking bugs, *Rhodnius prolixus* (Hemiptera: Reduviidae) (Eichler and Schaub, 2002) and house crickets, *Acheta domestica* (Orthoptera: Gryllidae) (Santo-Domingo *et al*., 1998) approximately has 10^9 bacterial cells within their guts, while the common fruit fly, *Drosophila melanogaster* (Diptera: Drosophilidae) (Ren *et al*., 2007) has 10^6 bacterial cells.

Sap-feeding insects such as psyllids, whiteflies, aphids and mealybugs have little to no gut microbes but instead have intracellular symbionts (Bauman, 2005; Engel and Moran, 2013). The primary function of these prokaryotic endosymbionts is to synthesise essential amino acids required by the host. Four different endosymbionts, each with a different bacterium, serves this purpose within the different hosts (Bauman, 2005). For example, the symbiosis between aphids and the bacterium, *Buchnera aphidicola* are one of the best-described endosymbionts (Moran and Baumann, 2000; Shigenobu *et al*., 2000; Bauman, 2005). This symbiont consists of genes enabling it to provide amino acids and vitamins to its host, which are inadequate in its diet. According to phylogenetic analysis this symbiotic relationship dates back 200 million years ago and led to the co-speciation of these two organisms (Shigenobu *et al*., 2000; Bauman, 2005).

A study conducted on the gut bacteria of ground beetles (Carabidae) revealed three bacterial species not previously associated with insects namely *Hafnia alvei*, *Caedibacter* sp., and *Enterobacter* B-14 (Lundgren *et al*., 2007). Lundgren and co-workers (2007) also isolated

potential pathogens such as *Burkholderia fungorum*, *Serratia marcescens* and *H. alvei*. The latter is a key component in regulating the microbial community dynamics within an insect gut. *Serratia marcescens* is a facultative anaerobe that maintains the anaerobic conditions by consuming oxygen at the periphery of the insect gut. This is essential for the survival of the strict anaerobes responsible for cellulose digestion within Formosan termites (Lundgren *et al*., 2007). *Serratia marcescens* was also isolated from the gut of *P. xylostella*, where it showed the highest chitinase activity of all the isolated bacterial strains (Indiragandhi *et al*., 2007). The production of chitinase is vital for host nutrition, therefore the presence of this bacterium also aids in the growth and development of larvae. Indiragandi *et al.* (2007) fed *P. xylostella* larvae leaves treated with *S. marcescens*, this caused these larvae to grow more rapidly than those not fed with the treated leaves.

Ground beetles also harbour microbes that are known to catabolise aromatic hydrocarbons generally found in insecticides. The *Enterobacter* strain B-14 isolated by Lundgren and co-workers (2007) degrades chlorpyrifos and organophosphate insecticides (Singh *et al*., 2004: Lundgren *et al*., 2007), which these beetles encounter regularly within agro-ecosystems. Singh *et al.* (2004) found that *Enterobacter* B-14 is a soil bacterium that utilises chlorpyrifos as a carbon source.

Most members of the family Enterobacteriaceae (Class: Gammaproteobacteria) produce digestives enzymes which aids in the nutrition of the insect host (Lundgren *et al*., 2007; Engel and Moran, 2013). *Enterobacter agglomerans* isolated from the apple maggot fly, *Rhagoletis pomonella*, (Diptera: Tephritidae) produces urease, which assist its host in nitrogen recycling (Lauzon *et al*., 2000).

It has been suggested that some *Enterococcus sp.* including *E. avium* produces acetate that is responsible for decreasing the gut pH (Dillon and Dillon, 2004; Cappellozza *et al*., 2011). Other bacteria such as *Bacillus* sp., *Microbacterium* sp. and *Klebsiella* sp. may also produce acid from various carbon sources (Anand *et al*., 2010; Gu *et al*., 2010; Suzuki and Hamada, 2012). In lepidopteran species a high pH is required for microbial toxins, such as *B. thuringiensis*-toxin to activate, therefore the production of acetate can aid in resistance development against insecticides (Broderick *et al*., 2004; Xiang *et al*., 2006).

(B) Archaea

Archaea represent only a small percentage of the total prokaryotic community in the gut of insects (Hongoh, 2010; Ohkuma, 2008). The most common archaeal type found are methanogens such as *Methanobrevibacter cuticularis* and *Methanobrevibacter curvatus* that have been isolated form the hindguts of termites (Isoptera) (Leadbetter *et al.*, 1998; Hongoh, 2010). Methanogens produce methane from H_2 and CO_2 and is tolerant to anaerobic conditions. It is believed that they play a role in lignocellulose fermentation in termite guts by utilising H_2. *Methanobrevibacter* spp. are present on the gut epithelium and in the cells of gut protists such as *Dinenympha parva* and *Spirotrichonympha leidyi* (Hongoh, 2010). *Methanobrevibacter* species was also isolated from the hindgut of the European cockchafer, *Melolontha melolontha* (Coleoptera: Scarabaeidae) (Egert *et al.*, 2005).

(C) Protists

Protists are unique to termites and the wood-feeding cockroach, *Cryptocercus* (Cryptocercidae) (Hongoh, 2010). However, protists are usually absent within higher termites (Ohkuma *et al.*, 1999; Wu *et al.*, 2012). Lower termites (wood-feeding) can harbour between 10^3 and 10^5 protistan cells within a single gut (Hongoh, 2010). The symbiosis between lower termites and the cellulolytic flagellated protists within their guts plays an essential role in their ability to degrade lignocellulose and consequently, in their nutrition (Tokuda *et al.*, 2007; Ohkuma, 2008). Lignocellulose primarily consists of lignin, cellulose and hemicellulose (Wu *et al.*, 2012). Therefore, gut protists play a vital role in the survival of termites on a lignocellulose diet. First, cellulose are partly degraded by the indigenous enzyme, cellulase. The product then moves to the hindgut, where it is ingested by the gut protists that are responsible for further degradation. Together the endogenous enzymes and gut protists can degrade cellulose almost completely (Ohkuma, 2008).

(D) Fungi

Fungi that inhabit insect guts play vital roles in their nutrition (Suh *et al.*, 2005; Vega and Dowd, 2005). These relations can either be casual or highly specific and obligated. Fungi can either occur extra- or intercellular within insects. Those associated with insects produce enzymes that improve digestion and nutrition and they may produce amino acids, vitamins and sterols. They are also responsible for the detoxification of toxic plant metabolites, which are part of an insect's diet (Dowd, 1989; Suh *et al.*, 2003; Vega and Dowd, 2005). Fungi commonly associated with bark beetles are *Ophiostoma*, *Ceratocystiopsis*, *Grosmannia*, and *Ceratocystis*

from the phylum Ascomycetes. Adult beetles have specialised structures on their exoskeleton called mycangia, for carrying fungi. This enables the fungi to gain access to inaccessible plant resources and to live on recently killed trees (Six and Wingfield, 2011).

Ectosymbiotic yeasts can also produce pheromones. *Hansenula capsulata* and *Pichia pinus* are responsible for converting *cis*- and *trans*-verbenol to verbenone. The latter is an anti-aggregation pheromone that occurs in the galleries of bark beetles, *Dendroctonus ponderosae* (Coleoptera: Curculionidae) (Vega and Dowd, 2005). Galleries are constructed under the bark, in the phloem layer of woody plants. This is where the beetles lay eggs and where their offspring feed and develop (Six and Wingfield, 2011).

Associations between *Drosophila* species and yeasts are well known (Starmer *et al.*, 1986; Vega and Dowd, 2005). In most cases the yeasts offer nutritional benefits to the insect at an extracellular level. It also produces pheromones and detoxifies plant metabolites within these flies. Some yeasts associated with *Drosophila* spp. are capable of metabolising decaying plant tissues, as in the case of *Candida ingens* that processes toxic fatty acids present in cactus tissues (Starmer *et al.*, 1986; Vega and Dowd, 2005). *Candida sonorensis* and *Cryptococcus cereanus* metabolises 2-propanol which is also present in cactus tissues that are toxic to *Drosophilla* spp. (Vega and Dowd, 2005).

Cigarette beetles, *Lasioderma serricorne* (Coleoptera: Anobiidae) primarily feed on tobacco, seeds, straw and pepper, which contain a variety of toxins. Tobacco contains toxins such as nicotine and a number of harmful esters including scopolamine, polyphenolics and flavonoid glycosides. These beetles are able to survive because of their symbiosis with intracellular fungi, which are responsible for the detoxification of these substances as well as providing nutrients for the insect host (Dowd, 1989; Suh *et al.*, 2003; Vega and Dowd, 2005). The yeast-like fungi are present in specialised tissues (mycetomes) that occur where the fore-and midgut joins (Dowd, 1989).

2.6.3. Lepidopteran gut community structure

The broad feeding range of certain lepidopteran species creates a challenging environment for microorganisms. The high alkalinity and diverse chemistry of the midgut attracts specific attention to the microbial communities they harbour (Gringorten *et al.*, 1993). Several studies have reported the presence of bacteria within this insect group, but knowledge about their function and role in insect development is limited (Broderick *et al.*, 2004). To better understand

the association between Lepidoptera and their microbial communities, several authors have surveyed the midguts of different species (Broderick *et al.*, 2004; Xiang *et al.*, 2006; Indiragandhi *et al.*, 2007; Anand *et al.*, 2010; Belda *et al.*, 2011; Priya *et al.*, 2012; Hammer *et al.*, 2014).

Differences in gut communities of three different *P. xylostella* populations were established by Indiragandhi and co-workers (2007). A field-collected population were compared with lab-reared insecticide-resistant and susceptible *P. xylostella* populations. The gut microbiota of the resistant population was more complex, with isolates from *Pseudomonas* spp., *Stenotrophomonas* spp., *Acinetobacte*r spp. and *S. marcescens*. Larvae from the susceptible population harboured isolates from *Brachybacterium* spp., *Acinetobacter* spp. and *S. marcescens*, while the field-collected population had the lowest microbial diversity. Qualitative differences of the respective gut bacteria suggest that the microbial community is a representation of the gut environment. Species belonging to *Pseudomonas* and *Stenotrophomonas* are well known for their ability to degrade insecticides and their absence therefore led to insecticide susceptibility in *P. xylostella*. Toxic compounds also play a selective role in enriching microbial populations (Indiragandhi *et al.*, 2007).

During an evaluation of the gut microbes in laboratory-reared and field-collected bollworms (*H. armigera*) it was shown that the field population harboured a more complex community structure than the laboratory population (Xiang *et al.*, 2006). Seven bacterial groups were isolated from the field-collected larvae, while only three were present in the laboratory population. The bacterial groups and abundance in which they occurred are shown in Table 2.1. These differences in diversity might be the result of variations in food availability and environmental factors, which laboratory and field populations encounter (Mead *et al.*, 1988; Xiang *et al.*, 2006). In a previous study by Mead *et al.* (1988) on the gut microbiota of migratory grasshoppers, *Melanoplus sanguinipes* (Orthoptera: Acrididae) it was also shown that field populations had a more complex community structure than laboratory populations.

Differences in the microbial diversity of individual bollworm larvae from field populations suggest that their internal biology can respond to changing external conditions, such as the consumption of novel microbes or phytochemicals (Xiang *et al.*, 2006). Field populations are more complex due to the fact that larvae are exposed to a larger variety of microbes in their natural environment. This cause variations in their gut microbial community that enables them to expand the range of phytochemicals which they can consume, since they possess greater diet related plasticity due to the alteration in their community structure (Patankar *et al.*, 2001).

Table 2.1: Comparison of gut microbes in laboratory reared and field-collected (*H. armigera*) larvae described by Xiang and co-workers (2006). The abundance in which these groups were isolated is also presented.

	Bacterial group %	Isolated species
Field-collected population	*Lactobacillales* 39 %	*Enterococcus avium* *Enterococcus casseliflavus* *Enterococcus gallinarum* *Lactococcus lactis* *Leuconostoc citreum*
	Actinobacteria 3 %	*Actinomyces* sp.
	Proteobacteria 1.5 %	*Pseudomonas mevalonii*
	Alfaproteobacteria 4.5 %	*Gluconobacter cerinus* *Sphingomons* sp. uncultured *Caulobacter* sp.
	Betaproteobacteria 5.5 %	*Bordetella* sp. *Comamonas testosteroni*
	Gammaproteobacteria 30,5 %	*Acinetobacter* sp. *Acinetobacter anitratus* *Acinetobacter venetianus* *Aquamonas fontana* *Stenotrophomonas maltophilia*
	Cytophaga-Flavobacterium-Bacteroides 6 %	Phenanthrene-degrading bacterium *Bacteriodes* bacterium *Flavobacterium* sp. *Chryseobacterium meningosepticum*
Laboratory population	*Lactobacillales* 98 %	*Enterococcus avium* *Enterococcus casseliflavus* *Lactococcus lactis* *Leuconostoc citreum*
	Gammaproteobacteria 1 %	*Acinetobacter* sp. *Acinetobacter anitratus* *Acinetobacter venetianus*
	Cytophaga-Flavobacterium-Bacteroides 1 %.	Uncultured bacterium

Several authors observed that diet alterations have an effect on the gut microbes associated with Lepidoptera (Broderick *et al*., 2004; Robinson *et al*., 2010; Belda *et al*., 2011; Hammer *et al*., 2014). The microbial community structure within the midguts of gypsy moth, *Lymantria dispar* (Lepidoptera: Erebidae) larvae fed with different diets varied greatly from one another. This included an artificial diet followed by one of four tree species namely white oak (*Quercus alba*), larch (*Larix laricina*), scrub willow (*Salix fragilis*) and quaking aspen (*Populus tremuloides*) (Broderick *et al*., 2004). Similarly, Belda and co-workers (2011) observed a clear connection between the diet and midgut microbes within *O. nubilalis* larvae.

Broderick and co-workers (2004) reported a relative simple community structure in *L. dispar,* with the presence of seven to 15 phylotypes. These authors isolated *Pseudomonas putida, Pantoea agglomerans, S. marcescens, Staphylococcus* spp. (*S. lentus, S. cohnii,* and *S.*

xylosus), *Enterococcus faecalis, Rhodococcus* sp., *Microbacterium* sp., *Agrobacterium* sp. and *Micrococcus* sp. (Broderick *et al*., 2004). *Enterococcus faecalis* were present in all the larvae regardless of the type of diet they were fed. This bacterium can aid its host in resistance against Bt toxins since it lowers the pH of the midgut through metabolic processes. Larvae consisting of low numbers of *E. faecalis* proved to be more susceptible to Bt toxins (Broderick *et al*., 2003).

2.6.4. Effect of geographical distribution of insects on gut microbe content

Corby-Harris and co-workers (2007) determined geographical differences of gut microbes between 11 fruit fly (*D. melanogaster*) populations. Four bacterial groups were isolated namely Proteobacteria, Firmicutes, *Bacteroidetes* and *Wolbachia*. They established differences between the microbial composition and richness among the various host populations, but did not recognise any relation between the microbial richness and abiotic factors such as temperature and latitude (Corby-Harris *et al*., 2007).

Mosquito populations from three different regions in Madagascar were compared to determine whether the environment could have an influence on the microbial communities they harbour. Males and females of two *Aedes* species (*A. albopictus* and *A. aegypti*) (Diptera: Culicidae) were analysed through DGGE, respectively. Bands from the genera *Bacillus*, *Acinetobacter, Agrobacterium*, *Enterobacter, Asaia, Delftia* and *Pseudomonas* were obtained. Diversity indices (Shannon and Simpson) indicated significant differences between the regions, while no variances in evenness occurred. It was concluded that the microbial community of the mosquitos are influenced by the various vegetation and animal hosts present within the respective regions (Zouache *et al*., 2011). However, three genera (*Acinetobacter*, *Asaia* and *Pseudomonas*) were consistently isolated from the two mosquito species which can also imply vertical inheritance of gut microbes through generations (Zouache *et al*., 2011).

2.7. Methods to identify gut community structure

The complexity of bacterial communities makes it challenging to identify the community structure of gut microbes. Cultivation-based techniques were initially used to identify bacterial groups present in different environmental samples (Xiang *et al.*, 2006). These techniques mainly consisted of biochemical tests in order to determine the physiological characteristics which are then used to identify the isolates (Conn and Pelczar, 1957).

Several studies used culture-dependent methods to survey the gut contents of insects such as wood-eating termites, *Reticulitermes flavipes* (Blattodea: Rhinotermitidae) (Schultz and Breznak, 1978; Potrikus and Breznak, 1980), craneflies, *Tipula abdominalis* (Diptera: Tipulidae) (Klug and Kotarski, 1980), sugar beet root maggots, *Tetanops myopaeformis* (Diptera: Ulidiidae) (Iverson *et al.*, 1984), mosquitoes (*Anopheles gambiae; A. funestus* and *Aedes aegypti*) (Lindh *et al.*, 2005; Gusmão *et al.,* 2007), silkworms (*B. mori*) (Anand *et al.*, 2010) and water beetle species namely *Agabus affinis* and *Hydroporus melanarius* (Coleoptera: Dytiscidae) (Schaaf and Dettner, 1997). Although these methods are unable to identify the entire spectrum of gut microbes, they provide an insight into the diversity of heterotrophic bacteria associated with insects (Mohr and Tebbe, 2006; Xiang *et al.*, 2006; Rani *et al.*, 2009). Heterotrophic bacteria utilise organic compounds as energy sources to grow (Allen *et al.*, 2004). These nutrients are incorporated into the various culturing media used to isolate and maintain microorganisms (Conn and Pelczar, 1957; Allen *et al.*, 2004).

Several studies of the community structure of gut microbes in insects have recently been done (De Vries *et al.*, 2001; Broderick e*t al.*, 2004; Delalibera *et al.*, 2007; Gusmão *et al.*, 2007; Rani *et al.*, 2009; Arias-Cordero *et al.*, 2012; Gupta *et al.*, 2012). Most of these studies made use of culture-dependent methods combined with molecular techniques. In these studies the gut is isolated and either sonicated (Broderick *et al.*, 2004; Robinson *et al.*, 2010) or vortexed (Klug and Kotarski, 1980; Schaaf and Dettner, 1996; Arias-Cordero *et al.*, 2012) in order to obtain the bacterial content. A dilution series was made and spread plated onto various agar plates (Klug and Kotarski, 1980; Bauer *et al.*, 2000; Zurek *et al.*, 2000; Broderick *et al.*, 2004; Rani *et al.*, 2009; Arias-Cordero *et al.*, 2012). Cultures are incubated aerobically at 37 °C (Rani *et al.*, 2009). Colony forming units are then categorised and counted according to their morphological traits (Schultz and Breznak, 1978; Klug and Kotarski, 1980; De Vries *et al.*, 2001; Gusmão *et al.*, 2007). This is based on the size, shape, texture, elevation and pigmentation of the colonies (Conn and Pelczar, 1957; Schaaf and Dettner, 1996; De Vries *et al.*, 2001; Broderick *et al.*, 2004; Delalibera *et al.*, 2007; Rani *et al.*, 2009). Representatives from the respective groups are selected and streaked onto new agar plates to obtain individual, pure colonies (Butler *et al.*,

2010; Broderick *et al.*, 2004; Lindh *et al.*, 2005). Isolates are then screened for additional differences by using Gram staining and standard biochemical tests (Conn and Pelczar, 1957; Schultz and Breznak, 1978; Davidson *et al.*, 2000; Zurek *et al.*, 2000). Through culture based techniques, isolates can be preserved until further analysis (Al-Awandhi *et al.*, 2013). It can be stored at different glycerol concentrations at -80 °C or -70 °C (Davidson *et al.*, 2000; Arias-Cordero *et al.*, 2012; Gupta *et al.*, 2012).

Based on morphological and physiological characteristics some bacterial species are indistinguishable (Bauer *et al.*, 2000; De Vries *et al.*, 2001). Therefore, alternative techniques are necessary to identify these microorganisms. Molecular techniques target the 16S gene present in all prokaryotes (Reeson *et al.*, 2003). 16S rRNA genes are used to identify and classify microorganisms through various molecular techniques (Xiang *et al.*, 2006).

Polymerase chain reaction (PCR) of 16S RNA genes, followed by sequencing is a well-known approach used to identify bacteria. Numerous authors have reported using this technique to study the microbiota associated with insects (Bauer *et al.*, 2000; Nayduch *et al.*, 2001; De Vries *et al.*, 2001; Broderick e*t al.*, 2004; Lindh *et al.*, 2005; Gusmão *et al.*, 2007; Rani *et al.*, 2009; Arias-Cordero *et al.*, 2012; Gupta *et al.*, 2012). Through PCR segments of the 16S rRNA gene or rDNA are amplified (Øvreas, 2000). After amplification PCR products are sequenced.

PCR-based techniques are used to investigate microbial communities present in the guts of several insect species belonging to Lepidoptera (Broderick e*t al.*, 2004; Brinkmann *et al.*, 2008; Allen *et al.*, 2009), Diptera (Lindh *et al.*, 2005; Corby-Harris *et al.*, 2007; Gusmão *et al.*, 2007; Rani *et al.*, 2009; Butler *et al.*, 2010; Gupta *et al.*, 2012), Coleoptera (Egert *et al.*, 2003; Delalibera *et al.*, 2007; Arias-Cordero *et al.*, 2012), Hymenoptera (Mohr and Tebbe, 2006), Thysanoptera (De Vries *et al.*, 2001), Isoptera (Berchtold and König, 1996) and Orthoptera (Dillon *et al.*, 2010).

With culture independent methods researchers are able to identify microorganisms that are not culturable under standard laboratory conditions (Handelsman, 2007). These techniques such as metagenomics provide new information on the structure and functions of microbial communities in various environments. Metagenomics involves direct isolation of DNA from environmental samples in which a complex pool of genomes is obtained from different organisms (Handelsman, 2004; Handelsman, 2007). Marker sequences such as the 16S rRNA gene sequences are amplified and then directly processed by next generation sequencing methods (Adams *et al.*, 2009; Powell *et al.*, 2014). This method is more sensitive, provides an enormous amount of data but is also more costly (Thomas *et al.*, 2012).

Culture-dependent methods should not be excluded when community structures are analysed. These methods are important in understanding the characteristics and properties of the isolated bacterial groups (Pontes *et al*., 2007). Gene sequences obtained from direct DNA extraction and cultivated bacteria of the same environmental sample are rarely identical (Lindh *et al*., 2005; Donachie *et al*., 2007; Pontes *et al*., 2007). Thus, to understand the full extent of the diversity within a microbial community, both culturing and molecular techniques should be applied (Donachie *et al*., 2007).

2.8. Approaches to evaluate microbial community structure

A better understanding of diversity within a community can be obtained by analysing the species richness and the relative abundance of the occurring microbes (Rani *et al*., 2009). Species richness refers to the number of species present within a community, but gives no information on the number of individuals represented in each species (evenness). Species within natural communities are never even (Studeny *et al*., 2011), therefore it is important to consider that some species are more prevalent than others before the community structure can be analysed. When richness and evenness of species are combined, the diversity is determined, which provides more insight into the community structure (Begon *et al*., 2006). Different diversity indices are available to calculate community diversity such as the Berger-Parker index, Fisher's alpha index, Simpson index and the Shannon index (Magurran, 2005; Dickson, 2009; Studeny *et al*., 2011).

The Shannon diversity index is one of the most widely used indices for analysing community structures (Studeny *et al*., 2011). Several studies made use of this index to determine community structures within insects such as the fruit fly (*D. melanogaster*; Cox and Gilmore, 2007), European cockchafer (*M. melolontha*; Egert *et al*., 2005), bollworm (*H. armigera*; Xiang *et al*., 2006), higher termites (*Nasutitermes* spp; Köhler *et al*., 2012), ground beetle, *Poecilus chalcites* (Coleoptera: Carabidae) (Lehman *et al*., 2009) and the common house fly, *Musca domestica* (Diptera: Muscidae) (Gupta *et al*., 2012). Studies such as Xiang *et al.* (2006), Rani *et al.* (2009) and Zouache *et al.* (2011) that were concerned with diversity differences in gut microbes from insects collected at different sites, made use of the Shannon diversity index.

Phylogenetics is the estimation of evolutionary descent of different species or genes from a common ancestor (Baldauf, 2003; Soltis and Soltis, 2003). In molecular phylogenetics a comparison is made between DNA or protein sequences from several species. By means of this approach the affiliation of species (or genes) to one another can be determined.

Phylogenetic trees depict these relationships and provide insight to speciation and gene duplication events (Baldauf, 2003). This is an effective and widely used approach for interpreting molecular data concerning biological diversity, construction of classifications, insight to evolution events as well as the origin, evolution and possible functions of genes and the proteins they encode (Baldauf, 2003).

A phylogenetic tree consists of branches and nodes. Branches are connected through nodes, thus a node signifies the point at which branches divide. Branches that originate from a mutual node are collectively called a clade. However, sequential clades may originate from each respective branch in the same continual manner. The accuracy of these groupings is supported by a bootstrap value. A bootstrap value is the proportion of replicates from which a clade is assembled. Generally, a value of 70 % and higher is considered as reliable groupings (Baldauf, 2003; Soltis and Soltis, 2003).

Phylogenetic analysis has been applied in several studies concerning the relation between insects and their gut microbes as well as the possible functions they may have. These include studies involving insect orders such as, Diptera (Corby-Harris *et al*., 2007; Rani *et al.,* 2009), Isoptera (Brennan *et al*., 2004; Hongoh *et al*., 2006), Homoptera (Fukatsu and Nikoh, 2000), Hymenoptera (Mohr and Tebbe, 2006), Coleoptera (Munteanu *et al*., 2014) and Lepidoptera (Brennan *et al*., 2004; Díaz-Mendoza *et al*., 2005; Priya *et al*., 2012).

CHAPTER 3
MATERIALS AND METHODS

3.1. Sample collection

Busseola fusca (Lepidoptera: Noctuidae) larvae were collected from 30 different geographical separate sites situated in the Eastern and Western maize production areas of South Africa. Larvae were collected from Bt and non Bt-maize plants within the same season (14 February - 22 March 2013). The coordinates for the various sampling sites were noterised. The location of each site is indicated in Figure 3.1. At the respective sites, maize plants with symptoms of stem borer damage were cut open in order to collect the larvae. The collected larvae (still in the stems) were kept cool until dissection in the laboratory. This was done within 48 hours of collection.

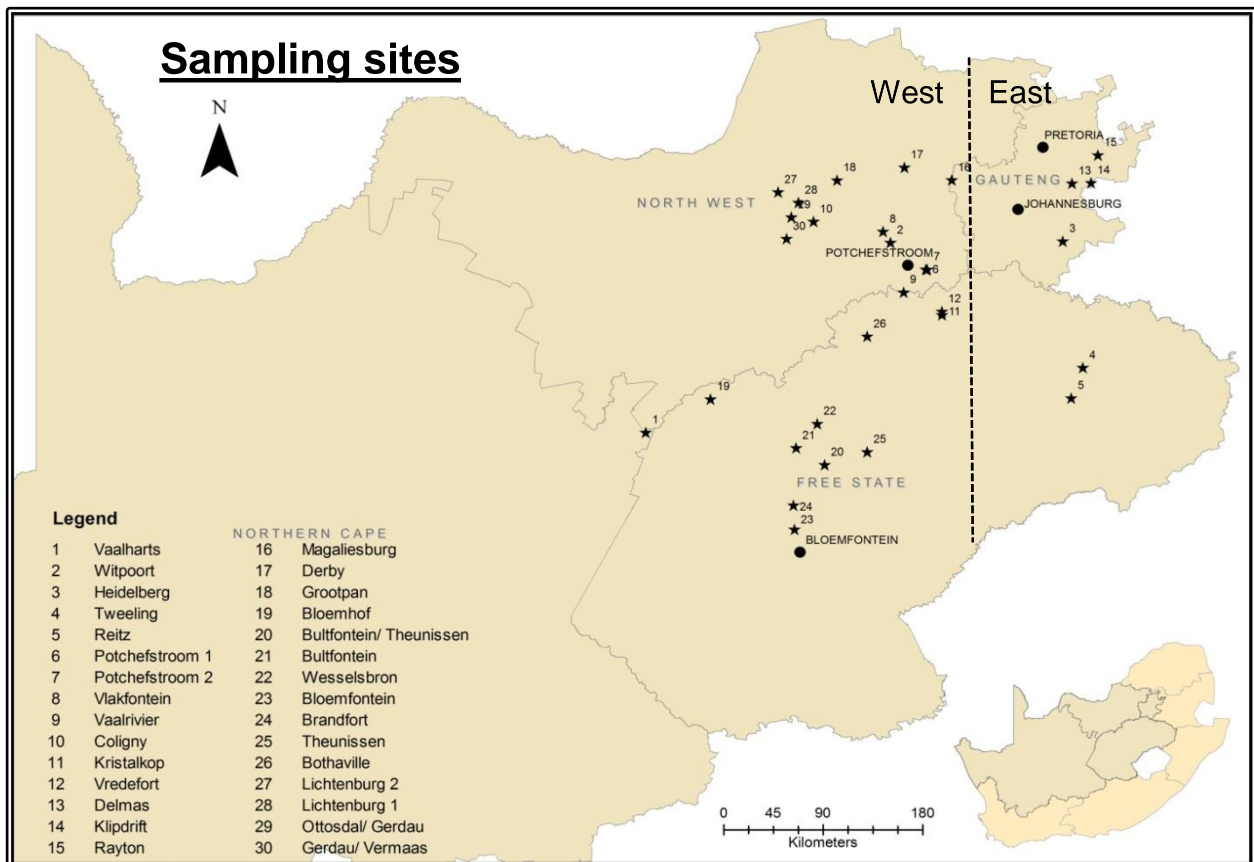

Figure 3.1: Sampling points at which *Busseola fusca* larvae were collected in South Africa.

3.2. Sample preparation

The collected larvae were taken to the North-West University were it was dissected in order to remove the midguts. Prior to dissection, the larvae were placed in 70 % ethanol (Schloss *et al.*, 2006). This was done to sterilise the outer surface of the larvae in order to avoid contamination by bacteria that may have colonised this surface. Dissections were conducted under aseptic conditions and the complete gut was removed (Figure 3.2 A-D). The larvae were pinned down with the dorsal side facing upwards (Peyronnet *et al.*, 1997; Figure 3.2 A). By using fine microdissection scissors the body walls were cut open from the last abdominal segment to the first thoracic segment (Figure 3.2 B).

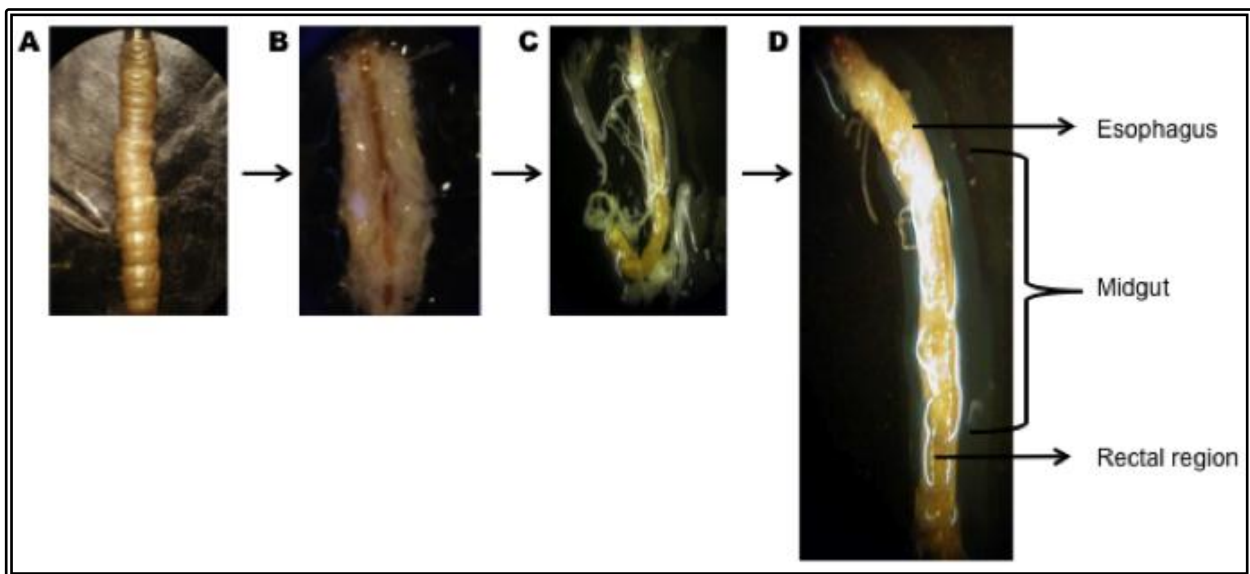

Figure 3.2 A-D: Aseptic dissections of *Busseola fusca* larvae.

Three midguts of larvae from the same location were pooled in one 1.5 ml sterile microfuge tube that contained 1 ml of distilled water. It was then sonicated with a PS-40 Ultrasonic cleaner (Jeken, Guangdong, China) for two minutes to disrupt the gut material in order to release the bacteria from the midgut (Broderick *et al.*, 2004; Robinson *et al.*, 2010; Priya *et al.*, 2012). Samples were then centrifuged for 15 seconds at 12 800 rpm in a MiniSpin microfuge (Eppendorf) to separate the bacteria from the gut material. Through centrifugation the heavier gut material sinks to the bottom, while the bacteria remain in a suspended state. The supernatants were used for immediate analysis.

3.3. Isolation of bacteria

The obtained supernatant contained the bacteria and was processed as follows. A 100 µl were used to make a dilution series of up to 10^{-5}. The rest of the supernatant was frozen at -80 ºC for later use. Each of the respective dilutions, as well as the stock solution, was spread-plated onto nutrient agar (Figure 3.3) (Lacey *et al.*, 2007; Tagliavia *et al.*, 2014; Özkan-Çakici *et al.*, 2014). Thus, for each locality there were six spread plates, each with a different concentration.

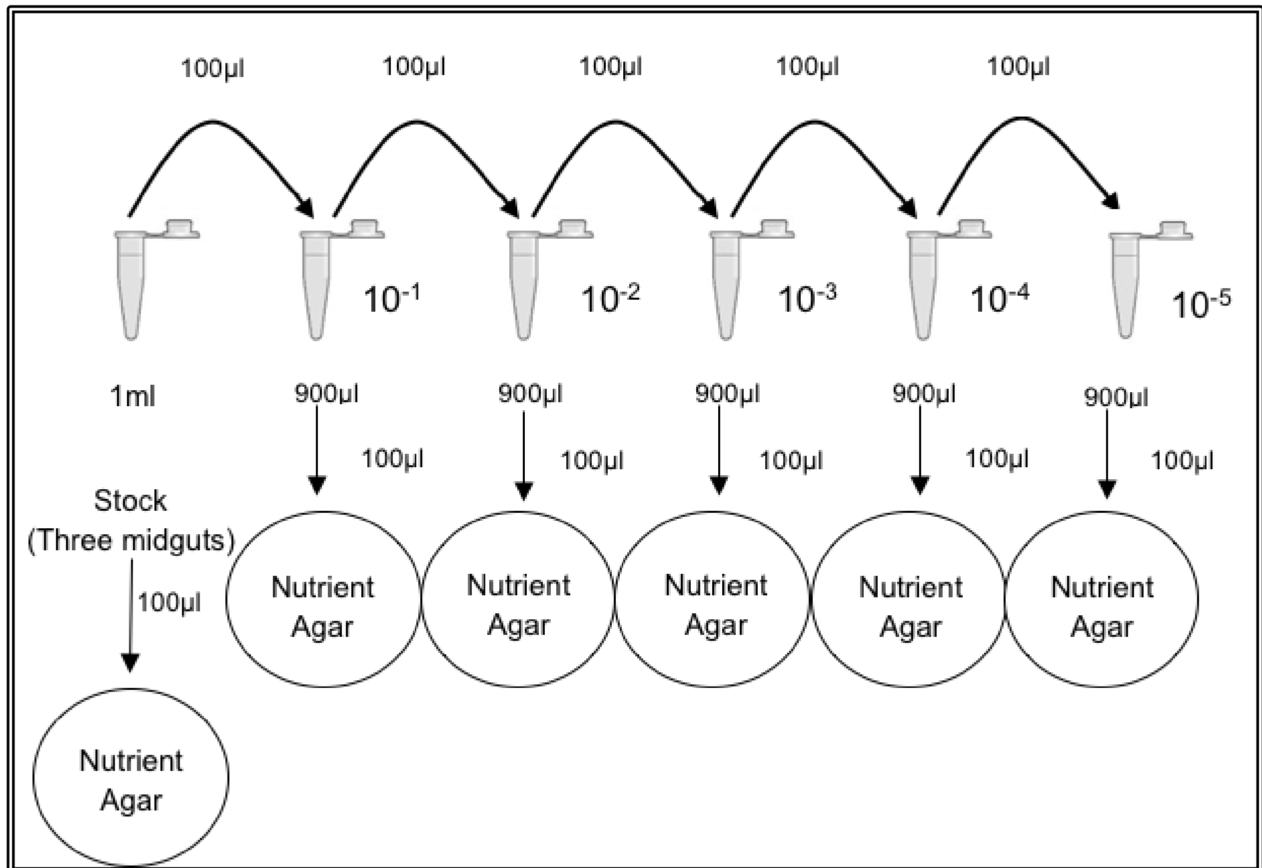

Figure 3.3: Illustration of the dilution series made from the supernatant.

Spread plates were incubated at 37 °C (Cox and Gilmore, 2007; Rani *et al.*, 2009). After a 24-hour incubation period the total number of colonies was counted. Various morphological traits of the colonies were recorded and the number of colonies with similar traits was counted. Morphotypes were identified based on four characteristics namely, the surface appearance and shape of the colony (Figure 3.4 A), the elevation of the colonies (Figure 3.4 B), the shape of the colonies edges (Figure 3.4 A) and the colour of the colonies (Figure 3.4 B) (Broderick *et al.*, 2004; Van der Hoeven *et al.*, 2008; Priya *et al.*, 2012). Based on these traits different morphotypes were identified. One of each morphotype was collected and streaked onto nutrient agar to obtain pure cultures. After several repetitions, Gram staining was conducted to

verify that the cultures were pure. Through Gram staining the bacteria were also divided into Gram-positive and Gram-negative bacteria.

A

Figure 3.4 A: Illustrates differences in the surface appearance and edges of the colonies. Both colonies a and b has a smooth surface appearance and colony c has a granular appearance. Colony a has round edges, while colonies b and c is considered irregular.

B

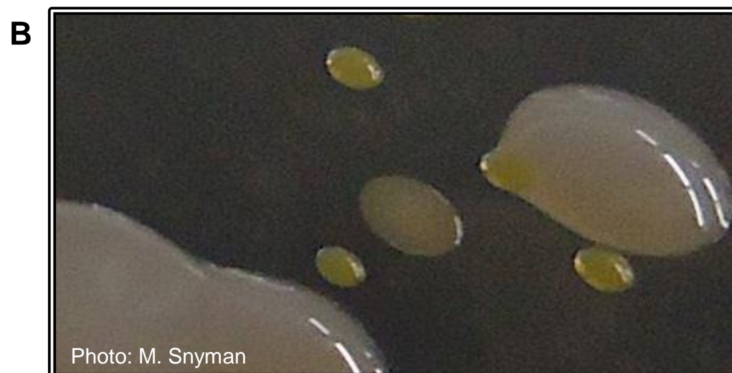

Figure 3.4 B: Elevation of the colonies and colour variances are demonstrated in the figure.

3.4. Gram stain

Gram staining is performed to distinguish between Gram-positive and Gram-negative bacteria. This procedure consists of four steps: (1) Primary stain, (2) mordant, (3) decolourising and (4) a counterstain (Bartholomew and Mittwer, 1952).

A drop of distilled water was first placed onto a sterile slide. The bacterial colony was transferred into the drop by using a sterilised inoculation loop. The suspended culture was air-dried followed by fixating the slide over a Bunsen burner for three seconds. The culture was primarily stained with crystal violet for one minute and rinsed off with water. Iodine was used as a mordant and was applied for one minute, after which the slide was decolourised with an acetone (30 %) - alcohol (70 %) mixture and rinsed with water to stop the decolourisation. Safranin was used as a counterstain and applied for one minute and rinsed thoroughly with water. Slides were left to dry before cultures were examined under a microscope. Gram-negative bacteria had a red / pink appearance whereas Gram-positive bacteria appeared purple (Sutton, 2006; Wiley *et al*., 2008).

3.5. DNA isolation

The pure isolates were grown in nutrient broth at 37 °C overnight prior to DNA extraction. DNA was isolated according to the method used by Towner *et al.* (1998). The method was modified as follows: 30 µl of PCR-grade water were added to 20 µl of pure culture and microwaved for two minutes. The tubes were immediately put on ice for two minutes and subsequently centrifuged for 90 seconds at 13 400 rpm. The tubes were put back onto ice and 1 µl of the supernatant was used for DNA amplification (Carstens *et al*., 2014).

3.6. DNA amplification

The 16S rDNA fragments were amplified by Polymerase Chain Reaction (PCR). The total volume of the reaction mixture was 25 µl, which consisted of 12.5 µl Master Mix [(0.05 U/µl *Taq* DNA Polymerase in reaction buffer, 0.4 mM of each dNTP, 4 mM $MgCl_2$) (Fermentas Life Science, US)], 9.5 µl nuclease free water (Fermentas Life Science, US), 1 µl of forward primer (27F), 1 µl of reverse primer (1492R; Lane, 1991; Allen *et al*., 2009) and 1 µl of DNA. Both the 27F and the 1492R primers have an amplification length of 1465 bp, which nearly amplifies the entire length of the gene (Frank *et al*., 2008). The 27F primer targets the 16S rRNA region (5'-AGAGTTTGATCMTGGCTCAG-3') and the 1492R primer targets the 16S rRNA region (5'-TACGGYTACCTTGTTACGACTT-3'; Lane, 1991). A C1000 Thermal Cycler (Bio-Rad, UK) was used to perform PCR under the following conditions: an initial step of 95 ºC for five minutes was followed by 35 cycles consisting of denaturing at 95 ºC for 30 seconds annealing at, 53 ºC for 30 seconds and extension at 72 ºC for one minute. This was then followed by a final extension of 72 ºC for ten minutes after which the reactions were briefly held at 12 ºC, removed from the thermocycler and stored at 4 ºC until further analysis could be conducted.

3.7. Agarose gel electrophoresis

The DNA quality was determined with agarose (WhiteSci, USA) gel electrophoresis (Bio-Rad, UK). Agarose electrophoresis was also used to determine if PCR reactions were successful. In both applications electrophoresis was for 45 minutes at 80 V using a 1 % w/v agarose gel. A 1 x TAE solution [40 mM Tris (Sigma Aldrich, US), 20 mM acetic acid, glacial (Merck,US) and 1 mM EDTA (Merck,US), pH 8.0] was used to prepare the gel as well as electrophoresis buffer. Three microliters of the DNA sample were mixed with 3 µl of 6 x Orange Loading Dye (Fermentas Life Science, US) containing GelRed and loaded into a well. For the genomic DNA application a 1 Kb and to determine the success of PCR amplifications a 100 bp molecular weight marker (O'GeneRuler, Fermentas Life Science, US) was loaded onto each gel. A horizontal Power Pac (UK) gel electrophoresis system (BioRad, US) was used to resolve the DNA and a ChemiDoc™ MP Imaging System (Bio-Rad, US) with Image Lab™ software (Version 4.0.1) was used to capture the gel images.

3.8. First clean-up

The first clean-up of the PCR products was done by using a PCR Cleanup Kit (Macherey-Nagel, Germany). This was done according to the manufacturer's instructions. Afterwards the DNA concentration (ng/µl) and quality ($A_{260\ nm}/A_{280\ nm}$) were determined by using a NanoDrop 1000 Spectrophotometer (Thermo Fischer Scientific, US). The products were stored at 4 ºC until further analysis could be conducted.

3.9. Sequencing

Products of the first clean-up were sequenced by the DNA Sequencing Facility of Stellenbosch University (South Africa) and Inqaba Biotech (South Africa) using the 27F primer. Chromatograms were viewed with Geospiza Finch TV (Version 1.4) software. Basic Local Alignment Search Tool (BLAST) searches were performed to compare the obtained sequences to those in the GenBank database, by using the National Centre for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/BLAST) website. The BLAST program used during this study was BLASTn 2.2.29+ (Altschul *et al.*, 1997).

3.10. Statistical and data analyses

In this study the bacterial diversity of the isolates were determined by using the Shannon diversity index, *H*.

$$H = -\sum_{i=1}^{S} P_i \, ln \, P_i$$

With *S* as the species richness and Pi as the proportion of species (Begon *et al.*, 2006). The number of different morphology groups present at a site, represents species richness. The abundance of colonies within these groups represents the P_i values. In the present study the average colonies that formed at the 10^{-1}-10^{-5} dilutions were determined and used as the P_i value. Shannon's diversity indices were calculated at each respective site, and used for further statistical analyses.

Parametric and non-parametric statistical analyses were performed on the data sets (Shannon diversity values) obtained by using STATISTICA 12 (StatSoft Inc ©., Tulsa, Oklahoma, USA, 2013). For parametric data one-way ANOVA was performed followed by the Tukey's Honest Significant Difference (HSD) test to determine statistical significant differences between the various diversity indices ($p < 0.05$).

CANOCO (Canoco for Windows Version 4.5, Biometris-Plant Research International, Wageningen, The Netherlands) was used to preform multivariate ordination analyses to determine differences in microbial structures (Ter Braak, 1994; Ter Braak and Verdonschot, 1995). A Canonical Correspondence Analysis (CCA) was performed using the species distribution data (Appendix 4) and the direct distances between the sampling sites (Appendix 5). Microsoft Windows Excel 2010 was used to calculate Shannon diversity values and to compose species composition and distribution figures.

Phylogenetic analysis was performed to determine the evolutionary history of species using the Neighbor-Joining method (Saitou and Nei, 1987). Accuracy of the groupings was determined by using the bootstrap test (1000 replicates; Felsenstein, 1985). Evolutionary distances were calculated using the Jukes-Cantor method (Jukes and Cantor, 1969). The evolutionary analyses were conducted in MEGA6 (Tamura *et al.*, 2013).

CHAPTER 4
RESULTS

4.1. Bacterial morphotypes

Busseola fusca (Lepidoptera: Noctuidae) larvae were collected from 30 geographically separated sites in the maize producing region of South Africa. The sites are located in four provinces namely, Free State, Gauteng, North-West Province and the Northern Cape. Conversations with farmers gave more insight on whether the larvae were collected from Bt or non-Bt maize (refuge area). Collected larvae were taken to the North-West University to remove and identify the midgut contents. During transportation the larvae were kept cool in the stems from which they were collected. Dissections were done within 48 hours after collection.

Bacteria were isolated using the procedures described in Section 3.3. This entailed making a serial dilution of the midgut contents and spread-plating it onto nutrient agar, then after incubation at 37 °C (Rani *et al.*, 2009), determining the total number of colonies as well as the morphologically different types. The morphological characteristics that were taken into account included texture, shape, colour and elevation and was done according to De Vries *et al.* (2001), Broderick *et al.* (2004), Delalibera *et al.* (2007), Rani *et al.* (2009) and Priya *et al.* (2012). A total of 135 morphotypes were identified. Morphotypes and their respective characteristics are listed in Appendix 1. The number of colony forming units (CFUs) in the respective morphology groups was recorded for each site. Composition as well as the CFUs of morphotypes differed at each of the sites. The variations of morphotypes as observed in the midgut contents of *B. fusca* larvae obtained from the various sampling sites are depicted in Figure 4.1. These variations are represented as the relative percentage that each morphotype comprises of the total number of morphotypes at each site.

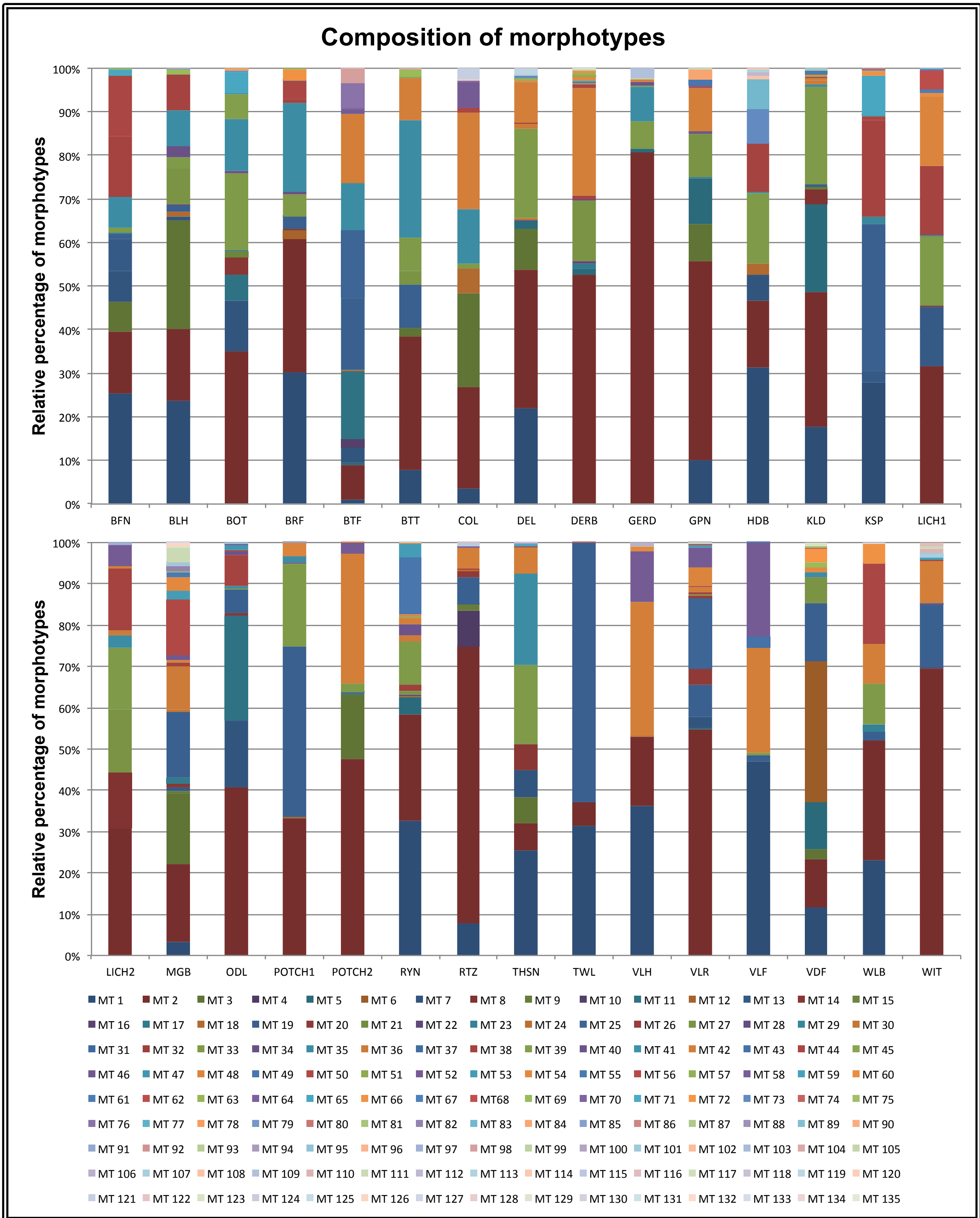

Figure 4.1: The various morphotypes that occurred at each site respectively. Morphotype 2 (MT2) was mostly present in high percentages and occurred at 28 of the 30 sites, while several other morphotypes were isolated in low numbers only at one site such as MT21, MT24, MT26, MT43 and MT50.

The composition of morphotypes occurring at the respective sites is complex, which makes it difficult to identify associations between the morphotypes and sampling sites. However, several patterns can be seen from Figure 4.1 Morphotype 2 occurred at all of the sampling sites, except at Kristalkop (KSP) and Vlakfontein (VLF). Both morphotypes 33 and 42 were present at 21 of the 30 sites. At Magaliesburg (MGB), 28 different morphotypes were identified, while only four occurred at Tweeling (TWL). None of the morphotypes were present at all of the sampling sites.

Information regarding the sampling sites is presented in Table 4.1.Twelve sites were situated in the Free State, 12 in the North-West, five in Gauteng and one in the Northern Cape. These four provinces form the maize producing region in South Africa. Twenty six of the maize fields at which *B. fusca* larvae were collected were dry land. The four sampling sites with irrigation were in the Free State (two), Gauteng (one) and the Northern-Cape (one). Shannon diversity indices for these sites were below two in all four cases. However, the results presented in this study show that irrigation had no influence on the midgut content of *B. fusca* larvae.

Shannon diversity indices (*H*) were determined for each sampling site as described in Section 3.10; these values are presented in Table 4.1 Magaliesburg, located in Gauteng had the highest diversity value (2.359) and Gerdau in the North-West province the lowest value (0.570). In the Free State, Bloemfontein showed the highest diversity (2.125) and Tweeling the lowest (0.929). Coligny (1.911) had the highest diversity and Gerdau the lowest in the North-West. None of the sampling sites in the North-West province had a Shannon diversity value above two. In Gauteng, Klipdrift had the lowest diversity value (1.461) and Magaliesburg the highest. Vaalharts was the only sampling point in the Northern Cape and had one of the lowest values (1.401).

Several other studies made use of the Shannon diversity index to compare bacterial diversities associated with insects (Xiang *et al*., 2006; Rani *et al*., 2009; Zouache *et al*., 2011; Arias-Cordero *et al*., 2012). Xiang and co-workers (2006) reported average Shannon values of 2.61 for field-collected and 1.47 for lab-reared *Helicoverpa armigera* (Lepidoptera: Noctuidae) larvae. Possible reasons for diversity differences includes environmental factors (Xiang *et al*., 2006; Rani *et al*., 2009; Zouache *et al*., 2011) and diet alterations (Broderick *et al*., 2004; Robinson *et al*., 2010; Belda *et al*., 2011; Priya *et al*., 2012).

Table 4.1: Details regarding the various sampling sites as well as the Shannon diversity index calculated at each site.

Province (Eastern or Western production area)	Site Name	Site Number	Site Abbreviation	Bt/Non-Bt	Irrigation	Shannon diversity Index
Free State (W)	Bloemfontein	23	BFN	NA	X	2.12544
Free State (W)	Bloemhof	19	BLH	Bt + Non-Bt	✓	1.99442
Free State (W)	Bothaville	26	BOT	Non-Bt	X	1.94905
Free State (W)	Brandfort	24	BRF	NA	X	1.66463
Free State (W)	Bultfontein	21	BTF	Bt	X	2.07633
Free State (W)	Bultfontein/Theunisen	20	BTT	Bt + Non-Bt	X	1.74725
Free State (W)	Kristalkop	11	KSP	NA	X	1.2527
Free State (E)	Reitz	5	RTZ	NA	X	1.20419
Free State (W)	Theunisen	25	THN	Bt	X	1.99249
Free State (E)	Tweeling	4	TWL	NA	X	0.92853
Free State (W)	Vredefort	12	VRF	Bt	X	1.94557
Free State (W)	Wesselsbron	22	WSB	Bt	✓	1.67869
North-West (W)	Coligny	10	COL	Non-Bt	X	1.91138
North-West (W)	Derby	17	DERB	Bt	X	0.84756
North-West (W)	Gerdau/Vermaas	30	GERD	NA	X	0.57047
North-West (W)	Grootpan	18	GPN	Bt	X	1.40567
North-West (W)	Lichtenburg	28	LICH1	NA	X	1.63497
North-West (W)	Lichtenburg (Mafikeng Road)	27	LICH2	Bt	X	1.66979
North-West (W)	Ottosdal\Gerdau	29	ODL	NA	X	1.53799
North-West (W)	Potchefstroom1	6	POTCH1	NA	X	1.31709
North-West (W)	Potchefstroom2	7	POTCH2	NA	X	1.25119
North-West (W)	Vaalrivier	9	VLR	NA	X	1.01312
North-West (W)	Vlakfontein	8	VLF	NA	X	1.32673
North-West (W)	Witpoort	2	WIT	NA	X	1.04961
Gauteng (E)	Delmas	13	DEL	Bt + Non-Bt	✓	1.49784
Gauteng (E)	Heidelberg	3	HDB	NA	X	2.06896
Gauteng (E)	Klipdrift	14	KPD	NA	X	1.46109
Gauteng (W)	Magaliesburg	16	MGB	NA	X	2.35952
Gauteng (E)	Rayton	15	RYN	NA	X	1.76515
Northern Cape (W)	Vaalharts	1	VLH	Bt	✓	1.40145

Shannon diversity index data were statistically analysed using STATISTICA 12 (StatSoft Inc ©, 2013). This data were compared to several factors regarding the sampling site such as the type of production system (irrigation or dry land), position of the site (Eastern or Western part of production area) and whether the larvae were collected from Bt or non-Bt maize. The mean Shannon diversity values of the different sites were compared to each factor respectively, in order to determine whether it might have influenced the microbial diversity. The results in Table 4.2 show no statistically significiant (p = 0.05) differences between either of these factors. The study was not designed to identify factors that might influence the microbial community thus, sampling sites were chosen at random. However, from the data presented in Table 4.2 is appears that the occurrence and distribution of bacterial morphotypes are not influenced by irrigation, locality or the type of maize from which larvae were collected.

Table 4.2: Comparison between the mean Shannon diversity values and the type of maize, production area and the type of production system, respectively (Means ± standard error, superscript characters denote statistical significant differences, p = 0.05, Tukey's Unequal HSD).

Comparison	Shannon value (Mean ± Standard error)
Type of maize	
Bt	1.627 ± 0.143 [a]
non-Bt	1.930 ± 0.018 [a]
Production area	
West	1.579 ± 0.090 [a]
East	1.488 ± 0.164 [a]
Type of production system	
Irrigation	1.643 ± 0.130 [a]
Dry land	1.716 ± 0.109 [a]

4.2. Identification of bacterial morphotypes

4.2.1. Gram staining and DNA isolation

Among the morphotypes 57 % were Gram-positive and the rest were Gram-negative (43 %). The pure cultures representing the various morphotypes were subjected to DNA isolation, PCR amplification of the 16S rRNA gene fragments, sequencing of the amplicons followed by identification through BLASTn searches to determine the identity. Genomic DNA was successfully isolated from the 135 pure isolates by using the method described in Section 3.5. Figure 4.2 represents a 1 % (w/v) agarose gel stained with GelRed, which indicates the quantity and quality of the isolated DNA.

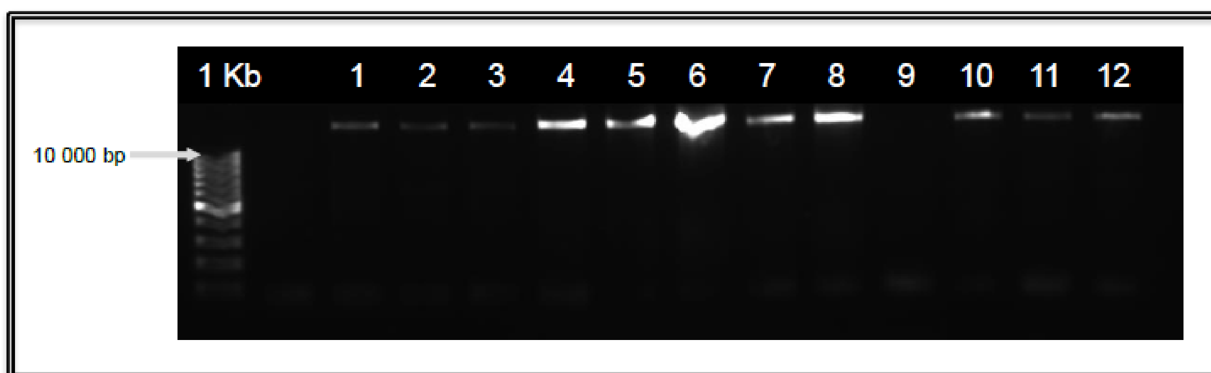

Figure 4.2: Agarose gel image of isolated DNA from pure cultures of the selected morphotypes. A 1 Kb molecular weight marker (O'GeneRuler, Fermentas Life Science, US) was loaded into lane 1.

The results in Figure 4.2 indicate that sufficient DNA was isolated for PCR amplification and that the DNA was not fragmented. These results were confirmed by determining the A_{260nm}/A_{280nm} ratio and DNA concentrations by using a NanoDropTM 1000 Spectrophotometer (Section 3.8). A ratio above 1.9 and below 1.7 indicates RNA or protein contamination, respectively. Thus, a ratio of 1.8 represents good DNA quality (Santella, 2006). The A_{260nm}/A_{280nm} ratios varied between 1.44 and 2.30, and had an average of 1.79. This also indicated that DNA was of good quality and suitable for PCR. The average DNA concentration was 38.5 ng/µl, which ranged from 4.99 ng/µl to 211.56 ng/µl and was also sufficient for PCR amplification.

4.2.2. DNA amplification

Isolated bacterial genomic DNA was amplified by using the PCR conditions described in Section 3.6. The amplification products were of the expected size of approximately 1500 bp (Figure 4.3).

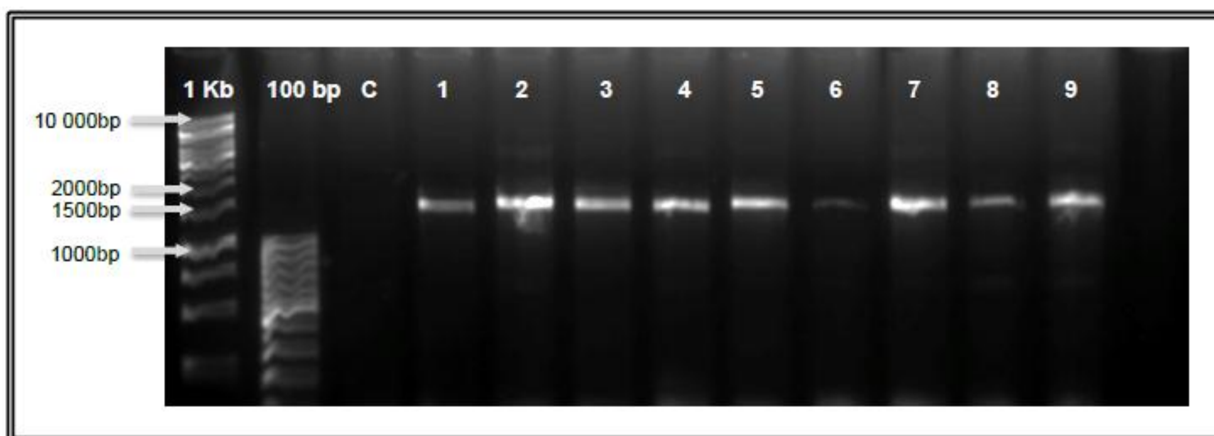

Figure 4.3: Image of a 1 % (w/v) agarose gel illustrating the amplified products of nine pure cultures (lanes 1 to 9) from the selected morphotypes. The first two lanes contain the 1 Kb and 100 bp molecular weight markers (O'GeneRuler, Fermentas Life Science, US), respectively, and lane C is the no template control.

The DNA concentration was almost similar for all the samples represented in Figure 4.3, except for lanes 6 (MT99) and 8 (MT114). The concentration of lane 6 was too low for sequencing analysis and was therefore repeated. However, the product yield for lane 8 was sufficient. From Figures 4.2 and 4.3 it is also evident that the fragments were amplified without any non-specific fragments. The other gels had similar results. These fragments were purified and sequenced as described in Sections 3.8. and 3.9.

4.2.3. 16S rRNA gene sequencing analysis

Obtained sequences (Section 3.9) were edited and submitted to the BLASTn program at the NCBI website. On average, 770 nucleotides were used to compare the query sequences to those within GenBank for similarities. The sequencing results together with the accession numbers assigned to the identified bacteria are presented in Appendix 2. The assigned accession numbers range from KJ742441 – KJ742566. Three of the identified organisms were not assigned accession numbers due to the quality of the sequences. Morphotypes 6, 29, 36, 106, 111 and 129 were not successfully identified because growth stopped during the streakplate purification process, and are therefore also excluded from further analysis. High sequence similarities (≥ 95 %) were obtained for 90 % of the isolates. Apart from nine isolates, all the expected (E) values were below zero, which indicates that the identities were significant. For 15 isolates, similarities between 74 % and 94 % were obtained, thus making them potential novel species. However, further analysis is required to support this deduction.

Three phyla were identified namely Firmicutes, Actinobacteria and Proteobacteria. Isolates belonging to Firmicutes and Actinobacteria are Gram-positive and members from the phylum Proteobacteria are Gram-negative. A total of 55 species belonging to 23 genera were isolated during this study. Figure 4.4 illustrates the genera found in each of the different phyla. The most abundant phylum was Firmicutes (44 %), followed by Proteobacteria (36 %) and Actinobacteria (20 %). A similar community structure was isolated from the midgut of *H. armigera* larvae, through culturable methods (Priya *et al.*, 2012). A representation of the species isolated within each phylum is illustrated in Figures 4.5 - 4.7; and the classification of species is shown in Appendix 3. Abbreviations for species names used in Figures 4.4 - 4.7 and 4.12 are available in Appendix 2.

Members from Firmicutes, Actinobacteria and Proteobacteria are associated with numerous insect species. They have been isolated from species such as *Aedes albopictus* (Diptera: Culicidae) (Moro *et al.*, 2013), *Apis mellifera* (Hymenoptera: Apidae) (Hendriksma *et al.*, 2013), *H. armigera* (Priya *et al.*, 2012), *Melolontha hippocastani* (Coleoptera: Scarabaeidae) (Arias-Cordero *et al.*, 2012), *Culex quinquefasciatus* (Diptera: Culicidae) (Chandel *et al.*, 2013) and *Manduca sexta* (Lepidoptera: Sphingidae) (Brinkmann *et al.*, 2008).

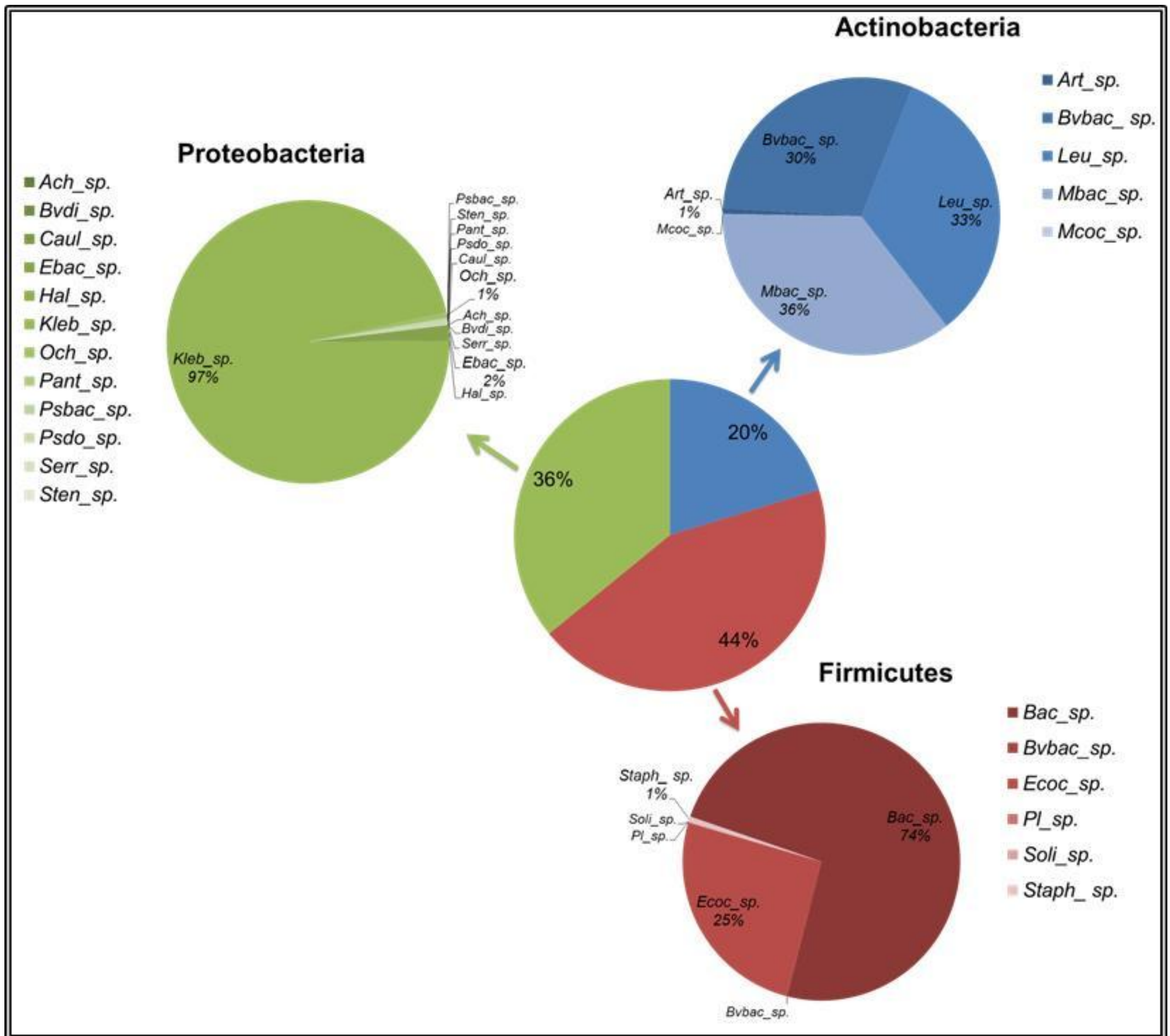

Figure 4.4: Distribution of genera within the respective phyla.

The phylum Proteobacteria is the most diverse group with isolates belonging to the class Gammaproteobacteria, Alfaproteobacteria and Betaproteobacteria. Gammaproteobacteria accounted for 99 % of the isolates within this group, while the remaining 1 % belonged to Alfaproteobacteria and Betaproteobacteria. The latter only consist of two *Achromobacter* species, while Alfaproteobacteria comprised of four species belonging to *Brevundimonas*, *Caulobacter* and *Ochrobactrum*. Isolates from Gammaproteobacteria belong to eight genera that include *Enterobacter* spp., *Halomonas* spp., *Klebsiella* spp., *Pantoea* spp., *Pseudochrobactrum* spp., *Pseudomonas* spp., *Serratia* spp. and *Stenotrophomonas* spp. From these species the most abundant were *Klebsiella pneumoniae* (70 %), *K. oxytoca* (20 %) and

K. variicolla (6 %). *Klebsiella pneumoniae* appears to be a dominant member within the midgut of *B. fusca* larvae. Figure 4.5 represents all of the species belonging to Proteobacteria.

Firmicutes represents the largest group (44 %) of isolates. This group includes 16 species belonging to six genera, with the most abundant being *Enterococcus* spp. (25 %) and *Bacillus* spp. (74 %) (Figure 4.6). Eight *Bacillus* species were identified, all in relatively low numbers except for *Bacillus thuringiensis* (65 %) and *Bacillus subtilis* (5 %). Species belonging to *Enterococcus* includes *E. gallinarum* (19 %), *E. casseliflavus* (6 %) and *E. mundtii*. Isolates from *Planomicrobium* spp., *Solibacillus* spp. and *Staphylococcus* spp. were also identified from the midgut contents. Figure 4.6 shows the ratios in which these species occurred.

In contrast to Proteobacteria and Firmicutes, the species distribution was relatively more evenly spread in Actinobacteria, and not dominated by one species. In this group the three prominent species included *Microbacterium paraoxydans* (32 %), *Brevibacterium frigoritolerans* (29 %) and *Leucobacter chromiiresistens* (21 %). Five genera with a total of 16 species were identified within this phylum (Figure 4.7). The genera includes *Arthrobacter* spp., *Brevibacterium* spp., *Leucobacter* spp., *Microbacterium* spp., and *Micrococcus* spp. Six *Leucobacter* species and five *Microbacterium* species were identified that accounted for 33 % and 36 % of the phylum, respectively. Isolates from Actinobacteria represented 20 % of the microbial community isolated from the midguts of *B. fusca* larvae (Figure 4.4). Species belonging to this phylum are illustrated in Figure 4.7 with the percentage representation also shown.

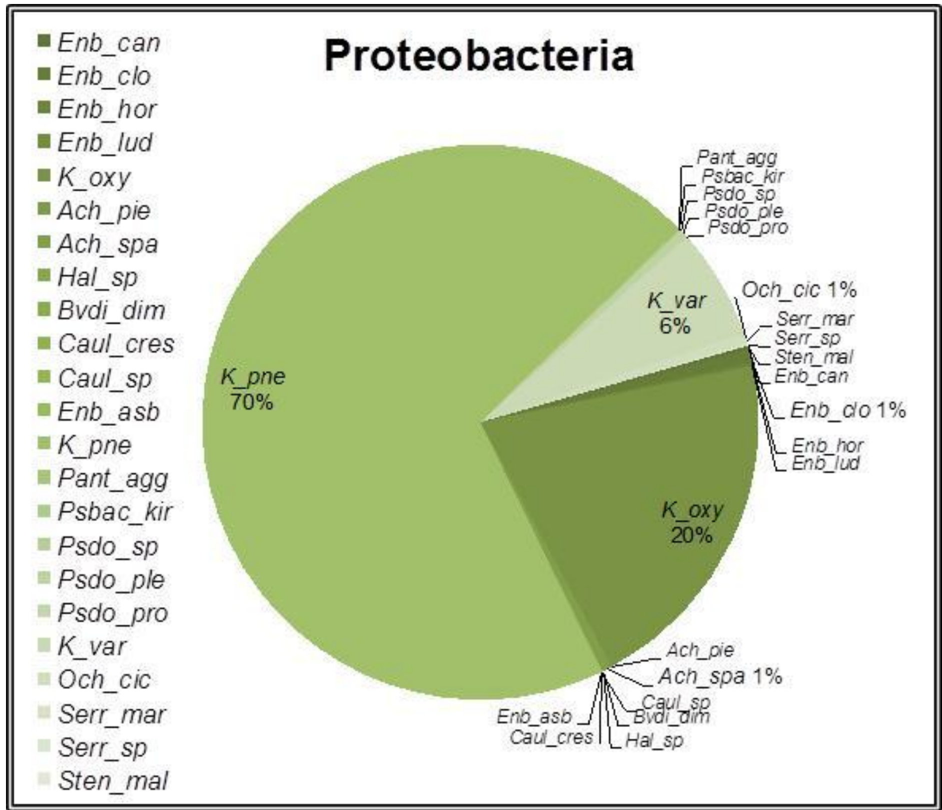

Figure 4.5: Phylum Proteobacteria with percentage representation of different species isolated from *Busseola fusca* larvae.

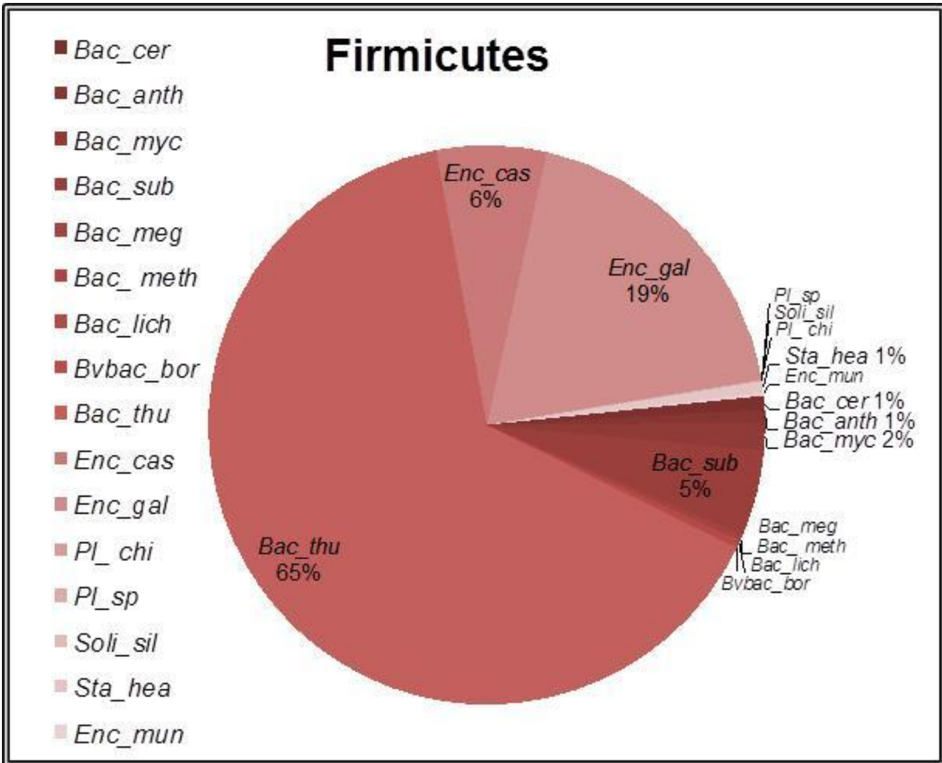

Figure 4.6: Phylum Firmicutes with percentage representation of different species isolated from *Busseola fusca* larvae.

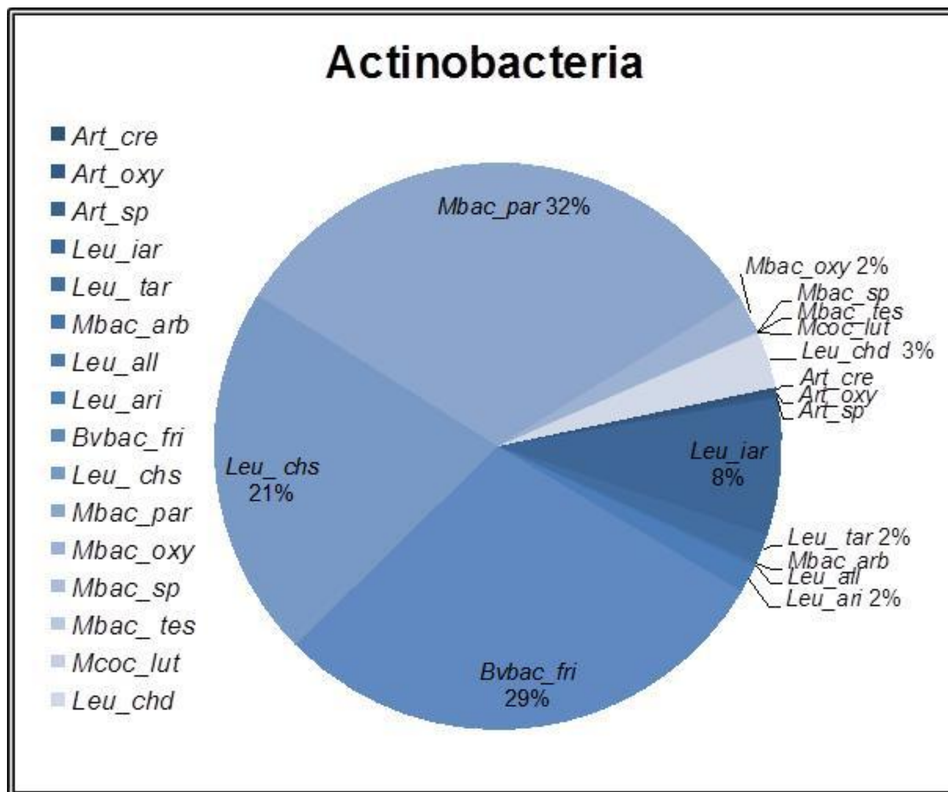

Figure 4.7: Phylum Actinobacteria with percentage representation of different species isolated from *Busseola fusca* larvae.

Overall, among the five main families in the midgut of *B. fusca*, *Enterococcaceae* and *Bacillaceae* were represented by 36 % and 32 % respectively. *Microbacteriaceae, Micrococcaceae* and *Brevibacteriaceae* made up the rest, consisting of 6 %, 15 % and 11 % respectively (Figure 4.9 B).

4.3. Phylogenetic analysis

Sequences were reviewed and carefully selected to perform phylogenetic analysis. Sequences were compared to 16S rRNA gene sequences within the GenBank database through BLASTn searches. Only those with high sequence similarities (≥ 97 %) were used for phylogenetic analysis. Sequences with similar identifications were grouped together to form a bacterial complex and assigned a specific code, which was used in the phylogenetic analyses. Table 4.3 provides the sequencing results together with the codes assigned to the respective complexes as well as the number of isolates within each complex. For example, in Table 4.3 M90 represents morphotype 90 and there were ten representatives of this species in this complex.

The dataset assembled for phylogenetic analyses contained sequences of typed strains of the different species that were retrieved from the GenBank database (http://www.ncbi.nlm.nih.gov/). By including these sequences a better understanding of the evolutionary relationships between species are given. The constructed phylogenetic tree is illustrated in Figure 4.10.

The GenBank identifications from Table 4.3 correspond to the sequencing results in Section 4.2.3. A total of 20 species belonging to 15 genera were identified. These species represent the phyla Firmicutes, Actinobacteria and Proteobacteria as well. Figure 4.8 illustrates the species distribution within these phyla; Firmicutes (49 %) represents the largest group of isolates, followed by Proteobacteria (34 %) and then Actinobacteria (17 %) with the least amount of isolates.

Table 4.3: Sequencing results and percentage of similarity (BLASTn, NCBI) as well as the assigned complex names and the number of isolates it entails.

Complex name (no. of isolates)	**GenBank ID**	**Similarity (%)**
M 100 (1)	*Brevundimonas diminuta*	100
M 89 (1)	*Caulobacter* sp.	99
M 55 (2)	*Arthrobacter scleromae*	99
M 58 (4)	*Microbacterium testaceum*	100
M 101 (6)	*Leucobacter alluvii*	100
M 50 (1)	*Leucobacter salsicius*	97
M 113 (3)	*Lactococcus lactis*	100
M 22 (13)	*Enterococcus gallinarum*	99
M 132 (1)	*Solibacillus silvestris*	99
M 110 (1)	*Planomicrobium chinense*	100
M 3 (1)	*Bacillus simplex*	99
M 95 (10)	*Bacillus thuringiensis*	99
M 73 (1)	*Staphylococcus haemolyticus*	99
M 90 (7)	*Bacillus licheniformis*	99
M 74 (1)	*Brevibacillus borstelensis*	99
M 79 (20)	*Klebsiella variicola*	99
M 115 (1)	*Pseudomonas protegens*	100
M 54 (1)	*Pseudomonas mosselii*	99
M 67 (2)	*Pseudomonas geniculata*	99
M 117 (1)	*Achromobacter marplatensis*	99

Firmicutes represents the most diverse group of isolates and comprise of nine species. In contrast to the species distribution of Firmicutes represented in Figure 4.6, *E. gallinarum* (34 %) was the most abundant species, followed by *B. thuringiensis* (26 %). Seven species are members of Proteobacteria, with *K. variicola* (74 %) as the dominant species. While *K. pneumoniae* was the dominant species from this phylum (Figure 4.5) in Section 4.2.3. Actinobacteria consist of four species belonging to three main species, *M. paraoxydans*, *B. frigoritolerans* and *L. chromiiresistens* making up 32 %, 29 % and 21 % of the identified species.

Most of the species identification in this analysis was identical to that in Section 4.2.3. There was however, some variation. This was due to the more stringent analysis that was conducted in the present section (Section 4.3). All the same genera were identified.

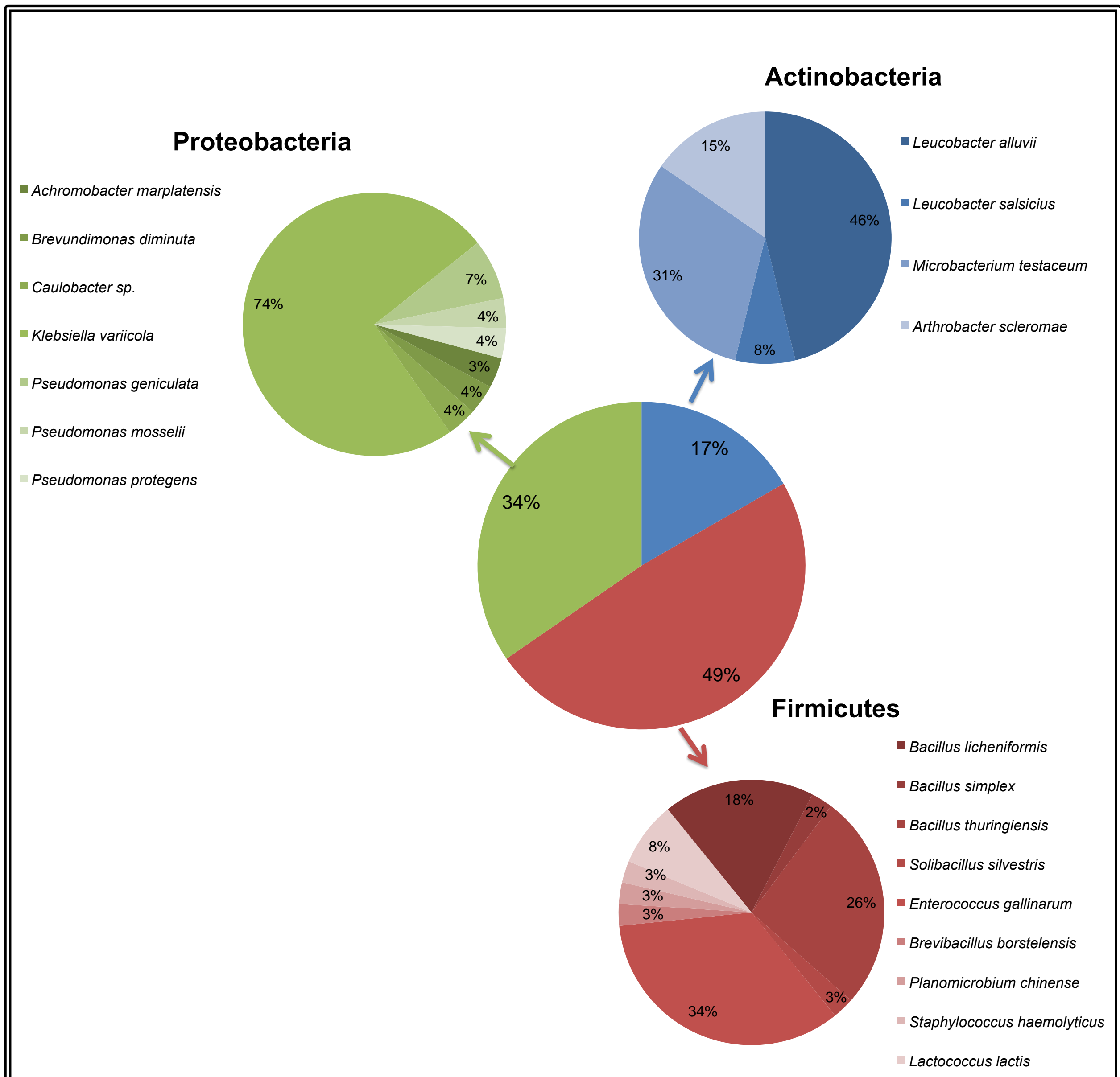

Figure 4.8: Species distribution for the isolates that were selected for phylogenetic analysis.

A total of 11 families are represented by the species selected for the phylogenetic analysis. These families are in accordance with those previously referred to in Section 4.2.3. The major bacterial families that occurred in both analyses are *Bacillaceae, Enterococcaceae, Enterobacteriaceae* and *Microbacteriaceae* (Figures 4.9 A and B). Families illustrated in Figure 4.9 B consist of all the isolated bacteria with sequence similarities ranging between 74 % and 100 %, whereas Figure 4.9 A entail species with similarities above 97 %.

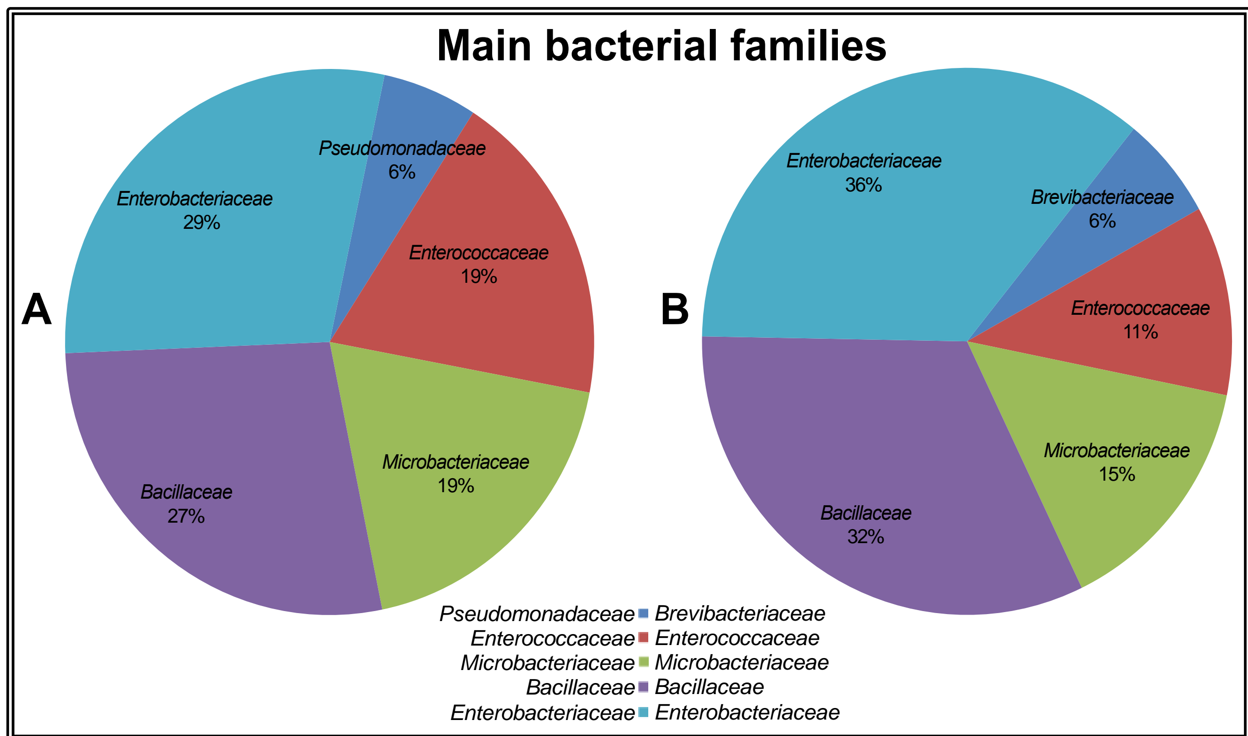

Figure 4.9 A-B: Compares the main families identified from (A) the selected sequences (≥ 97 %) and, (B) the total number of isolated sequences (≥ 74 %). The composition and structure of the major families is similar in both cases, however, inconsistency occurred in 6 % of the structure (*Brevibacteriaceae* and *Pseudomonadaceae*), which is expected since microbial communities are dynamic.

The selected sequences were further analysed and the evolutionary history inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 1.78136777 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor, 1969) and are in the units of the number of base substitutions per site. The analysis involved 85 nucleotide sequences that were selected from the total number of morphotypes. All ambiguous positions were removed for each sequence pair. There were a total of 794 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al.*, 2013). Figure 4.10 shows the evolutionary relationships of the isolates obtained from the midgut of *B. fusca*.

The 16S rRNA gene sequences of the isolated bacteria associated with three main groups namely Firmicutes, Proteobacteria and Actinobacteria. Within these groups multiple clades were observed, with supporting bootstrap values shown at the collective nodes (Figure 4.10). Species from the same genus generally group together, as a result of the similarities within their gene sequences. This is evident from Figure 4.10, as the isolated bacterial groups (Table 4.3) formed distinctive clades with species from the same genus, which was overall supported by high bootstrap values (≥ 94 %). Representative sequences for the main identified species were downloaded from GenBank and included in the analysis.

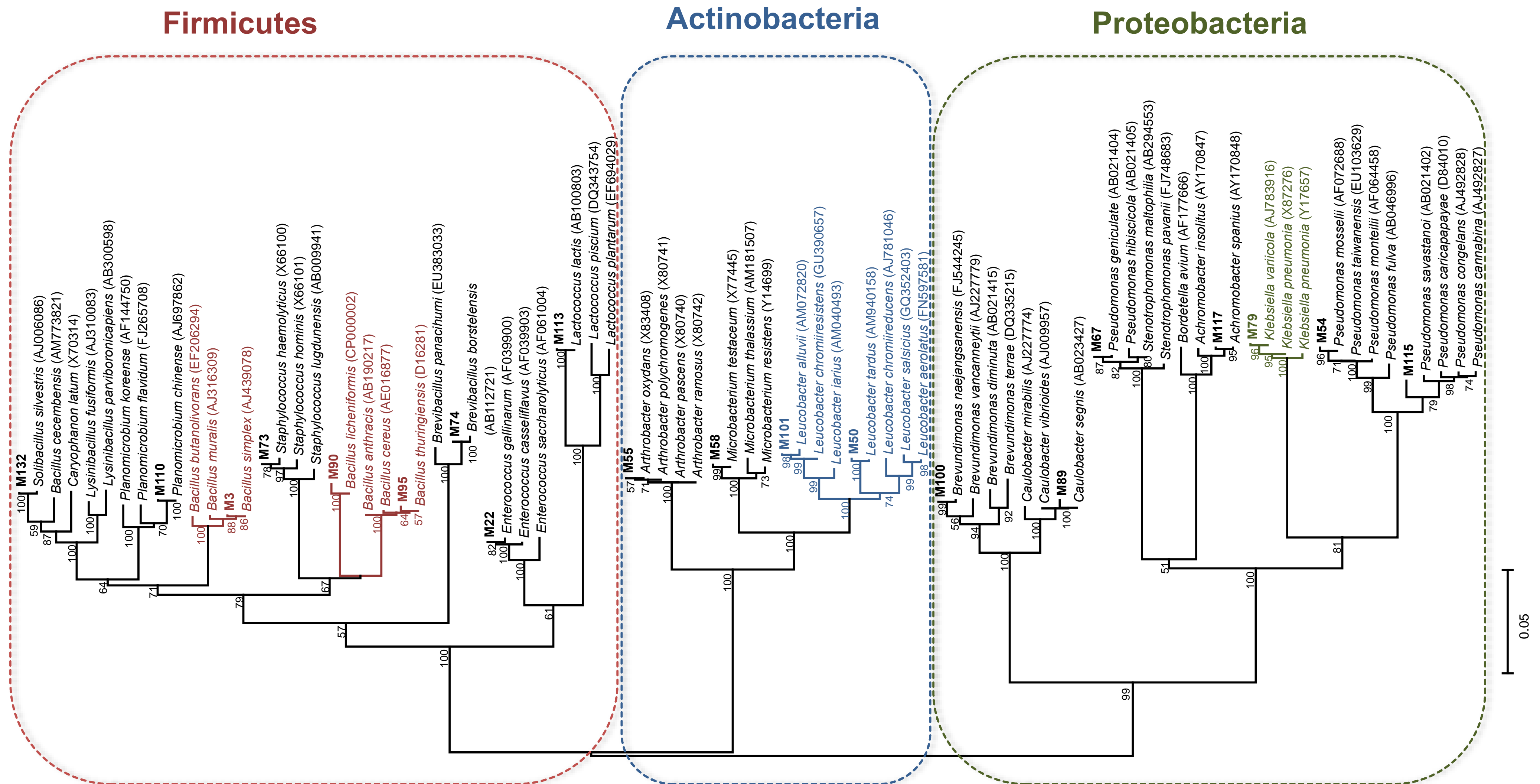

Figure 4.10: Neighbor-Joining tree constructed from partial 16S rRNA gene sequences collected from *Busseola fusca* larvae. Obtained Bootstrap confidence values (1000 replicates) are given at the branch point. Entries include the selected bacterial groups (Table 4.3) together with species names and accession numbers obtained from the GenBank database.

Some of the relationships in Figure 4.10 are briefly discussed: The M79 sequences clustered within a *K. variicola* (AJ783916) and *K. pneumoniae* (X87276) complex. There is strong bootstrap support (96 %) for the M79 and *K. variicola* cluster. The latter cluster also had 96 % support to *K. pneumoniae* sequences. Affiliation of the M101 (*Leucobacter alluvii*) complex and *Leucobacter salsicius* (M50) to the *Leucobacter* cluster are supported by 98 % and 100 % bootstrap, respectively. Two separate clades are observed within the *Bacillus* genus. *Bacillus simplex* (M3) clustered with *B. simplex* (AJ439078) and *B. muralis* (AJ316309) with bootstrap support of 86 % and 88 %, respectively. The M95 complex clustered with *B. thuringiensis* (D16281) with low bootstrap support (57 %). Support for M95 complex and *B. thuringiensis* to *Bacillus cereus* (AE016877) was 64 %. However, these species clustered with *Bacillus anthracis* (AB190217) with a bootstrap support of 100 %, indicating that they are members of the *Bacillus* genus.

4.4. Species distribution

Shannon diversity indices were again calculated based on the average CFUs at each site after species identifications were obtained from sequencing results. This diversity data were analysed in the same manner as for the morphotypes, which is presented in Table 4.1. The mean Shannon values were compared between the different factors as was done with the morphotypes. No statistically significant ($p < 0.05$) differences were observed between diversity of the dry lands (1.599 ± 0.070^a) and irrigation (1.519 ± 0.108^a), Bt (1.650 ± 0.084^a) and non-Bt (1.893 ± 0.041^a) maize and between the Western (1.510 ± 0.080^a) and Eastern (1.312 ± 0.094^a) parts of the sampling area. Although these results show no effect of the different factors on the microbial communities, trends were observed which showed the possibility of differences (Table 4.4). This observation may be incentive for further research.

Table 4.4: Comparison between the mean Shannon diversity values and the type of maize, production area and the type of production system, respectively (Means ± standard error, superscript characters denote statistical significant differences, p = 0.05, Tukey's Unequal HSD).

Comparison	Shannon value (Mean ± Standard error)
Type of maize	
Bt	1.650 ± 0.084 [a]
non-Bt	1.893 ± 0.041[a]
Production area	
West	1.510 ± 0.080 [a]
East	1.312 ± 0.094 [a]
Type of production system	
Irrigation	1.519 ± 0.108 [a]
Dry land	1.599 ± 0.070 [a]

A comparison of species diversity between the different sites, which are situated over four provinces, was made. The differences were noted and summarised (Appendix 4). Six of the species only occurred in larvae collected from the Free State and the North-West Province, five were observed in larvae from the Free State and Gauteng as well as five from Gauteng and the North-West Province. Eighteen species were present in the Free State, Gauteng and the North-West Province, whereas only five occurred in larvae collected from all four provinces.

Brevibacillus borstelensis occurred at three different sites, all located in the Free State, similarly *Caulobacter crescentus* occurred in three respective sites all situated in the North-West Province. *Bacillus thuringiensis* and *K. pneumoniae* were isolated at 29 of the 30 sampling sites, and *K. oxytoca* at 26 sites. Thirteen of the species were unique to one sampling site. In the North-West Province two different species occurred at the Vaalriver site, and in Gauteng three different species were present at Magaliesburg. This information is summarised in Table 4.5.

Table 4.5: Species unique to one sampling site.

Province	Site	Species
Free State	BLH	*E. hormaechei*
	BOT	*B. methylotrophicus*
	BTF	*S. silvestris*
	THN	*S. marcescens*
	DERB	*A. piechaudii*
North-West	VLR	*A. spanius; Caulobacter sp.*
	WIT	*P. chinense*
Gauteng	DEL	*Pseudomonas sp.*
	MGB	*L. tardus; M. luteus; P. agglomerans*
Northen Cape	VLH	*Halomonas sp.*

4.5. Geographical differences

A Canonical correspondence analysis (CCA) was performed using the species distribution data (presented in Appendix 4) and the direct distances (Appendix 5) between each site in order to compare the microbial communities present in larvae collected at different geographical sites. Species occurring in more than 10 % of the sampling sites were selected to complete the ordination (Figure 4.11).

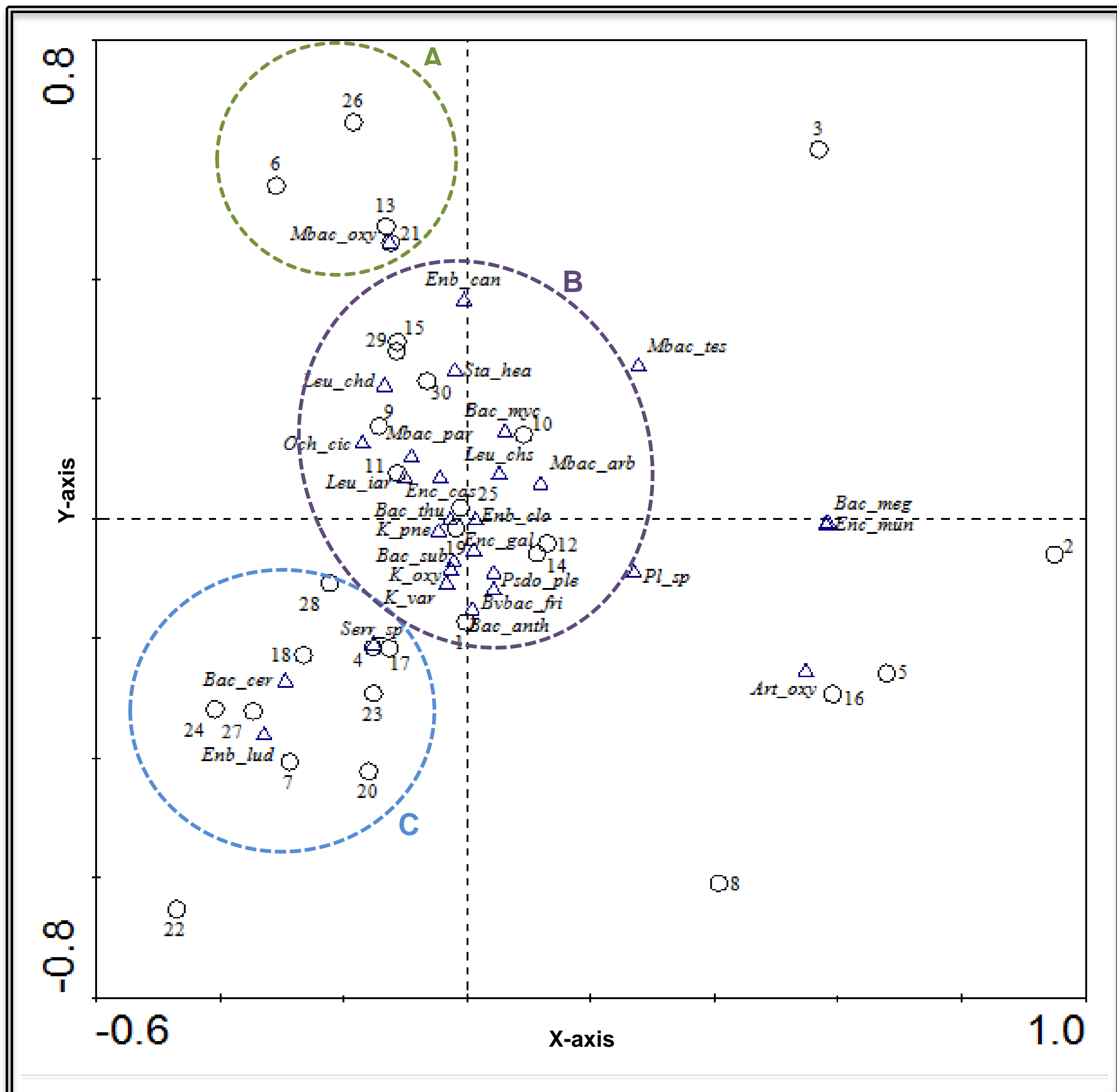

Figure 4.11: Canonical Correspondence Analysis illustrating the species composition at the different sites in relation to the direct distance between the respective sites. The sampling sites are represented by numbers 1-30 (refer to Table 4.1). The eigenvalues for the first two axes were 0.221 (x-axis) and 0.173 (y-axis), respectively.

Sites clustered into three distinct groups based on species distribution and the distance between the respective sampling sites. The CCA revealed differences in species composition between the respective groups as well as consistencies within the microbial structure. Species occurring within all three groups can be considered as indigenous members of *B. fusca* larvae such as *Klebsiella oxytoca* (*K_oxy*), *Enterococcus gallinarum* (*Enc_gal*) and *Brevibacterium frigoritolerans* (*Bvbac_fri*). The occurrence of *Bacillus thuringiensis* (*Bac_thu*) and *Klebsiella pneumoniae* (*K_pne*) at all of the sites within the three clusters suggests they are dominant species.

Species and sites situated in group B (Figure 4.11) are closely located to one another, which imply that the species composition of these sites is more similar to one another than it is to sites within groups A and C. Since most of the species and sites are present within this group, it indicates that the geographical distances between the sites had little influence on the gut microbes. Species not accounted for within all of the groups such as *Microbacterium oxydans* (*Mbac_oxy*: group A) and *Serratia sp.* (*Serr_sp*: group C) may only be temporal inhabitants of the midgut.

The observations from the CCA ordination (Figure 4.11) are in accordance with literature. Priya and co-workers (2012) showed that the type of host plant has a greater influence on the gut microbes than the locality. During the study *H. armigera* larvae were collected from different host plants at the same site and from the same host plants situated in different regions. The results showed more variations in microbial diversity if larvae were collected from different host plants rather than from the same plants at different localities (Priya *et al.*, 2012). Similar observations were made in the present study.

4.6. Summary of results

In this study *B. fusca* larvae were collected at 30 different maize fields in South Africa. Midgut contents were obtained and spread plated onto nutrient agar. The CFUs were characterised according to morphological traits and counted within each morphology group. A total of 135 different morphotypes were identified.

Microbial diversity was determined at each site by using the Shannon diversity index, based on the average CFUs for each morphotype. A comparison was made between the mean Shannon diversity values and the type of production system (irrigation or dryland), position of the site (Eastern or Western part of sampling area) and whether the larvae were collected from Bt or non-Bt maize, respectively. This was to determine whether these factors might have an influence on the gut microbes of *B. fusca* larvae. Statistical analyses revealed no significant ($p = 0.05$) differences between the morphotypes that occurred at the respective sites.

One representative was selected from each morphology group for 16S rRNA gene sequencing. Gram staining was performed to ensure that pure cultures were used during identification. Isolates were subjected to DNA isolation, PCR amplification of the 16S rRNA genes, sequencing of the amplicons, followed by BLASTn searches to obtain identities. Identification results revealed that 55 species belonging to 23 genera from Firmicutes, Proteobacteria and Actinobacteria were isolated from the midgut of *B. fusca* larvae. Fourteen of the isolates are potential novel species, since it had sequence similarities of 92 % and lower. Firmicutes represented the largest group of isolates, followed by Proteobacteria, that were the most diverse group, and the group with the least amount of isolates was Actinobacteria. The occurrence and distribution of species within the respective phyla revealed the dominance of *B. thuringiensis* and *K. pneumoniae*.

A phylogenetic tree was constructed from the partial 16S rRNA gene sequences obtained from the midgut of *B. fusca* larvae. Three distinct clusters occurred within the phylogenetic tree, which signifies the three isolated phyla. Bootstrap values are generally above 70 %, indicating reliable groupings.

Shannon diversity indices were calculated for the species data and similar results were obtained as for the morphotype data, indicating that the irrigation, position of site and type of maize from which larvae were collected had no effect on the gut microbes. A different approach was then used in an attempt to find a correlation between the microbial structure and the respective sampling sites. A CCA ordination was performed using the species distribution data

and the direct distances between each sampling site. In this analysis, three definite clusters were observed, suggesting a relation between the gut microbes and the site at which larvae were collected. After closer analysis of the CCA it was concluded that the geographical distances between the sites have no significant effect on the gut microbes associated with the sites. The ordination results did however reveal that species composition at certain sites is more similar to one another than to other sites.

The study was not designed to investigate the effect of various factors on the microbial diversity, but to determine the microbial structure within the midgut. This is the first in depth report on the microbial structure within the midgut of *B. fusca* larvae. Although no significant differences in microbial structure were observed between geographical areas, it does present the need for further investigation.

CHAPTER 5
DISCUSSION

5.1. Introduction

The aim of this study was to further our knowledge about the bacteria associated with *Busseola fusca* (Lepidoptera: Noctuidae) larvae. Larvae were collected from 30 different sites in the maize producing region of South Africa. Dissections were performed in order to remove the midguts as described in Section 3.2. Culture-dependent methods were used to identify the microbial community structure within the midguts. The midgut contents were spread-plated onto nutrient agar and colonies were selected based on morphology. A total of 135 bacterial morphotypes were selected and purified through streak plating. DNA was successfully isolated from the bacteria (Section 3.5) and subsequently used for PCR amplification (Section 3.6). This was followed by sequencing, and BLASTn searches to identify the isolated bacteria (Section 3.9).

5.2. Bacterial morphotypes

Bacteria were successfully obtained from the midguts of *B. fusca* larvae by means of the procedure described in Section 3.3. The midgut contents were serially diluted and spread-plated onto nutrient agar to isolate heterotrophic bacteria. In literature, studies concerning the isolation of gut bacteria from insects made use of a wide variety of growth media such as tryptone-yeast extract (Klug and Kotarski, 1980), brain heart infusion (Schaaf and Dettner, 1996), TSA agar (Broderick *et al.*, 2004; Delalibera *et al.*, 2007; Rani *et al.*, 2009; Priya *et al.*, 2012), CMC agar (Delalibera *et al.*, 2007) and LB agar (Rani *et al.*, 2009; Zouache *et al.*, 2011). These authors used agar media best fitted to accomplish the aim of their respective studies. In a study by Anand *et al.* (2010) Berg's agar that contained different substrates were used to determine the ability of bacteria from the gut of *Bombyx mori* (Lepidoptera: Bombycidae) to degrade various carbohydrate substrates. The aim of the present study was to isolate all genera of bacterial families, therefore nutrient agar (Lacey *et al.*, 2007; Kat *et al.*, 2010; Munteanu *et al.*, 2014; Tagliavia *et al.*, 2014; Özkan-Çakici *et al.*, 2014) was used instead of selective agar. De Vries and co-workers (2001) compared the growth and colony morphology of bacteria grown on ten different agar media, which included TSA, LB and nutrient agar, however, no meaningful differences were observed.

Several studies concerning the isolation of gut microbes through culture-dependent methods used incubation temperatures of 21 °C to 30 °C (Klug and Kotarski, 1980; De Vries *et al.*, 2001; Broderick *et al.*, 2004; Delalibera *et al.*, 2007; Van der Hoeven *et al.*, 2008; Priya *et al.*, 2012). These temperatures are relative to the conditions within insect guts. In the present study spread plates were incubated at 37 °C for 24 hours (Macovei and Zurek, 2006; Cox and Gilmore, 2007; Rani *et al.*, 2009). The higher incubation temperature may have caused certain bacterial families to have been under represented or not at all.

Initially, the isolated heterotrophic bacteria were categorised according to their morphological traits (Section 3.3). These included the surface appearance and shape of the colony, elevation of the colonies, the shape of colony edges and the colony colour (Conn and Pelczar, 1957; Schaaf and Dettner, 1996; De Vries *et al.*, 2001; Broderick e*t al.*, 2004; Delalibera *et al.*, 2007; Gusmão *et al.*, 2007; Van der Hoeven *et al.*, 2008; Rani *et al.*, 2009; Kat *et al.*, 2010; Priya *et al.*, 2012; Munteanu *et al.*, 2014). A total of 135 different morphology groups were identified, and afterwards one representative of each group was selected for 16S rRNA identifications (Sections 3.5-3.9). Several studies have made use of this approach to characterise gut bacteria within insects (Broderick e*t al.*, 2004; Lindh *et al.*, 2005; Rani *et al.*, 2009; Butler *et al.*, 2010; Gupta *et al.*, 2012).

The Shannon diversity index (*H*) was used to calculate the diversity of the culturable bacteria that were isolated at the respective sites (Table 4.1). Through this index the species richness and evenness are combined in order to quantify the diversity, which is expressed as a number (Begon *et al.*, 2006; Rani *et al.*, 2009; Chandel *et al.*, 2013). The bacterial diversity in the midgut of *B. fusca* larvae ranged from 0.570 to 2.359. This is similar to the bacterial diversity reported for other lepidopteran species namely, *Helicoverpa armigera* (Lepidoptera: Noctuidae), 2.61 to 1.47 (Xiang *et al.*, 2006) and *Pieris rapae* (Lepidoptera: Pieridae), 0.7 to 2.0 (Robinson *et al.*, 2010). Shannon indices reported for gut microbes within other insects include: (1) 1.16 to 2.45 - *Aedes albopictus* and *A. aegypti* (Diptera: Culicidae) (Zouache *et al.*, 2011); (2) 1.74 to 2.14 (laboratory reared) and 2.75 to 3.49 (field-collected) - *Anopheles stephensi* (Diptera: Culicidae) (Rani *et al.*, 2009); (3) 2.37 to 2.72 - larvae and adult *Melolontha hippocastani* (Coleoptera: Scarabaeidae) (Arias-Cordero *et al.*, 2012); (4) 1.54 to 3.87 – *Nasutitermes corniger* and *N. takasagoensis* (Isoptera: Termitidae) (Köhler *et al.*, 2012); (5) 1.634 to 1.905 – adult *Dastarcus helophoroides* (Coleoptera: Bothrideridae) and 2.597 to 2.731 – *D. helophoroides* larvae (Wang *et al.*, 2014). The bacterial diversity of Lepidoptera seems erratic in comparison to other insect orders, since it shows more variation between the highest and lowest diversity values reported.

The aim of this study was to describe the community structure within the midgut of *B. fusca* larvae. However, comparisons were also made between the microbial communities at each site, to determine whether differences may occur. The mean Shannon diversity values were used in combination with several factors regarding the sites to screen for potential variances. Diversity values were compared to the sites orientation in the maize production area (West or East), the presence or absence of irrigation and the type of maize (Bt vs non-Bt) from which larvae were collected. Statistical analyses revealed no significant (p = 0.05) differences between the community structures at the various sampling sites (Table 4.2). However, variations between species richness and composition were observed (Figure 4.1).

Diet alterations have been shown to influence the gut microbes associated with lepidopteran species (Broderick *et al.*, 2004; Robinson *et al.*, 2010; Belda *et al.*, 2011; Priya *et al.*, 2012). The larvae used during the current study were all collected from maize and no differences were therefore expected to occur between the microbial communities. The maize itself was different though in terms of being Bt or non-Bt maize. No statistically significant (p = 0.05) differences were observed, however, small variations did occur between the Shannon diversity values at the respective sites (Table 4.2). An increased lignin content in Bt maize plants compared to non-Bt maize plants has been reported (Saxena and Stotzky, 2001; Poerschmann *et al.*, 2005). Several insect orders have the ability to digest lignin by means of the gut microbiota they harbour (Dillon and Dillon, 2004; Genta *et al.*, 2006; Schloss *et al.*, 2006; Gibson and Hunter, 2010; Engel and Moran, 2013). It is possible that variations in plant composition may cause bacteria to adapt, causing a shift in the gut microbes (Santo-Domingo *et al.*, 1998; Anand *et al.*, 2010). Jung and Sheaffer (2004) reported contradictory results, as they did not observe any increase or decrease of lignin content within Bt plants compared to non-Bt plants. Thus, further studies are required before any conclusions in this regard can be drawn.

Sampling sites were situated in the Western and Eastern parts of the maize producing region of South Africa. Information regarding the biotic and abiotic factors at the various sites was not taken into account, since the aim of the study was to describe the bacterial community composition in *B. fusca* and whether there are geographical differences. Variations in species richness and composition of the bacterial morphotypes may differ between the respective sites. Microbial variation can result from changes in temperature, soil type, agricultural practices (such as irrigation), maize variety, pollution and other environmental influences (Horner-Devine *et al.*, 2004). However, Shannon diversity indices for this study revealed no significant differences, as in the case of several other studies (Corby-Harris *et al.*, 2007; Priya *et al.*, 2012; Chandel *et al.*, 2013). Geographical differences in species richness were observed between 11 *Drosophila melanogaster* (Diptera: Drosophilidae) populations, but no relation between species

richness and temperature or latitude was recognised. The authors stated the importance that more environmental factors should be taken into account in order to determine a correlation between the microbial differences and the site (Corby-Harris *et al.*, 2007). The observation that variations may or may not occur in species richness and composition at different geographical areas, shows the need for further studies regarding this phenomenon. Such investigations should attempt to explain the specific environmental factors, if any, which may lead to these variations.

5.3. Identification of bacterial morphotypes

5.3.1. Gram staining, DNA isolation and amplification

Gram staining was performed on all of the selected morphotypes prior to identification, to ensure that the bacterial cultures were pure but also to determine bacterial morphology and accordingly to divide the isolates into Gram-negative and Gram-positive bacteria (Beveridge, 1999). Numerous studies use Gram staining as a routine method when describing microbes associated with insects (Schultz and Breznak, 1978; Potrikus and Breznak, 1980; Schaaf and Dettner, 1997; Davidson *et al.*, 2000; Zurek *et al.*, 2000; Marchini *et al.*, 2002; Gusmão *et al.*, 2007; Van der Hoeven *et al.*, 2008; Rani *et al.*, 2009; Kat *et al.*, 2010; Munteanu *et al.*, 2014).
Pure cultures were grown in nutrient broth at 37 °C overnight prior to DNA extraction. DNA was successfully isolated with the method described in Section 3.5, and amplified through PCR (Section 3.6). This is a modified version of the method described by Towner *et al.* (1998), which has been proven to be successful (Carstens *et al.*, 2014). Extracted DNA was amplified by using the primers 27F and 1492R (Lane, 1991; Broderick *et al.*, 2004; Rani *et al.*, 2009). The PCR reaction conditions were related to previously described conditions (Rani *et al.*, 2009). The DNA quality was determined through gel electrophoresis (Figures 4.2 and 4.3). The product size was approximately 1500 bp (Janda and Abbott, 2007), giving confirmation that the 16S rRNA region was successfully amplified (Yadav *et al.*, 2012).

Several studies concerning the microbial communities associated with insects, integrated culture-dependent methods with molecular techniques (De Vries *et al.*, 2001; Broderick *et al.*, 2004; Delalibera *et al.*, 2007; Gusmão *et al.*, 2007; Rani *et al.*, 2009; Arias-Cordero *et al.*, 2012; Gupta *et al.*, 2012). Through this combined approach the gut microbes of several insect species have been identified. These include *Ips pini* (Coleoptera: Curculionidae) (Delalibera *et al.*, 2007), *Musca domestica* (Diptera: Muscidae) (Macovei and Zurek, 2006; Gupta *et al.*, 2012), *A. stephensi* (Rani *et al.*, 2009), *A. aegypti* (Zouache *et al.*, 2011) and Lepidoptera species such as *P. rapae* (Robinson *et al.*, 2010), *Lymantria dispa*r (Lepidoptera: Erebidae)

(Broderick *et al*., 2004), *Ostrinia nubilalis* (Lepidoptera: Pyralidae) (Belda *et al*., 2011) and *Manduca sexta* (Lepidoptera: Sphingidae) (Van der Hoeven *et al*., 2008).

5.3.2. 16S rRNA gene sequencing analysis

The amplified products were sequenced by the DNA Sequencing Facility of Stellenbosch University (South Africa) and Inqaba Biotech (South Africa), by using the 27F primer. All of the isolates selected for identification were not successfully identified due to a lack of growth during streaking. A total of 129 sequences (96.3 %) were edited and subjected to BLASTn searches for identification. Sequence similarities of ≥ 95 % were obtained for 114 of the isolates, which is considered as accurate identifications. For these sequences the E values were below zero, indicating that the identities were significant. Fifteen isolates that may potentially represent a new species or genera were identified (sequence similarities of ≤ 94 %) (Ma *et al*., 2007). Further analyses are required before this can be certain, for this reason BLASTn searches with the highest similarity were considered as the identification.

Members from Firmicutes, Actinobacteria and Proteobacteria were identified during this study. The most abundant phylum was Firmicutes consisting of 44 % of the isolates, however, Proteobacteria (36 %) were the most diverse group involving 12 genera. The smallest group of isolates belonged to Actinobacteria (20 %) and consisted of five genera. Bacteria from these phyla are generally associated with insect species (Brinkmann *et al*., 2008; Arias-Cordero *et al*., 2012; Priya *et al*., 2012; Hendriksma *et al*., 2013; Moro *et al*., 2013). A similar microbial composition was observed in the midgut of *H. armigera*, with Firmicutes as the most abundant group, followed by Proteobacteria and Actinobacteria that had the least amount of isolates (Priya *et al*., 2012). In contrast, Proteobacteria (92.2 %) were predominant in the gut of *P. rapae*, while Firmicutes represented only 3.1 % of the microbial structure. In this survey no members belonging to Actinobacteria were isolated (Robinson *et al*., 2010).

Firmicutes represents a diverse group of bacteria belonging to three classes namely Bacilli, Clostridia and Erysipelotrichi (Ludwig *et al*., 2011a). Several of these bacteria have the ability to produce lactic acid, acetone, butanol and ethanol through utilising various carbon sources, such as plant materials (Gu *et al*., 2010). The phylum Firmicutes include several families of which *Bacillaceae, Paenibacillaceae, Planococcaceae, Staphylococcaceae* and *Enterococcaceae* were isolated during the present study (Figure 4.6).

Proteobacteria is a group of Gram-negative bacteria divided into six different classes. In the present study members of Gammaproteobacteria, Alfaproteobacteria and Betaproteobacteria

were isolated. The main family isolated from this phylum in the present study was *Enterobacteriaceae* that includes the genera *Enterobacter* and *Klebsiella* (Figure 4.5).

Actinobacteria consists of phenotypically diverse organisms with various morphological characteristics. Gram staining results are variable and species can be facultatively anaerobic or strictly aerobic or anaerobic. Most species are chemo-organotrophs and saprophytic, and several species are pathogenic (Goodfellow, 2012). Three families from this phylum were isolated from *B. fusca* namely, *Micrococcaceae, Brevibacteriaceae, Microbacteriaceae* (Figure 4.7).

5.4. Phylogenetic analysis

Sequences with high similarities (≥ 97 %) were used to construct a Neighbor-Joining phylogenetic tree (Figure 4.10). The Neighbor-Joining method is based on the principal of minimum evolution. It identifies operational taxonomic units (OTUs) that will minimise the branch length at each point where OTUs cluster. This method can be applied to various evolutionary distance data sets and provides the correct tree topology (Saitou and Nei, 1987).
Neighbor-Joining trees have been used in various studies that analysed the gut microbiota associated with insects such as *D. melanogaster* (Corby-Harris *et al*., 2007; Cox and Gilmore, 2007), *Mastotermes darwiniensis* (Blattodea: Mastotermitidae) (Berchtold and König, 1996), *Sesamia nonagrioides* (Lepidoptera: Noctuidae) (Díaz-Mendoza *et al*., 2005), *Antonina crawii* (Homoptera: Pseudococcidae) (Fukatsu and Nikoh, 2000), *Apis mellifera* (Hymenoptera: Apidae) (Hamdi *et al*., 2013; Kim *et al*., 2014), *Spodoptera exigua* (Lepidoptera: Noctuidae) (Hernández-Martínez *et al*., 2010), *Plutella xylostella* (Lepidoptera: Plutellidae) (Indiragandhi *et al*., 2007), *Thaumetopoea pityocampa* (Lepidoptera: Thaumetopoeidae) (Kat *et al*., 2010) and *A. stephensi* (Rani *et al*., 2009).

Figure 4.10 shows the evolutionary relationships of the partial 16S rRNA gene sequences obtained from the midgut. The isolated species belonged to Firmicutes (47 %), Proteobacteria (33 %) and Actinobacteria (20 %). Within these groups, species from the same genus clustered together based on sequence similarities. Single clusters formed between the selected bacterial groups (Table 4.3) and the representative sequences downloaded from GenBank. The majority of these groupings were supported with high bootstrap values. Single clusters that formed between the majority of the bacterial groups (Table 4.3) and the representative sequences were supported with bootstrap values above 78 %, which is considered as reliable groupings

(Baldauf, 2003; Soltis and Soltis, 2003). Thus, the sequence alignment results are supported by the phylogenetic analysis.

The *Bacillus* genus is divided into the *Bacillus simplex* (M3) and *B. thuringiensis* (M95) clades. *Bacillus simplex* (M3) clustered with *B. simplex* (AJ439078) and *B. muralis* (AJ316309) with bootstrap support of 86 % and 88 %, respectively. However, the M95 complex and *B. thuringiensis* (D16281) cluster had low bootstrap support (57 %). This cluster had 64 % bootstrap support to *Bacillus cereus* (AE016877). The affiliation between *B. thuringiensis*, *B. cereus* and the M95 complex had 100 % bootstrap support with *B. anthracis*, which indicated that they are members of the *Bacillus* genus.

Bacillus thuringiensis forms part of the closely related *B. cereus sensu lato*, which also includes *B. anthracis* and *B. mycoides* (Helgason *et al*., 2000). Some authors suggested that this group should be considered as one species because of the extremely close similarities between them (Helgason *et al*., 2000; Schmidt *et al*., 2011). Members of the *B. cereus sensu lato* cannot be differentiated based on 16S rDNA sequence analysis alone (La Duc *et al*., 2004; Stenfors Arnesen *et al*., 2008), although they have different phenotypes and pathological effects (Helgason *et al*., 2000). Coding genes that are usually present on plasmids can be used to functionally distinguish between these species. The only difference between *B. thuringiensis* and *B. cereus* is the production of delta-endotoxins during sporulation (Stenfors Arnesen *et al*., 2008). If the plasmids containing coding genes for insecticidal toxins are absent, no distinction can be made between these species (Helgason *et al*., 2000). *Bacillus anthracis* can however be separated from *B. cereus* with PCR based methods (La Duc *et al*., 2004). Biochemical characteristics can separate *Bacillus* species from one another. Frequently used tests include motility (*B. mycoides* and *B. cereus* can be differentiated by rhizoidal colony shape), haemolysis and carbohydrate fermentation, as *B. cereus* does not ferment mannitol (Stenfors Arnesen *et al*., 2008). Therefore, further examination of the *B. thuringiensis* strains isolated during this study is required.

5.5. Potential function of gut microbes

Insects often depend on microorganisms for basic biological functions, especially on gut microbes (Engel and Moran, 2013; Powell *et al.*, 2014). The complexity of microbial communities differs greatly between insect species and among individuals. The structure of gut microbes is shaped according to the diet, location and lifestyle of the insect host (Robinson *et al.*, 2010; Engel and Moran, 2013). Symbiotic relationships with bacteria are known to (1) enhance nutrition by upgrading nutrient poor diets, aiding in digestion and the detoxification of harmful compounds, (2) develop and maintain immune system through promoting host development and fitness and by protecting against predators, parasites, and pathogens, (3) affect efficiency as disease vectors, (4) and govern mating and reproductive systems within insects (Dillon *et al.*, 2002; Azambuja *et al.*, 2004; Broderick *et al.*, 2004; Rajagopal, 2009; Gullan and Cranston, 2010; Engel and Moran, 2013; Gimonneau *et al.*, 2014; Tagliavia *et al.,* 2014). However, these studies have been mostly confined to termite and aphid species, which entail large complex communities (Potrikus and Breznak, 1980; Leadbetter *et al.*, 1998; Lundgren *et al.*, 2007; Tokuda *et al.*, 2007; Ohkuma, 2008; Hongoh, 2010; Wu *et al.*, 2012). Insects that have complex structures within their digestive tracts (paunches, diverticula and caeca) such as termites are more prone to large microbial communities, than insects with no specialised structures such as Lepidoptera (Dillon and Dillon, 2004).

Insects can accumulate gut microbes through various methods. This can either be from their environment, in which they can ingest bacteria, or through vertical transmission from mother to offspring (Gimonneau *et al.*, 2014). In Lepidoptera, bacteria can be transmitted to future generations by means of their eggs (Martin and Mundt, 1972; Cappellozza *et al.*, 2011). It was reported that *Enterococcus* species are transferred from the mother moths to larvae via the eggs in *M. sexta* (Brinkmann *et al.*, 2008).

Symbiotic relationships between Lepidoptera and their gut microbes are not well understood. However, several attempts have been made to better understand the interactions between these organisms, and the possible role it may have in insect survival (Broderick *et al.*, 2004; Xiang *et al.*, 2006; Van der Hoeven *et al.*, 2008; Anand *et al.*, 2010; Broderick *et al.*, 2009; Robinson *et al.*, 2010; Belda *et al.*, 2011; Priya *et al.*, 2012). Overall, these studies suggested that microbes are responsible for enhancing nutrition by aiding in digestion, providing nutrients and by detoxifying harmful food components (Broderick *et al.*, 2004; Genta *et al.*, 2006; Xiang *et al.*, 2006; Brinkmann *et al.*, 2008; Anand *et al.*, 2010; Li *et al.*, 2011; Engel and Moran, 2013; Özkan-Çakici *et al.*, 2014). During the present study several bacteria were isolated that may

enhance the nutrition of *B. fusca*. These included *Bacillus* spp., *Enterobacter* spp., *Enterococcus* spp., *Klebsiella* spp. and *Brevibacillus borstelensis* (Section 5.5.1).

Several acid producing bacteria were isolated during the present study such as *Bacillus* spp., *Enterococcus* spp., *Arthrobacter* spp., *Microbacterium* spp. and *Brevibacterium frigoritolerans* (Table 5.1). These bacteria may influence the effectiveness of Bt maize against *B. fusca* by altering the pH within the gut. The alkalinity within the midgut of Lepidoptera is mainly part of the specificity that Bt toxins have to these insects (Broderick *et al.*, 2006). A decrease in the pH of the midgut may prevent the activation of Cry toxins, thus reducing the effectiveness of Bt in killing larvae. Members of Lactobacillales are mostly lactic acid bacteria (LAB), such as *Enterococcus* and *Lactococcus*. These microbes are able to convert carbohydrates into lactic acid during fermentation. Some species produce additional by-products that include acetic acid, ethanol and carbon dioxide (CO_2). Acid production can cause the pH value of an environment to decrease (Mead *et al.*, 1988; Dillon and Dillon, 2004; Cappellozza *et al.*, 2011). Other bacterial groups are also able to produce various acids from organic compounds, which may also result in a decrease in pH. If the microbes isolated during this study are indeed able to reduce the pH within the gut, they might be responsible for resistance within *B. fusca* to Bt maize. Therefore, further investigation is required to determine whether these bacteria are able to influence the gut pH. Additionally, it is also of importance to establish how the larvae acquire these bacteria, as this will aid in preventing further resistance evolution within these insects.

A better understanding of the interactions between the gut microbes as well as their biochemical characteristics is important for assessing the functional role of the gut community within resistance development. Several bacterial groups isolated during the current study such as *Serratia* spp., *Pseudomonas* spp., *Staphylococcus* spp., *Stenotrophomonas maltophilia* and *Bacillus subtilis* are able to produce alkaline compounds, which can neutralise acidic environments caused by LAB. Although acid producing bacteria may have a potential role in resistance development, other bacteria are able to produce alkaline compounds that will in return increase the pH value within the gut (Cotter and Hill, 2003), thus, providing an alkaline environment that will enable the activation of Bt toxins. These bacteria release ammonia (NH_3), which is produced by urease hydrolysis, nitrite ammonification and by the degradation and decarboxylation of amino acids (Weise *et al.*, 2013). After ammonia binds to protons, ammonium (NH_4) is produced that will increase the pH of the environment (Cotter and Hill, 2003). In terms of CFUs, the acid producing bacteria are predominant compared to bacteria that may have the potential to neutralise the gut environment (94 % vs 6 %), if it should be too acidic. However, further studies are required before any conclusions can be drawn regarding the influence of gut microbes on the gut pH of *B. fusca.*

Various isolates from this study were previously described as insect pathogens and may thus be able to induce mortality within *B. fusca* larvae in certain circumstances (Section 5.5.5). This observation may also suggest that mortality within *B. fusca* larvae might be as a result of bacterial pathogens within the gut, and not from Bt toxins. Although gut microbes are generally non-pathogenic (Tagliavia *et al.*, 2014), several isolates are known to cause disease within insects. A better understanding of these pathogens may aid in the development of biological control, for instance microbial pesticides are considered as a key element in controlling agricultural pests (Secil *et al.*, 2012). Microbes that can be used in biological control include natural disease-causing microorganisms such as viruses, nematodes, fungi, protozoa and bacteria (Özkan-Çakici *et al.*, 2014). Such bacteria also refer to opportunistic pathogens that may infect and rapidly reproduce within the haemolymph of insects after gaining access through wounds (Secil *et al.*, 2012). The development of resistance in several pest species against *B. thuringiensis* adds to the need of finding new control agents. Numerous studies have been conducted with the aim to identify potentially new bacterial strains that can be used as microbial insecticides (Bora *et al.*, 1994; Inglis *et al.*, 2000; Lacey *et al.*, 2007; Özkan-Çakici *et al.*, 2014). The observation that gut bacteria may or may not be responsible for insect mortality requires extensive research before any conclusions can be drawn in this regard. Future studies attempting to determine the role of bacteria in mortality of *B. fusca* should also consider the interactions these microbes may have with other inhabitants of the gut. More information regarding this may aid in controlling insect pests in the future.

The dynamic nature of microbial communities may cause bacteria to have different functions under certain conditions. For example, *Serratia marcescens* can be beneficial to its host when occurring in low numbers (Mano and Morisaki, 2008), but may also be pathogenic when it occurs in high numbers (Inglis *et al.*, 2000). Figure 5.1 is a summary of the potential roles gut microbes may have within *B. fusca* larvae. The information presented in this diagram was compiled from various articles regarding the possible roles of gut microbes in other insects, especially Lepidoptera species. In Figure 5.1, 32 bacterial species are listed together with the characteristics responsible for promoting certain functions. A total of 55 species, including all of the bacteria shown in Figure 5.1, were isolated in the current study from *B. fusca* larvae. The isolated bacteria may therefore have similar functions within the biology and survival of *B. fusca* larvae.

Table 5.1: Acid producing bacteria isolated from the midgut of *B. fusca* as well as the substrates from which acid is produced.

Acid producing bacteria	Substrate from which acid is produced	Reference
Bacillus spp.	Various carbon sources including carbohydrates, lipids and proteins	Gu *et al.*, 2010 Logan and De Vos, 2011
Brevibacillus borstelensis	D-Fructose	Shida *et al.*, 1995
Planomicrobium chinense	Glucose	Dai *et al.*, 2005
Enterococcus spp. *E. casseliflavus*	Various carbon sources, but mainly from glucose D-Raffinose	Cappellozza *et al.*, 2011 Ludwig *et al.*, 2011b
Arthrobacter spp. *A. creatinolyticus* *A. oxydans*	 Glycerol Various sugars	 Busse *et al.*, 2012 Busse, 2012
Microbacterium spp. *M. arborescens*	Various carbon sources Sucrose, D-xylose and L-arabinose	Suzuki and Hamada, 2012 Suzuki and Hamada, 2012
Klebsiella pneumoniae	Glucose, lactose, sorbitol and monnitol	Anand *et al.*, 2010
Brevibacterium frigoritolerans	D-fructose, trehalose, L-arabinose, D-glucose and D-xylose	Li *et al.*, 2014

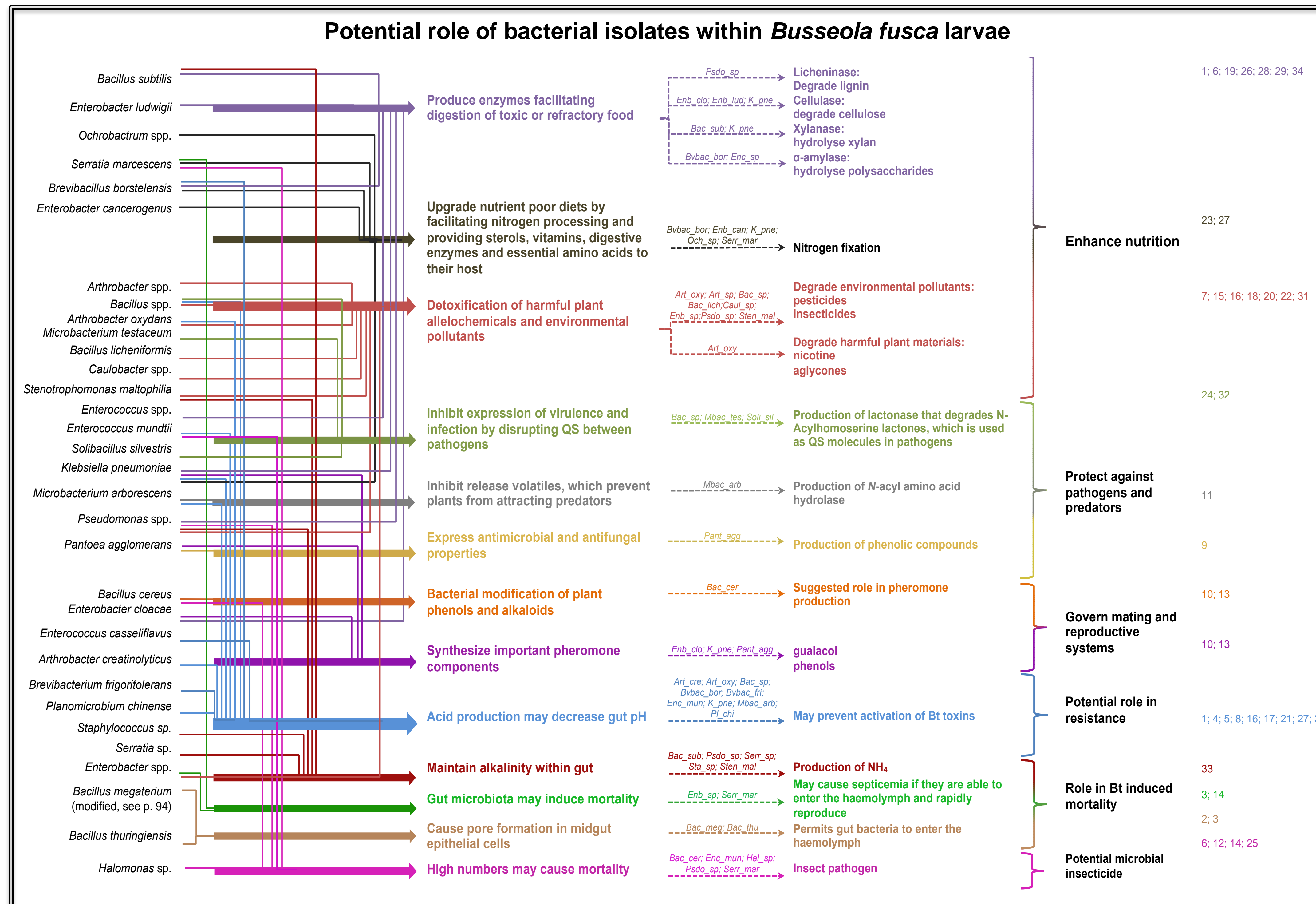

Figure 5.1: Summary of the potential roles the isolated gut microbes may facilitate in the lifestyle of *B. fusca* larvae. [Refer to Appendix 2 for the abbreviations used in this diagram. Information was compiled from: (1) Anand *et al.*, 2009; (2) Bora *et al.*, 1994; (3) Broderick *et al.*, 2006; (4) Busse *et al.*, 2012; (5) Busse, 2012; (6) Cappellozza *et al.*, 2011; (7) Chen *et al.*, 2011; (8) Dai *et al.*, 2005; (9) Dillon and Dillon, 2004; (10) Dillon *et al.*, 2002; (11) Engel and Moran, 2013; (12) Fedhila *et al.*, 2010; (13) Gullan and Cranston, 2010; (14) Inglis *et al.*, 2000; (15) Kallimanis *et al.*, 2007; (16) Li *et al.*, 2011; (17) Li *et al.*, 2014; (18) Liu *et al.*, 2007; (19) Liu *et al.*, 2011; (20) Liu *et al.*, 2014; (21) Logan and De Vos, 2011; (22) Lundgren *et al.*, 2007; (23) Mano and Morisaki, 2008; (24) Morohoshi *et al.*, 2012; (25) Özkan-Çakici *et al.*, 2014; (26) Rhee *et al.*, 2014; (27) Shida *et al.*, 1995; (28) Suen *et al.*, 2010; (29) Suribabu *et al.*, 2014; (30) Suzuki and Hamada, 2012; (31) Tang and You, 2012; (32) Wang *et al.*, 2010; (33) Wiese *et al.*, 2013; (34) Yadav *et al.*, 2012].

From Figure 5.1 it appears as if the isolated gut microbes mainly assist *B. fusca* larvae in enhancing nutrition. This is in accordance with observations from other studies regarding the role of gut microbes in different Lepidoptera species (Genta *et al.*, 2006; Brinkmann *et al.*, 2008; Anand et al., 2010; Li *et al.*, 2011; Engel and Moran, 2013; Özkan-Çakici *et al.*, 2014). Bacteria such as *Bacillus subtilis* (Rhee *et al.*, 2014), *Brevibacillus borstelensis* (Suribabu *et al.*, 2014) *Microbacterium oxydans*, *Klebsiella pneumoniae, Enterobacter cloacae* and *Enterobacter ludwigii* (Yadav *et al.*, 2012) as well as species belonging to *Pseudomonas* (Suen *et al.*, 2010) and *Enterococcus* (Cappellozza *et al.*, 2011) produces enzymes that facilitate digestion of refractory or toxic food compounds. Many species belonging to *Arthrobacter* (Kallimanis *et al.*, 2007), *Enterobacter* (Lundgren *et al.*, 2007), *Pseudomonas* (Tang and You, 2012) and *Stenotrophomonas* (Liu *et al.*, 2014) are also able to detoxify plant allelochemicals and environmental pollutants. Detoxification of harmful plant materials is required before larvae can assimilate it, while the neutralisation of chemical pollutants may aid in the survival of larvae. Gut microbes isolated during this study were also suggested to provide essential nutrients to their host insects in previous studies. Bacteria isolated in this study include *Enterobacter cancerogenus*, *Pantoea agglomerans*, *K. pneumoniae*, *Serratia marcescens*, *Ochrobactrum* sp. (Mano and Morisaki *et al*, 2008) and *B. borstelensis* (Shida *et al.*, 1995). The majority of the species isolated during the current study that may aid in the nutrition of *B. fusca* are members of the family *Enterobacteriaceae.* These include *Enterobacter* spp., *Serratia* spp., *Pantoea* spp. and *Klebsiella* spp. that represented 36 % of total community structure in *B. fusca* (Figure 4.9 B).

Isolates from this study may also protect *B. fusca* from invasive pathogens and from predators. *Microbacterium arborescens* were shown to inhibit the production of plant volatiles, which will prevent the plant from attracting predators, and therefore may promote the survival of larvae (Engel and Moran, 2013). Bacteria such as *Microbacterium testaceum* and *Solibacillus silvestris* may also disrupt cell-to-cell communication between pathogens, which may prevent these microbes from expressing virulence. *Bacillus* and *Arthrobacter* species may have a similar role in protecting larvae from pathogens (Wang *et al.*, 2010; Morohoshi *et al.*, 2012).

Broderick and co-workers (2006) reported that Bt-induced mortality depends on the interaction between *B. thuringiensis* and the indigenous enteric gut bacteria. *Bacillus thuringiensis* is responsible for pore formation that enables the gut bacteria to enter the haemocoel that leads to septicaemia in larvae. Non-spore forming bacterial pathogens is otherwise not capable of entering the haemocoel on their own (Lacey *et al.*, 2007). Such bacteria isolated from *B. fusca* in the current study includes *S. marcescens* (Inglis *et al.*, 2000), *Enterococcus mundtii* (Cappellozza *et al.,* 2011) as well as species belonging to *Brevibacterium* (Steinhause, 2012), *Klebsiella* (Özkan-Çakici *et al.*, 2014) and *Pseudomonas* (Inglis *et al.*, 2000). The activation of

Bt toxins requires high alkalinity and as mentioned, several acid producing bacteria have been isolated from the midgut of *B. fusca*, which may decrease the gut pH (Table 5.1). These bacteria may therefore aid in resistance evolution of *B. fusca* to Bt maize.

Figure 5.1 and the potential functions described above gives a brief summary of the potential functions that the isolates from this study may have in the midgut of *B. fusca* larvae. Sections 5.5.1.-5.5.5. provides a more detailed description of these functions within Lepidoptera as well as other insect orders.

5.5.1. Enhancing nutrition

Gut microbes have different methods of enhancing the nutrition of insects. This may include the production of digestive enzymes to facilitate degradation of complex molecules, provide essential compounds for direct assimilation or bacteria can detoxify harmful compounds to enable digestion (Minard *et al.*, 2013).

5.5.1.1. Aid in digestion

Several bacteria were isolated that may aid in the digestion of *B. fusca* larvae. Since their diet consist of maize plants it is vital for these larvae to acquire the necessary enzymes in order to digest refractory plant materials such as lignin and cellulose. Bacterial species belonging to *Bacillus*, *Enterobacter*, *Enterococcus*, *Klebsiella* and *Brevibacillus* is known to produce several digestive enzymes (Anand *et al.*, 2010; Özkan-Çakici *et al.*, 2014). Together these species represented 79 % of the gut microbes isolated during this study. From Figure 5.1 it is apparent that *B. fusca* depends on the symbiotic relationship with its gut microbes for the facilitation of digestion.

Insects belonging to Lepidoptera have a diet that consists mainly of plant materials that may be toxic or refractory to digestion (Appel and Maines, 1995; Pordesimo *et al.*, 2005). Plant cell walls consist of lignin, cellulose and hemicellulose, which include polysaccharides such as xylan, glucuronoxylan, arabinoxylan, glucomannan, xyloglucan, mannose, galactose, rhamnose, and arabinose (Roy *et al.*, 2003; Genta *et al.*, 2006; Rhee *et al.*, 2014). Other components include glycosides, tannins, phenols and allelochemicals. Digestive enzymes such as cellulose, xylanase, xylosidase and α-amylase that are responsible for hydrolysis of these polysaccharides are not all secreted by the insects themselves. Instead, most digestive enzymes are produced through their microbial symbionts (Roy *et al.*, 2003; Brennan *et al.*, 2004; Janson *et al.*, 2007; Anand *et al.*, 2010; Engel and Moran, 2013). Xylanolytic bacteria include *Aeromonas* sp., *Bacillus* sp., *Citrobacter* sp., *Klebsiella* sp., *Pseudomonas* sp. and

Proteus sp. Isolates from these species are associated with insects such as *Samia Cynthia pryeri* (Lepidoptera: Saturniidae) (Roy *et al*., 2003) and *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae) (Butera *et al*., 2012).

Heterotrophic bacteria obtain carbon and energy sources from metabolising organic compounds such as sugars, alkaloids, polysaccharides, proteins and carbohydrates. The type of metabolism differs among bacterial groups. In aerobic conditions microbes use oxygen as a terminal electron acceptor, this is known as oxidation. Anaerobes use organic carbon as electron acceptors during fermentation instead of oxygen. Several organisms are able to ferment organic compounds under anaerobic and aerobic respiration. These organisms are facultatively anaerobic. Oxygen levels within Lepidoptera species can be either anaerobic or aerobic, although relative low oxygen levels has been reported (Johnson and Barbehenn, 2000). The gut conditions are therefore suitable for aerobes, anaerobes and facultative anaerobes. *Bacillus* species represented 32 % of the microbial community isolated from *B. fusca* (Figure 4.9 B). These species are well adapted to survive and colonise environments such as insect guts because they are mostly aerobic or facultative anaerobic, but strict aerobic species have also been described. Additionally, these species are capable of growing in high alkaline environments (Logan and De Vos, 2011; Schmidt *et al*., 2011). The family *Enterococcaceae* consists of mostly facultatively anaerobic bacteria and comprised 11 % of the gut microbes isolated during this study (Figure 4.9 B).

Bacillus subtilis is known to have the ability to utilise xylan (Rhee *et al.*, 2014), and has been used as a model species to study the regulatory mechanisms involved in xylose catabolism in microorganisms (Gu *et al*., 2010). This bacterium has been isolated from the guts of herbivorous insects such as termites (Liu *et al*., 2011). These authors suggested that *B. subtilis* promotes digestion within their insects hosts by producing essential digestive enzymes that hydrolyse xylan namely xylanase (Liu *et al*., 2011). Insects can obtain *B. subtilis* through their environment, as this is a natural occurring bacterium (Mano and Morisaki, 2008). This bacterium was obtained from *B. fusca* larvae at 50 % of the sampling sites.

Several Bacillus species are considered as normal inhabitants of the insect gut and have been isolated from insects such as *Solenopsis invicta* (Hymenoptera: Formicidae) (Gunawan *et al*., 2008), *Dastarcus helophoroides* (Wang *et al*., 2014), *R. ferrugineus* (Tagliavia *et al*., 2014), *Porcellio scaber* (Isopoda: Crustacea) (Swiecicka and Mahillon, 2005), *Limonius canus* (Coleoptera: Elateridae) (Lacey *et al*., 2007) and from mosquito larvae including *A. aegypti*, *C. quinquefasciatus*, *Anopheles coluzzii* and *A. gambiae* (Diptera: Culicidae) (Luxananil *et al*., 2001; Gusmão *et al*., 2007; Gimonneau *et al*., 2014). These bacteria are also associated with Lepidoptera species including *B. mori* (Anand *et al*., 2010), *H. armigera* (Priya *et al*., 2012), *O.*

nubilalis (Secil *et al*., 2012), *Spodoptera littoralis* (Lepidoptera: Noctuidae) (Özkan-Çakici *et al*., 2014), *Diatraea grandiosella* and *D. crambidoides* (Lepidoptera: Crambidae) (Inglis *et al*., 2000). In the current study eight *Bacillus* species were isolated, these include *Bacillus anthracis*, *B. cereus*, *B. licheniformis*, *B. megaterium*, *B. methylotrophicus*, *B. mycoides* and *B. thuringiensis*. The family Bacillaceae represented 32 % of the microbial community in the midgut.

Brevibacillus borstelensis produces α-amylase (Anand *et al*., 2010; Suribabu *et al*., 2014), and it is capable of nitrate reduction (Shida *et al*., 1995). Therefore, B. borstelensis may promote digestion and the availability of nutrients in *B. fusca* larvae. Although a few colonies of this bacterium was isolated at only three of the sampling sites (Kristalkop, Vredefort and Tweeling), *B. borstelensis* may be indigenous to larvae from these sites. Most of the CFUs obtained from this bacterium were isolated from larvae collected at Kristalkop (nr. 11) and Vredefort (nr. 12), which are in close proximity to each other (Figure 3.1). No literature were found describing any relation between *B. borstelensis* and insects, however, it is known that *B. borstelensis* is present in several soil types. It is possible this bacterium was ingested by the larva and is therefore only a provisional inhabitant of *B. fusca*.

Brevibacillus borstelensis was described by Shida and co-workers (1995) as *Bacillus borstelensis* and afterward transferred to the genus *Brevibacillus* in 1996 (Shida *et al.,* 1996). Members are strictly aerobic, Gram-positive, spore-forming, motile rods with optimal growth at 30 ^{o}C and pH 5.5 - 5.6. Acid is produced from D-Fructose but neither gas nor acid is produced from other sugars. Bacteria are oxidase negative, catalase positive, reduces nitrate to nitrite and tested negative for hydrogen sulphide (H_2S) production and indole (Shida *et al*., 1995).
Species belonging to *Klebsiella* and *Enterobacter* form part of the family *Enterobacteriaceae* and represented 36 % of the total microbial community structure isolated in the current study. Members from these genera were isolated at each sampling site, which may indicate that they are indigenous to the midgut. This is supported by literature affirming the typical associations between insects and species belonging to *Klebsiella* and *Enterobacter* (Mano and Morisaki, 2008; Özkan-Çakici *et al*., 2014). The affiliation between these organisms can even date back several decades, which may explain the predominance of these genera. However, these species are typically present within the environment and therefore it is possible that larvae may have obtained these microbes from their environment.

Several species belonging to *Enterobacter* were isolated during this study namely *Enterobacter asburiae, E. cancerogenus, E. cloacae, E. hormaechei* and *E. ludwigii*. These species were present at 60 % of the sampling sites and may assist *B. fusca* larvae in their digestion processes. Members belonging to *Enterobacter* are generally not insect pathogens,

but rather symbionts aiding in the survival of insect hosts (Özkan-Çakici *et al*., 2014). Several species are frequently associated with the degradation of cellulose (Anand *et al*., 2010). Strains of *E. ludwigii* and *E. cloacae* isolated from decomposing wood samples showed cellulase activities (Yadav *et al*., 2012). These two species have been isolated from insects such as *R. ferrugineus* (Tagliavia *et al*., 2014), *S. littoralis* (Özkan-Çakici *et al*., 2014), *L. dispar* (Broderick *et al*., 2006), *D. grandiosella*, *D. crambidoides* (Inglis *et al*., 2000), *Harpalus pensylvanicus* and *Anisodactylus sanctaecrucis* (Coleoptera: Carabidae) (Lundgren *et al*., 2007). *Enterobacter cloacae* were reported as the dominant bacterial species in the gut microbiota of *Caledia captiva* (Orthoptera: Acrididae) (Mead *et al*., 1988).

In this study *K. oxytoca*, *K. pneumoniae* and *K. variicola* were isolated. Associations with Lepidoptera species include *B. mori* (Anand *et al*., 2010; Khyade and Marathe, 2012) and *S. littoralis* (Özkan-Çakici *et al*., 2014) as well as other insects such as *L. canus* (Lacey *et al*., 2007). *Klebsiella* species are closely associated with insects and usually non-pathogenic. Insects can ingest these bacteria from their environment, for example as endophytes (Mano and Morisaki, 2008). These species aid in the digestion and the physical development of its host (Özkan-Çakici *et al*., 2014). Several studies have shown that *K. pneumoniae* has the ability to utilise cellulose and xylan (Anand *et al*., 2010; Özkan-Çakici *et al*., 2014).

Enterococcus casseliflavus, E. gallinarum and E. mundtii were isolated in this study and represented 11 % of the total community. As mentioned these bacteria produce lactic acid from various carbon sources that may decrease the pH within the midgut (Cappellozza *et al*., 2011), enabling the activation of Bt toxins (Broderick *et al*., 2006). Additionally, these bacteria may also assist *B. fusca* in the digestion of different plant materials. Brinkmann and co-workers (2008) reported the dominance of *Enterococcus* species in terms of metabolic processes in *M. sexta* larvae.

An early study by Martin and Mundt (1972) embodied the extent to which *Enterococcus* species are associated with insects. During this study enterococci were either randomly or consistently isolated from 213 insect species belonging to several insect orders (Martin and Mundt, 1972). Some *Enterococcus* species are typically associated with plants such as *E. casseliflavus*, permitting insects to obtain these bacteria from their environment. These bacteria are among the most frequently isolated bacteria from insect guts, and are considered as indigenous species (Brinkmann *et al.,* 2008). Members of *Enterococcus* are associated with Lepidoptera species such as *B. mori* (Inglis *et al*., 2000), *H. armigera* (Priya *et al*., 2012), *M. sexta* (Brinkmann *et al.,* 2008), *D. grandiosella* and *D. crambidoides* (Inglis *et al*., 2000).

5.5.1.2. Improving quality of nutrient poor diets

In the current study, several nitrogen-fixing bacteria were isolated such as *Enterobacter* sp., *Klebsiella* sp., *Ochrobactrum* sp. and *Serratia* sp. These bacteria may provide nitrogen sources as well as other essential nutrients to *B. fusca*. *Enterobacter* species such as *E. cancerogenus* are mostly capable of nitrogen fixation (Mano and Morisaki, 2008; Tagliavia *et al.*, 2014). Brinkmann *et al.* (2008) reported that *Enterobacter* species are indigenous to the midgut of *M. sexta* larvae.

Klebsiella species are frequently isolated as endophytes, and can therefore be ingested by feeding larvae. *Klebsiella pneumoniae* is able to fix atmospheric nitrogen (Mano and Morisaki, 2008) and may therefore enhance nutrition within *B. fusca* larvae. This genus is closely associated with insects and usually non-pathogenic. It has been isolated from different Lepidoptera species (previously mentioned: p. 85) as well as from *Rhynchophorus ferrugineus* (Tagliavia *et al.*, 2014). *Serratia marcescens* is another endophyte capable of nitrogen fixation (Mano and Morisaki, 2008). It has been isolated from several insects including *O. nubilalis* (Secil *et al.*, 2012), *A. coluzzii* and *A. gambiae* (Gimonneau *et al.,* 2014), *L. canus* (Lacey *et al.*, 2007) and *S. littoralis* (Özkan-Çakici *et al.*, 2014).

Insects rely on symbiosis with microorganisms in order to meet their nutritional requirements. Their diet either lacks adequate amounts of nutrients, or they are unable to obtain enough nutrients from their diet due to poor digestion (Brinkmann *et al.,* 2008; Lundgren and Lehman, 2010). Bacteria aid in sulphate assimilation, nitrogen processing and fatty acid metabolism, they also provide sterols, vitamins, digestive enzymes and essential amino acids to their host. Insects are unable to synthesise sterols, which are important regulators of developmental processes, and precursors of steroid hormones as well as vital in lipid biostructures (Behemer and Nes, 2003). Therefore, microorganisms are vital in insect nutrition and survival (Janson *et al.*, 2007; Engel and Moran, 2013).

Well-known symbionts of insects also include species from *Wigglesworthia* and *Buchnera. Wigglesworthia glossinidia* is a midgut symbiont of the tsetse fly (Diptera: Glossinidae) that produces B vitamins, which is absent in the fly's diet of blood (Geiger *et al.*, 2009). Aphids (Hemiptera: Aphididae) obtain amino acids from *Buchnera*, and wood-feeding termites (Isoptera) obtain fixed nitrogen from symbiotic nitrogen-fixing bacteria within their guts (Breznak, 2000). Nitrogen fixation is a process in which atmospheric nitrogen (N_2) is converted into ammonium (NH_4^+), which organisms are able to absorb (Logan and De Vos, 2011).

Most organisms, including bacteria, are dependent on iron since it is required in cellular processes such as aerobic respiration, amino acid and nucleotide biosynthesis (Daou *et al*., 2009; Logan and De Vos, 2011). Unlike other nutrients iron is not freely available and therefore bacteria developed specific mechanisms to obtain iron from their hosts (Ratlege and Dover, 2000). In insects, iron is stored and transported through carrier proteins such as transferrin, lactoferrin, ferritin or hemoproteins. Bacteria have developed two mechanisms to obtain iron from the iron-binding proteins within their host. By secreting siderophores that binds to the iron-binding proteins, iron is transferred from these proteins into the cytosol of the bacteria (Ratlege and Dover, 2000; Daou *et al*., 2009). After attaching to host iron-rich proteins by means of specialised surface receptors, bacteria interacts with membrane bound transporters and permeases, causing iron to transfer into the cytosol (Daou *et al*., 2009; Logan and De Vos, 2011). Daou and co-workers (2009) showed that *B. cereus* is able to acquire iron from haemoglobin, heme and ferritin through means of the surface protein, *IlsA* Iron-regulated leucine rich surface protein. This protein is restricted to the *B. cereus sensu lato* group, which includes *B. anthracis, B. thuringiensis* and *B. mycoides* (Daou *et al*., 2009). *Klebsiella* species also produce siderophores to obtain iron (Brisse *et al*., 2006). Iron is essential for colonisation and survival of bacteria. This mechanism enables bacteria to survive within hosts and facilitate the various functions to aid in the insect's survival and fitness. Species belonging to the *B. cereus sensu lato* group (Section 5.4) and *Klebsiella* were isolated from *B. fusca* larvae collected from all of the sampling sites. The predominance of these species may be a result of their ability to obtain iron from their host. In return, these species facilitate various roles that may promote the survival of *B. fusca* larvae. These potential roles are summarised in Figure 5.1.

5.5.1.3. Detoxification

Several microbial species are known to degrade environmental pollutants such as chlorophenols, xenobiotics, polycyclic aromatic hydrocarbons (PAHs), p-nitrophenol (PNP) and organophosphate compounds (Singh *et al*., 2004; Kallimanis *et al*., 2007; Liu *et al*., 2007; Sahoo *et al*., 2011; Busse *et al*., 2012). These compounds are abundant within the environment and are mainly used as herbicides, pesticides, fungicides, algaecides, insecticides and bactericides. By utilising these compounds as sole carbon and nitrogen sources, bacteria are able to reduce their toxicity. Biodegradation of harmful compounds has been observed in microbes such as *Acaligenes* spp., *Arthrobacter* spp., *Enterobacter* spp., *Caulobacter* spp., *Flavobacterium* spp., *Pseudomonas* spp., *Rhodococcus* spp., *Sphingomonas* spp. and *Stenotrophomonas* spp. (Lundgren *et al*., 2007; Kallimanis *et al*., 2007; Sahoo *et al*., 2011; Busse *et al*., 2012; Tang and You, 2012). The detoxification of insecticides and other harmful compounds, may add to an insects survival.

In the present study isolates of *Arthrobacter* sp., *Bacillus* sp., *Enterobacter* sp., *Pseudomonas* sp. and *Stenotrophomonas* sp. were identified. Pesticides may be applied when the Bt-maize is not sufficient in controlling *B. fusca* larvae. However, as mentioned in Section 2.2.4 chemical control of these larvae are redistricted by their feeding behaviour. If these bacteria are able to detoxify pesticides, then the larvae will be able to survive regardless of the control strategies. Thus, the presence of these isolates may aid in the survival of *B. fusca* and as a result making it more difficult to control.

Lundgren and co-workers (2007) reported the ability of an *Enterobacter* strain to degrade chlorpyrifos and organophosphate insecticides. The isolate utilises these harmful compounds as it sole carbon source (Singh *et al.*, 2004). *Enterobacter* species isolated from *B. fusca* larvae may also use harmful compounds as carbon sources, which may aid to the survival of larvae after insecticides are applied in maize fields.

Arthrobacters are ubiquitous and have been isolated from numerous environments, however it is considered as a soil bacterium (Busse *et al.*, 2012). Several *Arthrobacter* species have the potential to degrade harmful compounds mostly used in the production of insecticides, such as xenobiotics and PAHs including naphthalene, phenanthrene, nitrophenol, nitrocatechol, chlorobenzoate and chlorophenols (Kallimanis *et al.*, 2007; Sahoo *et al.*, 2011; Busse *et al.*, 2012). *Arthrobacter oxydans* is able to detoxify environments from the harmful effects of nicotine by utilising it as a sole carbon and nitrogen source (Li *et al.*, 2011). Although this bacterium was also isolated during the present study, no definite conclusion can be drawn in terms of the role it might have within *B. fusca*. *Arthrobacter oxydans* were present in larvae collected at 16 % of the sampling sites, which may imply that it was ingested from the environment and has no role or this bacterium may be limited to these sampling sites (see Appendix 4). Further investigations are required to determine the role *Arthrobacter* species may have within *B. fusca* larvae, and whether they are limited to a specific geographical area, and if they are, what the possible reasons might be.

Bacillus licheniformis and *Stenotrophomonas* sp. has the ability to degrade 3-phenoxybenzoic acid (3-PBA), which is a metabolite of the pesticides fenvalerate (Chen *et al.*, 2011) and cypermethrin (CY; Liu *et al.*, 2014). The latter is directly degraded by *Pseudomonas* sp. (Liu *et al.*, 2014). These bacteria have been associated with insects such as *M. sexta* (Brinkmann *et al.,* 2008), *Xylosandrus germanus* (Coleoptera: Curculionidae) (Kati and Kati, 2013), *R. ferrugineus* (Tagliavia *et al.*, 2014), *O. nubilalis* (Secil *et al.*, 2012) and *S. littoralis* (Özkan-Çakici *et al.*, 2014).

Stenotrophomonas maltophilia are Gram-negative bacteria and negative for H_2S production (Kati and Kati, 2013). It has been isolated from endophyte communities (Mano and Morisaki, 2008), and from insects such as *X. germanus* (Kati and Kati, 2013). Some species have the ability to degrade harmful pesticides such as PNP (Liu *et al*., 2007), CY (Liu *et al*., 2014), 3-PBA (Chen *et al*., 2011). Xiang and co-workers (2006) reported that *S. maltophilia* is one of the dominant members in the midgut of *H. armigera*. This is in contrast to the results obtained in the current study. *Stenotrophomonas maltophilia* was only isolated in very low numbers from larvae collected at two sites (Reitz and Vaalrivier), and therefore appears to have been a temporal inhabitant of the midgut. However, this bacterium may still have a role in the survival of *B. fusca* larvae since it may be present within their environment. In this manner *S. maltophilia* may detoxify harmful components that the larvae would have encountered otherwise. The scope of this study did not include the isolation of bacteria at the different sampling sites, no conclusions can therefore be drawn in this regard. Future investigations should also identify bacteria that occur within the environment where larvae are collected in order to determine whether these bacteria might have an indirect effect on the survival of *B. fusca.*

Insect diets may also contain toxic plant components, such as glucosides. Detoxification of these compounds is necessary before it can be hydrolysed. Gut microbes are considered to assist insects in neutralising these toxins, thus aiding in the digestion and adaptation of certain insect populations to new food sources (Engel and Moran, 2013). The activity of digestive enzymes was compared between *Tenebrio molitor* (Coleoptera: Tenebrionidae) larvae (mealworms) rid of gut microbes and consisting of gut microbes (Genta *et al*., 2006). No substantial differences were observed, suggesting that microbially derived enzymes are not essential within these insects. After exposing larvae to glucoside, variations occurred within enzyme profiles, and axenic (bacteria-free) larvae were not able to survive. Thus, the conclusion was drawn that gut microbes has a detoxifying role within mealworms (Genta *et al*., 2006).

5.5.2. Affect efficiency as disease vectors

Symbionts are directly and indirectly involved in the proficiency of an insect for being a parasite vector. By maintaining their immune system, microorganisms can improve the efficacy and vigour of insect vectors. Gut microbes can also facilitate the colonisation of parasites within insects, such as the *Enterobacteriaceae* member, *Sodalis glossinidius,* which produce *N*-acetyl glucosamine that favours the introduction of the parasite, *Trypanosoma* into the gut of the tsetse fly (Geiger *et al*., 2009).

The midgut microbiota of insect vectors can also inhibit the development of parasites. The elimination of the normal microbial community within *Anopheles* sp. showed that *Plasmodium falciparum* infection increased within the gut (Geiger *et al*., 2009). The production of prodigiosin (red pigment) by *S. marcescens* eradicates the *Trypanosoma cruzi* parasite in the gut of *Rhodnius prolixus* (Hemiptera: Reduviidae) vector (Azambuja *et al*., 2004).

From available literature, *B. fusca* is not considered as a disease vector, therefore this function will probably not apply to this insect. However, it provides additional information regarding the various roles that gut microbes may have within different insect orders.

5.5.3. Govern mating and reproductive systems within insects

Klebsiella pneumoniae, *P. agglomerans, E. cloaca*e and *E. casseliflavus* obtained from the midgut of *B. fusca* may also have a role in the production of phenols, which can be implemented as antimicrobial or pheromone components. Microbial metabolism of plant chemicals can produce essential compounds for the host insect. Gut microbes were shown to produce phenols from the digestion of allelochemicals. Phenols are also components of pheromones such as aggregation pheromones in *Schistocerca gregaria* (Orthoptera: Acrididae). Bacteria responsible for phenolic production in locusts are *P. agglomerans*, *K. pneumoniae*, *E. cloaca*e and *E. casseliflavus* (Dillon *et al*., 2002; Brisse *et al*., 2006). Aggregation pheromones increase the potential for mating, as it causes both male and female insects to gather at the source where it was released. Both male and female insects are able to release this pheromone (Gullan and Cranston, 2010). Aggregation pheromones have not been reported in Lepidoptera, however, these insects also release pheromones for reproductive proposes. *Danaus gilippus* (Lepidoptera: Nymphalidae) is only able to synthesise courtship pheromones after feeding on selected plants. These plants provide the required plant chemicals necessary to produce this pheromone. Similarly, *Creatonotus gangis* (Lepidoptera: Arctiidae) moths are able to produce pheromones after obtaining plant alkaloids from its host plant through the larval stages (Gullan and Cranston, 2010).

5.5.4. Develop and maintain host immune system

5.5.4.1. Protect against pathogens and predators

Competition among gut microbes to obtain nutrients can prevent colonisation of pathogens within the gut. Invasive species have to compete to obtain nutrients in order to survive. This process is referred to as colony resistance (CR; Dillon and Dillon, 2004; Rajagopal, 2009). As previously mentioned enteric gut microbes are required for *B. thuringiensis*-induced mortality (Broderick *et al.*, 2006), therefore CR in the microbial community of *B. fusca* may influence the effectiveness of Bt maize in controlling this pest. This phenomenon was demonstrated in *Homona magnanima* (Lepidoptera: Tortricidae) larvae, in which significantly more growth of *B. thuringiensis* was observed in aseptically reared larvae than in the larvae having the normal gut bacteria complex. Thus, the microbial community can inhibit or eliminate the growth of insect pathogens (Dillon and Dillon, 2004). This emphasises the importance of understanding the interactions between gut microbes and the outcomes it may have in terms of insect control.

Several bacteria produce phenolic compounds, which has antimicrobial and antifungal properties (Dillon and Dillon, 2004). Depending on the species, these compounds have different effects. Fungal diseases were able to infected axenic locusts (*S. gregaria*) whereas locusts consisting of their normal gut bacteria were not infected. Phenols were absent in the axenic (raised under sterile conditions) locusts, which suggest that gut microbes are responsible for producing antifungal phenols within the host insect. *Pantoea agglomerans* were found to produce one of the antifungal phenolic compounds associated with locusts reared under sterile conditions. Several bacterial species are required for complete protection against fungal diseases, since the presence of one species is inadequate. Antimicrobial phenols are selectively bactericidal and as a result, only indigenous bacteria are able to survive in most cases. Colony resistance also occurs against bacterial pathogens, as mentioned, such as *Serratia marcescens* (Dillon and Dillon, 2004).

In the present study *Solibacillus silvestris* and *Microbacterium testaceum* were isolated, both of which is known to degrade *N-Acyl*homoserine lactones (AHL; Wang *et al.*, 2010; Morohoshi *et al.*, 2012). A few Gram-positive bacteria have the ability to degrade AHL-lactones, which is used as quorum sensing (QS) signal molecules (cell-to-cell communication) in Gram-negative bacteria (Wang *et al.*, 2010). Collective behaviour within a bacterial population is stimulated through QS (Williams, 2007). The disruption of QS will constrain gene expression and growth within a microbial population. Species belonging to *Arthrobacter* sp., *Bacillus* sp., *Agrobacterium* sp., *Rhodococcus* sp., *Microbacterium* sp., *Solibacillus* sp. and *Chryseobacterium* sp. were found to produce AHL-lactonase, the enzyme responsible for AHL-degrading activity (Morohoshi *et al.*, 2012). Insect hosts can benefit from these bacteria,

because it may inhibit the expression of virulence and infection of invasive species through disrupting or manipulating the QS signals of these species (Wang *et al*., 2010). Therefore, AHL-degrading bacteria may promote its host defence against pathogens.

Microbacterium testaceum is an endophytic bacterium, commonly found within host plants without causing symptoms in these plants. Wang *et al*. (2010) reported the ability of *M. testaceum* to degrade AHL-lactones after isolating it from a potato leaf. Similarly, this bacterium may have a potential role in protecting its host from invasive pathogens. However, this bacterium was only recently associated with insects after Secil *et al*. (2012) obtained it from the lepidopteran maize pest, *O. nubilalis*. In addition, *M. testaceum* was isolated in low numbers during the current study. Therefore, in this study it seems more apparent that *M. testaceum* was obtained from the environment, and it may have no symbiotic relation with *B. fusca* larvae.

Solibacillus silvestris produces AHL-lactonase which is responsible for disrupting QS signalling of potential invasive species (Morohoshi *et al*., 2012). It was shown that *S. silvestris* are able to restrict the virulence of the plant pathogen *Pectobacterium carotovorum* subsp. *carotovorum* (Morohoshi *et al*., 2012). The results indicate that *S. silvestris* is not recurrent within the midgut structure; however, its AHL-degrading activity may aid *B. fusca* in antipathogenic activity.

Solibacillus silvestris was first described as *Bacillus silvestris* by Rheims *et al*. (1999). Other species belonging to the family *Planococcaceae* has been isolated from insects such as *D. helophoroides* (Wang *et al*., 2014). No literature is available on associations between *Solibacillus silvestris* and insects and this report of its occurrence in *B. fusca* larvae may be a first report.

Gut microbes may also protect the insect host from insect predators. Insect herbivores can either prevent or promote defence responses in plants through oral secretions when feeding. Some insects produce compounds such as *N*-acyl-amino acids that aids in digestion, but also induce plant responses that attracts predators. *Microbacterium arborescens* isolated in a previous study from *Spodoptera exigua* larvae were shown to synthesise *N*-acyl-amino acid hydrolase, which is able to prevent plant defence responses. Thus, preventing the attraction of predators and promoting the survival of the insect host (Engel and Moran, 2013). *Microbacterium arborescens* was also isolated during the present study. If this bacterium has the same function within *B. fusca* it may possibly promote the survival of these larvae by reducing the probability of natural enemies such as parasitoids detecting the feeding larvae. Stem borers, including *B. fusca*, are parasitised by several parasitoids as described in Section 2.2.4.

Several microbes, especially *Lactobacillus* species, demonstrate probiotic activity. This type of symbionts offers its host with benefits such as (1) stabilising the gut community, (2) producing antimicrobials, (3) inhibiting pathogenesis of harmful bacteria (4) and stimulating immune responses (Cappellozza *et al.*, 2011; Florou-Paneri *et al.*, 2013). Hosts will only benefit, after probiotic bacteria are established within the gut. However, these bacteria are easily disturbed through changes within the environment such as acidity, temperature and oxygen (Florou-Paneri *et al.*, 2013). Probiotic bacteria activity was also observed in *Enterococcus* sp. (Cappellozza *et al.*, 2011). During this study several species belonging to *Enterococcus* were isolated from the midgut (previously mentioned: p. 85), which may suggest similar functions within *B. fusca*.

5.5.4.2. Immune homeostasis

Homeostasis is maintained by eliminating pathogens without harming the indigenous gut microbiota (Lazzaro and Rolff, 2011; Buchon *et al.*, 2013). Insects are able to distinguish between harmful and beneficial bacteria by recognising molecules present in the cell walls of these bacteria, called microbe-associated molecular patterns (MAMPs; Lazzaro and Rolff, 2011). Thus, some insect species are able to control their gut microbiota for example *D. melanogaster* (Chandler *et al.*, 2011).

5.5.4.3. Development and fitness

Gut microbes can contribute to insect fitness and development either through direct interactions or as a result of their role in nutrient provision and digestion that facilitate the absorption of nutrients (Hosokawa *et al.*, 2006; Engel and Moran, 2013). Various roles have been suggested for *Enterococcus* species in insects including that they carry genes for antibiotic resistance (Macovei and Zurek, 2006), regulate parasite transmission (Azambuja *et al.*, 2005) and provide nutrients (Brinkmann *et al.,* 2008; Lundgren and Lehan, 2010). The presence of these species may facilitate in the life style of *B. fusca* in more than one way.

5.5.5. Insect pathogens

The importance of identifying insect pathogens was previously described in terms of developing microbial insecticides. A few known opportunistic pathogens have been isolated in this study. These are *Bacillus* sp., *Brevibacterium* sp., *Enterococcus* sp., *Klebsiella* sp., *Pseudomonas* sp. and *Serratia* sp.

Many *Bacillus* species, for example *B. cereus, B. sphaericus, B. lentimorbus, B. larvae* and *B. thuringiensis* are known as entomopathogenic bacteria (Fedhila *et al*., 2010; Özkan-Çakici *et al*., 2014). The latter is widely used to control agricultural pests belonging to several insect orders such as Lepidoptera, Diptera and Coleoptera (Federici, 1998; Broderick *et al*., 2006; Pigott and Ellar, 2007; Özkan-Çakici *et al*., 2014). *Bacillus thuringiensis* is a Gram-positive, spore-forming, motile bacterium generally associated with soil and plants (Federici, 1998; Ferré *et al*., 2008). In the absence of adequate nutrients this bacterium undergoes sporulation, which produces insecticidal toxins that are responsible for larval death (Knowles, 1994; Bravo and Soberón, 2008).

Bacillus thuringiensis was one of the dominant species identified in this study. This bacterium was present at all of the sampling sites (except at Vlakfontein) and represented 65 % of the isolates belonging to the phylum Firmicutes. Larvae could have merely ingested this bacterium from the environment due to its ubiquitous lifestyle. As mentioned, this bacterium will only produce insecticidal toxins when nutrients are unavailable. Thus, for as long as there are enough food sources available within the gut, *B. thuringiensis* will not sporulate (Knowles, 1994), and as a result will not induce toxicity to larvae. It is possible that *B. thuringiensis* is an indigenous member in the midgut, and because there is sufficient amount of nutrients and food sources available, it does not undergo sporulation. *Bacillus thuringiensis* may also be a symbiont in *B. fusca*.

Bacillus also includes several opportunistic pathogens such as *B. anthracis*. However, low mortality rates were observed in *Galleria mellonella* (Lepidoptera: Pyralidae) larvae infected with *B. anthracis* (Fedhila *et al*., 2010). *Bacillus megaterium* is an aerobic, spore-forming bacterium typically associated with soil and plants. It is frequently isolated from endophytic and rhizobacterial communities (Mano and Morisaki, 2008; Zou *et al*., 2010). Its general occurrence within insect habitats, make it apparent that *B. megaterium* is associated with several insect species. These include *L. canus* (Lacey *et al*., 2007) and *O. nubilalis* (Secil *et al*., 2012).

It has been suggested that bacteria that occur naturally within the gut or environment of insect pests can be used in biological control (Lacey *et al*., 2007; Özkan-Çakici *et al*., 2014). Early work by Lynch and co-workers (1976) showed the possibility of *B. megaterium* in controlling lepidopteran pests, after observing pathogenesis to the eggs of *O. nubilalis*. *Bacillus megaterium* strains can be modified in order to express insecticidal toxins from *B. thuringiensis* (Bora *et al*., 1994; Lacey *et al*., 2007). This modification of *B. megaterium* has been shown to be effective in controlling *H. armigera* (Bora *et al*., 1994). It is unlikely that the isolated *B. megaterium* strain in this study was pathogenic. Although this bacterium was present in low

numbers it may be a symbiont of *B. fusca* and therefore, has potential in biological control as it can be genetically transformed to express insecticidal toxins.

Although the genus *Brevibacterium* is rarely associated with insects, it has been reported to be pathogenic to insects. It was found that high numbers of *Brevibacterium* sp. caused mortality in various grasshopper species (Orthoptera: Caelifera) (Steinhause, 2012). These bacteria have been isolated from various habitats such as soil, sediment and marine environments. Kat and co-workers (2010) isolated a novel strain from the the lepidopteran, *Thaumetopoea pityocampa*, namely *Brevibacterium pityocampae*. Species from this genus were also isolated from *X. germanus* (Kati and Kati, 2013).

In this study *Brevibacterium frigoritolerans* were one of the predominant species isolated in *B. fusca.* It represented 6 % of the microbial composition and occurred at 37 % of the sampling sites. This is a Gram-positive, non-spore forming bacterium that produces acid from fructose, L-arabinose, D-glucose and D-xylose (Li *et al.*, 2014). As previously mentioned, acid production can cause a decrease in the gut pH, which may enable the activation of Cry toxins in the gut. However, *B. frigoritolerans* were found to be pathogenic to first instar larvae of *Anomala dimidiata* and *Holotrichia longipennis* (Scarabaeidae: Coleoptera) (Selvakumar *et al.*, 2011). Therefore, this bacterium may aid in digestion and possibility Bt-resistance by decreasing the pH, but it can also cause mortality especially if it is present in high numbers. Further research is required before any assumptions can be made regarding the role of *B. frigoritolerans* in *B. fusca*.

Based on rRNA gene sequences, it was found that *B. frigoritolerans* may have been misclassified and should rather be placed within the *Bacillus* group (Gelsomino *et al.,* 2004; Beesly *et al.*, 2010; Ludwig *et al.*, 2012; Ivy *et al.*, 2012).

Enterococcus mundtii is a pathogen of *B. mori* larvae, which is responsible for flacherie disease in larvae (Cappellozza *et al.*, 2011). This bacterium is able to rapidly colonise, in spite of the high alkalinity in the gut. Cappellozza and co-workers (2011) showed that *E. mundtii* are able to migrate into the haemocoel after reproducing within the gut. *Klebsiella* strains were also shown to cause mortality within Lepidoptera for example *S. littoralis* (Özkan-Çakici *et al.*, 2014).

Some *Pseudomonas* species for example *Pseudomonas aeruginosa* are pathogenic to insects (Fedhila *et al.*, 2010). Infections have been observed in several insects including *R. ferrugineus* (Banerjee and Dangar, 1995), *G. mellonella* (Jander *et al.*, 2000), *D. grandiosella*, *D. crambidoides* (Inglis *et al.*, 2000) and *S. littoralis* (Özkan-Çakici *et al.*, 2014).

S. marcescens is a nitrogen fixating bacterium that may aid its host in enhancing nutrition (Mano and Morisaki, 2008), although it is better known as a pathogen of various insect species. Some of the hydrolytic enzymes produced by *S. marcescens*, were found to be toxic to insects. Various authors observed high mortality rates after exposing different insect species to *S. marcescens*. These include *Rhagoletis pomonella* (Diptera: Tephritidae) (Lauzon *et al.*, 2003), *Oberia linearis* (Coleoptera: Cerambycidae) (Bahar and Demirbag, 2007), *Balaninus nucum* (Coleoptera: Curculionidae) (Sezen and Demirbag, 1999), *Rhynchites bacchus* (Coleoptera: Rhynchitidae) (Gokce *et al.*, 2010), *O. nubilalis* (Secil *et al.*, 2012), *D. grandiosella* and *D. crambidoides* (Inglis *et al.*, 2000). These bacteria can only induce mortality if they are able to enter the haemocoel, and rapidly increase (Lacey *et al.*, 2007; Özkan-Çakici *et al.*, 2014). If small numbers are present within the gut, it does not cause any immediate pathogenesis to its host.

Only a few isolates of *S. marcescens* were obtained from the midgut of *B. fusca*. It is possible that that the indigenous gut microbiota inhibit the growth of *S. marcescens* by means of CR (previously mentioned: p. 91), thus preventing infection from this bacterium. No conclusions can therefore be drawn on the potential role of this bacterium until the occurrence of pathogenesis is tested for *S. marcescens* to *B. fusca* larvae.

5.6. Species distribution

Busseola fusca larvae were collected from 30 geographically separate maize fields, and the microbial structure determined at each site. Variations in species richness and abundance at the respective sampling sites were observed. Microbial diversity was determined at each site by using the Shannon diversity index (Section 3.10). This index is widely accepted and generally used in diversity studies (Xiang *et al.*, 2006; Rani *et al.*, 2009; Robinson *et al.*, 2010; Zouache *et al.*, 2011; Arias-Cordero *et al.*, 2012; Köhler *et al.*, 2012; Chandel *et al.*, 2013; Wang *et al.*, 2014). The data were analysed in the same manner as for the morphotypes. Similarly, the mean Shannon values were used to determine whether ecological factors might influence the microbial diversity occurring at the respective sites (Table 4.4). Statistically, no significant ($p < 0.05$) differences were observed between the microbial structures identified at the various sites. However, variations in species richness and abundance occurred. In previous studies similar observations were reported when comparing microbial structures between different insect populations. These studies compared microbial diversities in *D. melanogaster* (Corby-Harris *et al.*, 2007) and mosquito populations (Zouache *et al.*, 2011) collected from geographically separate sites.

In literature, a rather simple community structure has been proposed for Lepidoptera larvae (Broderick *et al.*, 2004; Xiang *et al.*, 2006; Anand *et al.*, 2010; Robinson *et al.*, 2010; Hammer *et al.*, 2014). Fifty five bacterial species were identified from field-collected larvae during the present study. Several authors have reported higher microbial diversity within field-collected larvae in comparison with laboratory-reared larvae. It was reported that insects may encounter more microbes in natural environments than in controlled laboratory conditions (Xiang *et al.*, 2006; Belda *et al.*, 2011; Priya *et al.*, 2012; Hammer *et al.*, 2014). Additionally, Priya *et al.* (2012) established that the media and artificial diets used for laboratory experiments are an insufficient bacterial source for insects compared to a natural diet of crop plants. Several studies have reported similarities between gut microbiota and the microbes present within the environment (Zouache *et al.*, 2011; Priya *et al.*, 2012; Chandel *et al.*, 2013). Therefore, the variations in species richness and abundance observed during the present study may have resulted from the occurrence of different microbiota at the respective sampling sites.

5.6.1. Geographical differences

The gut microbes isolated from *B. fusca* larvae may affect several aspects regarding the behaviour and survival of this species (Section 5.5). However, environmental factors affecting the spatial variation and complexity of the microbial communities are required to better understand the interaction between these organisms. Although the scope of the study did not address the influence of environmental factors on gut microbes, it presented the need for further investigation.

A CCA was performed on the species distribution data (Appendix 4) and the direct distances (Appendix 5) between each site to determine whether geographical differences occurred between the microbial communities (Figure 4.11). The ordination diagram showed variations as well as consistencies in species composition between the respective sampling sites. The results from the CCA analysis suggest an overall similar community structure in *B. fusca* larvae, which is supported by similar Shannon diversity indices at the respective sampling sites. This is in accordance with the observations from the Shannon diversity analyses. The majority of the sites and species clustered together in Group B, this indicates that most of the sampling sites had a similar microbial composition. Refer to the script given in Section 4.5 in the results on Figure 4.11.

Interactions between species and their environment can be explained with ordination diagrams (Ter Braak, 1994; Lepš and Šmilauer, 2003). Ordination methods arrange points (representing sites) displaying similarities into distinctive groups. The distance between points indicates the degree of similarity between these points. Thus, sites with similar species compositions are

more closely grouped than conflicting sites (Ter Braak, 1994). Ordination methods are frequently used to interpret the association between organisms and their environment (Zouache *et al.*, 2011; Chandler *et al.*, 2011; Martinson *et al.*, 2012; Gimonneau *et al.*, 2014).

The results obtained from this study suggest that *B. fusca* larvae have a relative consistent microbial community structure over diverse geographical areas. Since all of the larvae were collected from maize plants, no diet alterations occurred between the individuals collected from the respective sampling sites. The species richness and abundance did vary between geographically separate sites. This may be as a result of different microbes present at the sites.

Most of the species located in Group B can be considered as indigenous members in the midgut. These include *Bacillus thuringiensis* (*Bac_thu*) and *Klebsiella pneumoniae* (*K_pne*) which both occurred in 97 % of the sites, *Klebsiella oxytoca* (*K_oxy*) which was isolated at 87 % of the sites and *Enterococcus gallinarum* (*Enc_gal*) which occurred in 80 % of the sampling sites. These species might have been obtained from the mother moths via the eggs. Vertical transmission of bacteria has been reported in different insect orders such as Coleoptera, Diptera and Lepidoptera (De Vries *et al.*, 2001; Brinkmann *et al.*, 2008). Brinkmann and co-workers (2008) reported that *Enterococcus* species in *M. sexta* larvae originated from the eggs. They also suggested that lepidopteran larvae in general could obtain various bacterial species by means of vertical transmission. Indigenous bacteria may therefore be obtained through this process in *B. fusca* as well.

Species located in Groups A and C, as well as species that are not situated in any of the groups may be transient bacteria. This refers to bacteria which are ingested together with food but do not colonise within the gut and therefore, are not transferred to the next generation (De Vries *et al.*, 2001). Thus, these species are only opportunistic inhabitants of the midgut. Possible transient species isolated from this study include *Serratia* sp. (*Serr_sp*) that was present in 17 % of the sites and *Microbacterium oxydans* (*Mbac_oxy*) that occurred at 13 % of the localities. Both of these species are known as endophytes, therefore larvae may have obtained these bacteria from their environment.

The observations from the present study are in accordance with a study conducted by Priya and co-workers (2012). In their study *H. armigera* larvae were collected from different host plants at the same site and from the same host plants at different sites. The gut microbes were compared between the respective larvae. The results showed more variations in microbial diversity when larvae are collected from different host plants rather than from the same plants at different localities. After analysing the leaf from which each larva was collected, it was found

that the microbial community was similar to that of the leaf phyllosphere. This suggests that the bacteria present on the leaf surfaces on which larvae feed, do influence the bacterial community in the midgut (Priya *et al.*, 2012).

Ecological factors may have a defining role in shaping the microbial community associated with insects. Zouache and co-workers (2011) reported an affiliation between the gut microbes and the habitat from which two mosquito species, *A. albopictus* and *A. aegypti*, were collected in Madagascar. The bacterial diversity within the mosquito populations was determined by using the Shannon diversity index. The differences that were observed was ascribed to the different vegetation and animals which mosquitos may encounter within the respective sites (Zouache *et al.*, 2011). As mentioned, insects can acquire gut microbes from their environment that may alter the microbial composition within the gut (Xiang *et al.*, 2006; Rani *et al.*, 2009; Priya *et al.*, 2012; Hammer *et al.*, 2014). Chandel and co-workers (2013) isolated several known soil and water bacteria from the midgut of *C. quinquefasciatus* collected from different localities. Similar to Zouache *et al.* (2011), microbial diversities differed among the sampling sites. It was suggested that the soil and water sources at the various sites, had a defining role in the microbial community structure in the respective mosquito populations (Chandel *et al.*, 2013). Thus, insects may encounter and obtain different microbes from different environments (Xiang *et al.*, 2006; Rani *et al.*, 2009; Belda *et al.*, 2011; Zouache *et al.*, 2011; Priya *et al.*, 2012; Hammer *et al.*, 2014).

CHAPTER 6
CONCLUSION AND RECOMMENDATIONS

6.1. Conclusion

The aim of the study was to further our knowledge on the microbial community structure within the midgut of *Busseola fusca* larvae.

The aim of this study was achieved by completing two specific objectives. The trends and conclusion for each objective will be discussed separately:

6.1.1. Identification of the microbial community structure within *Busseola fusca* larvae

Midgut contents were successfully isolated from *B. fusca* larvae as described in Section 3.3. Isolates were selected for identification based on colony morphology. Sequencing results revealed the presence of 55 species within the midgut that belonged to Firmicutes, Proteobacteria and Actinobacteria. Most of the isolated bacteria have been previously associated with other insect species, including various Lepidoptera species.

Gut microbes are known to facilitate the lifestyle and survival of various insect species. Although the symbiotic relation between lepidopteran species and their gut microbes are not well established, several studies have been conducted to better understand this affiliation. Figure 5.1, compiled from literature, provide a summary regarding the potential roles of gut microbes within these insects.

Overall, studies suggest that the microbial community within Lepidoptera is essential to the nutrition of these insects. The roles proposed for the isolated bacteria within other lepidopteran species are in accordance with the findings of this study. From Figure 5.1 it can be seen that most of the gut microbes may have a role in digestion, nutrient provision and / or detoxification of harmful components within the insect diet.

The ability of several bacterial species to alter their environment, may possibly aid in resistance occurring within *B. fusca* larvae. As mentioned, LAB such as *Enterococcus* species isolated in this study can decrease the pH value within the midgut, which might prevent the

activation of Bt toxins within larvae. Other bacteria may alter their environment by producing alkaline components that will increase the gut pH, which may facilitate toxin activation. Thus, it is of great importance to better understand the microbial interactions within the community structure of *B. fusca* larvae.

Several acid producing bacteria were isolated from the midgut of *B. fusca*, which should be of concern (Table 5.1). The diet of *B. fusca* larvae consists of plant material, which is rich in carbon sources such as cellulose, lignin and hemicellulose. These components consist of carbohydrates and sugars such as xylan, arabinose, xyloglucan, mannose, galactose and rhamnose. This is utilised by certain gut microbes that convert these carbohydrates and sugars into acid. The lifestyle of this pest provides essential carbon sources to its gut bacteria, which they convert into acid and other nutrients.

6.1.2. Comparison of microbial diversity at geographically separate sites

Shannon diversity indices were used to compare different factors that may have influenced the gut microbial diversity of the collected larvae. These factors included the type of production system, the type of maize as well as the production area from which larvae were collected. From this study no significant differences were observed between the microbial diversity of larvae collected from dry lands or irrigation, Bt and non-Bt maize and between the Western and Eastern parts of the sampling area (Section 4.4).

Canonical correspondence analysis revealed a relatively similar microbial composition among the respective sampling sites. However, trends were observed that suggest variations within species richness and abundance between the different sampling sites. Previous studies suggested that this observation is an indication that gut microbes may be obtained from the environment, for instance from leaf surfaces and within the stems where larvae feed. Therefore, some of the isolates may be indigenous within the midgut, while other bacteria only temporarily inhabit the gut.

It is important to understand the distribution and structure of microbial communities within insects and whether the gut community is influenced by the geographical distribution of the insects. A better understanding of the distribution of the insects and community structure of their gut microbiota may aid in the development of better insect control strategies.

6.2. Recommendations

The outcomes of the present study resulted in the following recommendations:

i) Biotic and abiotic environmental factors should be taken into account at different sampling sites in order to establish whether these factors may have an influence on the microbial diversity that occurs at the different sites. By determining the climate, agricultural practices, maize variety and the type of soil present within the environment from which larvae are collected, a better understanding of the possible influence that these factors may have can be obtained. In the current study variations were observed in the gut contents obtained from the different sampling sites in terms of bacterial composition and species richness. However, the environmental factors at these sites were not taken into account, therefore it was not possible to determine whether these variations were a result of the different environmental factors that larvae may have encountered at the respective sampling sites.

ii) The bacteria that colonise the leaves and stems from which *B. fusca* larvae are collected should be identified in order to determine which bacteria might be obtained from their environment. Co-speciation has occurred between some insects and certain indigenous gut microbes as a result of a specific role that these bacteria have within the insect host. On the other hand, some bacterial species are obtained from the environment. These microbes can either be known symbionts that are able to survive and colonise within the gut or they can be temporarily present without facilitating any beneficial role within the insects biology or survival. Therefore, the bacteria that may occur within the environment from which these insects are collected, should be determined in order to establish the affiliation between *B. fusca* and its gut microbes.

iii) Metagenomics and deep sequencing analyses will give more insight regarding the total microbial community structure in *B. fusca.* Through culture-independent methods the non-culturable bacteria will be identified. Additionally, by collecting more larvae at each sampling site and analysing the gut contents separately, a better understanding of the potential influence environmental factors may have on the microbial diversity and structure can be obtained.

iv) The biochemical characteristics of the bacteria isolated during this study should be investigated in order to determine their metabolic processes. This will provide information on the type of compounds that these bacteria may produce, and if it may cause pH variations within the gut. Several potential functions have been suggested for the bacteria isolated during this study, however further investigations are required before any conclusions can be drawn in this regard. Since various acid producing bacteria have been isolated during this study, it is

important to determine if these microbes are able to reduce the gut pH, and if this is the case, to establish whether it may have a potential role in resistance evolution of Lepidoptera species against Bt crops, for example *B. fusca* to Bt maize.

v) The isolated strains of *B. thuringiensis* and *B. cereus* should be characterised. These bacteria cannot be distinguished based on 16S gene sequences alone, but can be separated based on their ability to produce insecticidal toxins. The genes encoding for this ability occur in plasmids which is present on the cell membrane. During this study *B. thuringiensis* and *B. cereus* were isolated, and it is of importance to separate these species and to determine their affiliations with *B. fusca*.

REFERENCES

Adams, I. P., Glover, R. H., Monger, W. A., Mumford, R., Jackeviciene, E., Navalinskiene, M., Samuitiene, M. and Boonham, N. 2009. Next-generation sequencing and metagenomic analysis: a universal diagnostic tool in plant virology. *Molecular Plant Pathology* 10(4): 537-545.

Adesiyun, A. A. 1983. Some effects of intercropping of sorghum, millet and maize on infestation by lepidopterous stalk-borers, particularly *Busseola fusca. Insect Science and its Application* 4(4): 387-391.

Adeyemo, R. 1984. Maize-consumption expenditure of rural and urban workers: implications for Nigeria. *The Journal of Modern African Studies* 22(1): 163-166.

Akhurst, R. J., James, W. J., Bird, L. J. and Beard C. 2003. Resistance to the Cry1Ac δ-endotoxin of *Bacillus thuringiensis* in the cotton bollworm, *Helicoverpa armigera* (Lepidoptera: Noctuidae). *Journal of Economic Entomology* 96(4): 1290-1299.

Al-Awandhi, H., Dashti, N., Khanafer, M., Al-Mailem, D., Ali, N. and Radwan, S. 2013. Bias problems in culture-independent analysis of environmental bacterial communities: a representative study on hydrocarbonoclastic bacteria. *SpringerPlus* 2: 369-380.

Allen, H. K., Cloud-Hansen, K. A., Wolinski, J. M., Guan, C., Greene, S., Lu, S., Boeyink, M., Broderick, N. A., Raffa, K. F. and Handelsman, J. 2009. Resident microbiota of the gypsy moth midgut harbors antibiotic resistance determinants. *DNA and Cell Biology* 28(3): 109-117.

Allen, M. J., Edberg, S. C. and Reasoner, D. J. 2004. Heterotrophic plate count bacteria - what is their significance in drinking water? *International Journal of Food Microbiology* 92: 265-274.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. 1997. Gapped BLAST and PSIBLAST: a new generation of protein database search programs. *Nucleic Acids Research* 25(17): 3389-3402.

Anand, A. A. P., Vennison, S. J., Sanker, S. G., Pradhu, D. I. G., Vasan, P. T., Raghuraman, T., Geoffrey, C. J. and Vendan, S. E. 2010. Isolation and characterization of bacteria from the gut of *Bombyx mori* that degrade cellulose, xylan, pectin and starch and their impact on digestion. *Journal of Insect Science* 10(107). doi: 10.1673/031.010.10701.

Anklam, E., Gadani, F., Heinze, P., Pijnenburg. H. and Van den Eede, G. 2002. Analytical methods for detection and determination of genetically modified organisms in agricultural crops and plant-derived food products. *European Food Research and Technology* 214(1): 3-26.

Appel, H. M. and Maines, L. W. 1995. The influence of host plant on gut conditions of gypsy moth (*Lymantria dispar*) caterpillars. *Journal of Insect Physiology* 41(3): 241-246.

Arias-Cordero, E., Ping, L., Reichwald, K., Delb, H., Platzer, M. and Boland, W. 2012. Comparative evaluation of the gut microbiota associated with the below- and above-ground life stages (larvae and beetles) of the forest cockchafer, *Melolontha hippocastani*. *PLoS ONE* 7(12). doi: 10.1371/journal.pone.0051557.

Azambuja, P., Feder, D. and Garcia, E. 2004. Isolation of *Serratia marcescens* in the midgut of *Rhodnius prolixus*: impact on the establishment of the parasite *Trypanosoma cruzi* in the vector. *Experimental Parasitology* 107(1/2): 89-96.

Bagwan, J. D., Patil, S. J., Mane, A. S., Kadam, V. V. and Vichare, S. 2010. Genetically modified crops: Food of the future (review). *International Journal of Advanced Biotechnology and Research* 1(1): 21-30.

Bahar, A. A. and Demirbag, Z. 2007. Isolation of pathogenic bacteria from Oberea linearis (Coleoptera: Cerambycidae). *Biologia* 62: 13-18.

Bai, Y. Y., Yan, R. H., Ye, G. Y., Huang, F. N. and Chen, J. A. 2010. Effects of transgenic rice expressing *Bacillus thuringiensis* Cry1Ab protein on ground-dwelling Collembolan community in postharvest seasons. *Environmental Entomology* 39(1): 243-251.

Bailey, J., Scott-Dupree, C., Harris, R., Tolman, J. and Harris, B. 2005. Contact and oral toxicity to honey bees (*Apis mellifera*) of agents registered for use for sweet corn insect control in Ontario, Canada. *Apidologie* 36: 623-633.

Baldauf, S. L. 2003. Phylogeny for the faint of heart: a tutorial. *TRENDS in Genetics* 19(6): 345-351.

Banerjee, A. and Dangar, T. K. 1995. *Pseudomonas aeruginosa*, a facultative pathogen of red palm weevil, *Rhynchophorus ferrugineus. World Journal of Microbiology and Biotechnology* 11(6): 618-620.

Bartholomew, J. W. and Mittwer, T. 1952. The gram stain. *Microbiology and Molecular Biology Reviews* 16(1): 1-29.

Basset, Y., Cizek, L., Cuénoud, P., Didham, R. K., Guilhaumon, F., Missa, O., Novotny, V., Ødegaard, F., Roslin, T., Schmidl, J., Tishechkin, A. K., Winchester, N. N., Roubik, D. W., Aberlenc, H., Bail, J., Barrios, H., Bridle, J. R., Castaño-Meneses, G., Corbara, B., Curletti, G., Duarte da Rocha, W., De Bakker, D., Delabie, J. H. C., Dejean, A., Fagan, L. L., Floren, A., Kitching, R. L., Medianero, E., Miller, S. E., Gama de Oliveira, E., Orivel, J., Pollet, M., Rapp, M., Ribeiro, S. P., Roisin, Y., Schmidt, J. B., Sørensen, L. and Leponce, M. 2012. Arthropod diversity in a tropical forest. *Science* 338: 1481-1484.

Bauer, S., Tholen, A., Overmann, J. and Brune, A. 2000. Characterization of abundance and diversity of lactic acid bacteria in the hindgut of wood- and soil-feeding termites by molecular and culture-dependent techniques. *Archives of Microbiology* 173: 126-137.

Bauman, P. 2005. Biology of bacteriocyte-associated endosymbionts of plant sap-sucking insects. *Annual Review of Microbiology* 59: 155-189.

Beesley, C. A., Vanner, C. L., Helsel, L. O., Gee, J. E. and Hoffmaster, A. R. 2010. Identifcation and characterization of clinical *Bacillus* spp. isolates phenotypically similar to *Bacillus anthracis*. *FEMS Microbiology Letters* 313: 47-53.

Begon, M., Townsend, C. R. and Harper, J. L. 2006. In: Ecology: From individuals to ecosystems. 4th Ed. Oxford: Blackwell Publishing. Pp. 738.

Behmer, S. T. and Nes, W. D. 2003. Insect sterol nutrition and physiology: a global overview. *Advances in Insect Physiology* 31: 1-72.

Belda, E., Pedrola, L., Pereto, J., Martínez-Blanch, J. F., Montagud, A., Navarro, E., Urchueguía, J., Ramón, D., Moya, A. and Porcar, M. 2011. Microbial diversity in the midguts of field and lab-reared populations of the European corn borer *Ostrinia nubilalis*. *PLoS ONE* 6(6). doi:10.1371/journal.pone.0021751.

Ben-Dov, E. 2014. *Bacillus thuringiensis* subsp. *israelensis* and its dipteran-specific toxins. *Toxins* 6(4): 1222-1243.

Berchtold, M. and König, H. 1996. Phylogenetic analysis and *in situ* identification of uncultivated spirochetes from the hindgut of the termite *Mastotermes darwiniensis*. *Systematic and Applied Microbiology* 19: 66-73.

Beveridge, T. J. 1999. Structures of Gram-negative cell walls and their derived membrane vesicles. *Journal of Bacteriology* 181(16): 4725-4733.

Bora, R. S., Murty, M. G., Shenbagarathai, R. and Vaithilingam, S. 1994. Introduction of a Lepidoptera-specific insecticidal crystal protein gene of *Bacillus thuringiensis* subsp. *kurstaki* by conjugal transfer into a *Bacillus megaterium* strain that persists in the cotton phyllosphere. *Applied and Environmental Microbiology* 60: 214-222.

Bravo, A. and Soberón, M. 2008. How to cope with insect resistance to Bt toxins? *TRENDS in Biotechnology* 26(10): 573-579.

Bravo, A., Gill, S. S. and Soberón, M. 2007. Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. *Toxicon* 49: 423-435.

Bravo, A., Likitvivatanavong, S., Gill, S. S. and Soberón, M. 2011. *Bacillus thuringiensis*: A story of a successful bioinsecticide. *Insect Biochemistry and Molecular Biology* 41: 423-431.

Brennan, Y., Callen, W. N., Christoffersen, L., Dupree, P., Goubet, F., Healey, S., Hernández, M., Keller, M., Li, K., Palackal, N., Sittenfeld, A., Tamayo, G., Wells, S., Hazlewood, G. P., Mathur, E. J., Short, J. M., Robertson, D. E. and Steer, B. A. 2004. Unusual microbial xylanases from insect guts. *Applied and Environmental Microbiology* 70(6): 3609-3617.

Breznak, J. A. 2000. Ecology of prokaryotic microbes in the guts of wood-and litter-feeding termites. (*In* Abe, T., Bignell, D. E. and Higashi, M. *eds*. Termites: evolution, sociality, symbioses, ecology. Kluwer Academic Publishers: The Netherlands. Pp. 209-231).

Brink, D. E., Bezuidenhout, C. C. and Van den Berg, J. 2011. Diversity of gut-microorganisms in Bt-resistant and Bt-susceptible larvae of the maize stem borer (*Busseola fusca*) (Lepidoptera: Noctuidae). Potchefstroom: NWU. (Thesis-MSc).

Brinkmann, N., Martens, R. and Tebbe, C. C. 2008. Origin and diversity of metabolically active gut bacteria from laboratory-bred larvae of *Manduca sexta* (Sphingidae, Lepidoptera, Insecta). *Applied and Environmental Microbiology* 74(23): 7189-7196.

Brisse, S., Grimont, F. and Grimont, P. A. D. 2006. The genus *Klebsiella*. *Prokaryotes* 6: 159-196.

Broderick, N. A., Goodman, R. M., Handelsman, J. and Raffa, K. F. 2003. Effect of host diet and insect source on synergy of gypsy moth (Lepidoptera: Lymantriidae) mortality to *Bacillus thuringiensis* subsp. *kurstaki* by zwittermicin A. *Environmental Entomology* 32(2): 387-391.

Broderick, N. A., Raffa, K. F. and Handelsman, J. 2006. Midgut bacteria required for *Bacillus thuringiensis* insecticidal activity. *Proceedings of the National Academy of Sciences of the USA* 103(41): 15196-15199.

Broderick, N. A., Raffa, K. F., Goodman, R. M. and Handelsman, J. 2004. Census of the bacterial community of the gypsy moth larval midgut by using culturing and culture-independent methods. *Applied and Environmental Microbiology* 70(1): 293-300.

Broderick, N. A., Robinson, C. J., McMahon, M. D., Holt, J., Handelsman, J. and Raffa, K. F. 2009. Contributions of gut bacteria to *Bacillus thuringiensis*-induced mortality vary across a range of Lepidoptera. *BMC Biology* 7(11). doi:10.1186/1741-7007-7-11.

Buchon, N., Broderick, N. A. and Lamaitre, B. 2013. Gut homeostasis in a microbial world: insights from *Drosophila melanogaster*. *Nature Reviews Microbiology* 11: 615-626.

Busse, H. J. 2012. Phylum XXVI. Actinobacteria phyl. Nov. (*In* Goodfellow, M., Kämpfer, P., Busse, H. J., Trujillo, M. E., Suzuki, K., Ludwig, W. and Whitman, W. B., *eds*. Volume 5 of Bergey's Manual of Systematic Bacteriology. Springer: London. Pp. 571-578).

Busse, H. J., Wieser, M. and Buczolits, S. 2012. Phylum XXVI. Actinobacteria phyl. Nov. (*In* Goodfellow, M., Kämpfer, P., Busse, H. J., Trujillo, M. E., Suzuki, K., Ludwig, W. and Whitman, W. B., *eds*. Volume 5 of Bergey's Manual of Systematic Bacteriology. Springer: London. Pp. 578-624).

Butera, G., Ferraro, C., Colazza, S., Alonzo, G. and Quatrini, P. 2012. The culturable bacterial community of frass produced by larvae of *Rhynchophorus ferrugineus* Olivier (Coleoptera: Curculionidae) in the Canary island date palm. *Letters in Applied Microbiology* 54: 530-536.

Butler, J. F., Garcia-Maruniak, A., Meek, F. and Maruniak, J. E. 2010. Wild Florida house flies (*Musca domestica*) as carriers of pathogenic bacteria. *Florida Entomologist* 93(2): 218-223.

Calatayud, P. A., Le Rü, B., Van den Berg, J. and Schulthess, F. 2014. Ecology of the African maize stalk borer, *Busseola fusca* (Lepidoptera: Noctuidae) with special reference to insect-plant interactions. *Insects* 5: 539-563.

Calatayud, P., Guénégo, H., Le Rü, B., Silvain, J. and Frérot, B. 2007. Temporal patterns of emergence, calling behaviour and oviposition period of the maize stem borer, *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae). *Annales de la Société Entomologique de France (NS)* 43(1): 63-68.

Cannon, R. J. C. 2000. Bt transgenic crops: Risks and benefits. *Integrated Pest Management Reviews* 5: 151-173.

Cappellozza, S., Saviane, A., Tettamanti, G., Squadrin, M., Vendramin, E., Paolucci, P., Franzetti, E. and Squartini, A. 2011. Identification of *Enterococcus mundtii* as a pathogenic agent involved in the ‘‘flacherie’’ disease in *Bombyx mori* L. larvae reared on artificial diet. *Journal of Invertebrate Pathology* 106: 386-393.

Cardwell, K., Schulthess, F., Ndemah, R. and Ngoko, Z. 1997. A systems approach to assess crop health and maize yield losses due to pests and diseases in Cameroon. *Agriculture, Ecosystems and Environment* 65: 33-47.

Carpenter, J. E. 2011. Impact of GM crops on biodiversity. *GM Crops* 2(1): 7-23.

Carstens, A., Bartie, C., Dennis, R. and Bezuidenhout, C. 2014. Antibiotic resistant heterotrophic plate count bacteria and amoeba resistant bacteria in aquifers of the Mooi River, North West province, South Africa. *Journal of Water and Health.* In Press. doi:10.2166/wh.2014.226.

Chabi-Olaye, A., Nolte, C., Schulthess, F. and Borgemeister, C. 2005. Relationships of intercropped maize, stem borer damage to maize yield and land-use efficiency in the humid forest of Cameroon. *Bulletin of Entomological Research* 95: 417-427.

Chabi-Olaye, A., Schulthess, F., Poehling, H. M. and Borgemeister, C. 2001. Factors affecting the biology of *Telenomus isis* (Polaszek) (Hymenoptera: Scelionidae), an egg parasitoid of cereal stem borers in West Africa. *Biological Control* 21: 44-54.

Chandel, K., Mendki, M. J., Parikh, R. Y., Kulkarni, G., Tikar, S. N., Sukumaran, D., Prakash, S., Parashar, B. D., Shouche, Y. S. and Veer, V. 2013. Midgut microbial community of *Culex quinquefasciatus* mosquito populations from India. *PLoS ONE* 8(11). doi: 10.1371/journal.pone.0080453.

Chandler, J. A., Lang, J. M., Bhatnagar, S., Eisen, J. A. and Kopp, A. 2011. Bacterial communities of diverse *Drosophila* species: ecological context of a host–microbe model system. *PLoS Genetics* 7(9). doi: 10.1371/journal.pgen.1002272.

Cheeke, T. E., Rosentiel, T. N. and Cruzan, M. B. 2012. Evidence of reduced arbuscular mycorrhizal fungal colonization in multiple lines of Bt maize. *American Journal of Botany* 99(4): 700-707.

Chen, M., Zhao, J., Shelton, A. M., Cao, J. and Earle, E. D. 2008. Impact of single-gene and dual-gene Bt broccoli on the herbivore *Pieris rapae* (Lepidoptera: Pieridae) and its pupal endoparasitoid *Pteromalus puparum* (Hymenoptera: Pteromalidae). *Transgenic Research* 17: 545-555.

Chen, S. H., Yang, L., Hu, M. Y. and Liu, J. J. 2011. Biodegradation of fenvalerate and 3-phenoxybenzoic acid by a novel *Stenotrophomonas* sp. strain ZS-S-01 and its use in bioremediation of contaminated soils. *Applied Microbiology and Biotechnology* 90: 755-767.

Cominelli, E. and Tonelli, C. 2010. Transgenic crops coping with water scarcity. *New Biotechnology* 27(5): 473-477.
Conn, H. J. and Pelczar, M. J. 1957. Manual of microbiological methods. McGraw-Hill Book: New York.

Corby-Harris, V., Pontaroli, A. C., Shimkets, L. J., Bennetzen, J. L., Habel, K. E. and Promislow, D. E. L. 2007. Geographical distribution and diversity of bacteria associated with natural populations of *Drosophila melanogaster. Applied and Environmental Microbiology* 73(11): 3470-3479.

Cotter, P. D. and Hill, C. 2003. Surviving the acid test: responses of Gram-positive bacteria to low pH. *Microbiology and Molecular Biology Reviews* 67(3): 429-453.

Cox, C. R. and Gilmore, M. S. 2007. Native microbial colonization of *Drosophila melanogaster* and its use as a model of *Enterococcus faecalis* pathogenesis. *Infection and Immunity* 75(4): 1565-1576.

Daou, N., Buisson, C., Gohar, M., Vidic, J., Bierne, H., Kallassy, M., Lereclus, D. and Nielsen-LeRoux, C. 2009. IlsA, a unique surface protein of *Bacillus cereus* required for iron acquisition from heme, hemoglobin and ferritin. *PloS Pathogens* 5(11). doi: 10.1371/journal.ppat.1000675.

Davidson, E. W., Rosell, R. C. and Hendrix, D. L. 2000. Culturable bacteria associated with the whitefly, *Bemisia argentifolii* (Homoptera: Aleyrodidae). *Florida Entomologist* 82: 159-171.

De Maagd, R. A., Bravo, A. and Crickmore, N. 2001. How *Bacillus thuringiensis* has evolved specific toxins to colonize the insect world. *TRENDS in Genetics* 17(4): 193-199.

De Vaufleury, A., Kramarz, P. E., Binet, P., Cortet, J., Caul, S., Andersen, M. N., Plumey, E., Coeurdassier, M. and Krogh, P. H. 2007. *Pedobiologia* 51: 185-194.

De Vries, E. J., Breeuwer, J. A. J., Jacobs, G. and Mollema, C. 2001. The association of Western flower thrips, *Frankliniella occidentalis,* with a near *Erwinia* species gut bacteria: transient or permanent? *Journal of Invertebrate Pathology* 77: 120-128.

Delalibera, I., Vasanthakumar, A., Burwitz, B. J., Schloss, P. D., Klepzig, K. D., Handelsman, J. and Raffa, K. F. 2007. Composition of the bacterial community in the gut of the pine engraver, *Ips pini* (Say) (Coleoptera) colonizing red pine. *Symbiosis* 43: 97-104.

Deng, C., Peng, Q., Song, F. and Lereclus, D. 2014. Regulation of *cry* gene expression in *Bacillus thuringiensis*. *Toxins* 6(7): 2194-2209.

Dia, X., Wang, Y. N., Wang, B. J., Liu, S. J. and Zhou, Y. G. 2005. *Planomicrobium chinense* sp. nov., isolated from coastal sediment, and transfer of *Planococcus psychrophilus* and *Planococcus alkanoclasticus* to *Planomicrobium* as *Planomicrobium psychrophilum* comb. nov. and *Planomicrobium alkanoclasticum* comb. nov. *International Journal of Systematic and Evolutionary Microbiology* 55: 699-702.

Díaz-Mendoza, M., Ortego, F., García de Lacoba, M., Magaña, C., De la Poza, M., Farinós, G. P., Castaera, P. and Hernández-Crespo, P. 2005. Diversity of trypsins in the Mediterranean corn borer *Sesamia nonagrioides* (Lepidoptera: Noctuidae), revealed by nucleic acid sequences and enzyme purification. *Insect Biochemistry and Molecular Biology* 35: 1005-1020.

Dickson, P. R. 2009. Secondary diversity: Ecological and spectral dimensions of secondary succession following smallholder cultivation in the southern Yucatán. Massachusetts: Clark University (Dissertation- PhD).

Dillon, R. J. and Dillon, V. M. 2004. The gut bacteria of insects: Nonpathogenic interactions. *Annual Review of Entomology* 49: 71-92.

Dillon, R. J., Vennard, C. T. and Charnley, A. K. 2002. A note: gut bacteria produce components of a locust cohesion pheromone. *Journal of Applied Microbiology* 92: 759-763.

Dillon, R. J., Webster, G., Weightman, A. J. and Charnley, A. K. 2010. Diversity of gut microbiota increases with aging and starvation in the desert locust. *Antonie van Leeuwenhoek* 97: 69-77.

Donachie, S. P., Foster, J. S. and Brown, M. V. 2007. Culture clash: challenging the dogma of microbial diversity. *The International Society for Microbial Ecology Journal* 1: 97-99.

Dowd, P. 1989. In situ production of hydrolytic detoxifying enzymes by symbiotic yeasts in the cigarette beetle (Coleoptera: Anobiidae). *Journal of Economic Entomology* 82(2): 396-400.

Ebenebe, A. A., Van den Berg, J. and Van der Linde, T. C. 1999. Distribution and relative abundance of stalk borers of maize and sorghum in Lesotho. *African Plant Protection* 5*:* 77-82.

Egert, M., Stingl, U., Bruun, L. D., Pommerenke, B., Brune, A. and Friedrich, M. W. 2005. Structure and topology of microbial communities in the major gut compartments of *Melolontha melolontha* larvae (Coleoptera: Scarabaeidae). *Applied and Environmental Microbiology* 71(8): 4556-4566.

Egert, M., Wagner, B., Lemke, T., Brune, A. and Friedrich, M. W. 2003. Microbial community structure in midgut and hindgut of the humus-feeding larva of *Pachnoda ephippiata* (Coleoptera: Scarabaeidae). *Applied and Environmental Microbiology* 69(11): 6659-6668.

Ehler, L. E. 2006. Perspectives - Integrated pest management (IPM): definition, historical development and implementation, and the other IPM. *Pest Management Science* 62: 787-789.

Eichler, S. and Schaub, G. A. 2002. Development of symbionts in Triatomine bugs and the effects of infections with Trypanosomatids. *Experimental Parasitology* 100: 17-27.

Engel, P. and Moran, N. A. 2013. The gut microbiota of insects - diversity in structure and function. *FEMS Microbiology* 37: 699-736.

Fandohan, P., Hell, K., Marasas, W. F. O. and Wingfield, M. J. 2003. Infection of maize by Fusarium species and contamination with fumonisin in Africa. *African Journal of Biotechnology* 2(12): 570-579.

FAO (Food and Agriculture Organization). 1996. Rome declaration on world food security and world food summit plan of action. http://www.fao.org/DOCREP/003/W3613E/W3613E00.HTM. Date of access: 9 November. 2013.

FAOSTAT. 2013. Food and agricultural commodities production. http://faostat.fao.org/site/339/default.aspx. Date of access: 27 March. 2014.

Federici, B. A. 1998. Transgenic Bt crops and resistance: Broadscale use of pest-killing plants to be true test. *California Agriculture* 52(6): 14-20.

Federici, B. A., Park, H. and Bideshi, D. K. 2010. Overview of the basic biology of *Bacillus thuringiensis* with emphasis on genetic engineering of bacterial larvicides for mosquito control. *The Open Toxicology Journal* 3: 154-171.

Fedhila, S., Buisson, C., Dussurget, O., Serror, P., Glomski, I. J., Liehl, P., Lereclus, D. and Nielsen-LeRoux, C. 2010. Comparative analysis of the virulence of invertebrate and mammalian pathogenic bacteria in the oral insect infection model *Galleria mellonella*. *Journal of Invertebrate Pathology* 103: 24-29.

Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* 39: 783-791.

Ferré, J. and Van Rie, J. 2002. Biochemistry and genetics of insect resistance to *Bacillus thuringiensis*. *Annual Review of Entomology* 47: 501-533.

Ferré, J., Van Rie, J. and Macintosh, S. C. 2008. Chapter 3: Insecticidal genetically modified crops and insect resistant management. (*In* Romeis, J., Shelton, A. M. and Kennedey, G. G. *eds*. Integration of insect-resistant genetically modified crops within IPM programs. Springer. Pp. 450).

Firbank, L. G., Heard, M. S., Woiwod, I. P., Hawes, C., Haughton, A. J., Champion, G. T., Scott, R. J., Hill, M. O., Dewar, A. M., Squires, G. R., May, M. J., Brooks, D. R., Bohan, D. A., Daniels, R. E., Osborne, J. L., Roy, D. B., Black, H. I. J., Rothery, P. and Perry, J. N. 2003. An introduction to the farm-scale evaluations of genetically modified herbicide-tolerant crops. *Journal of Applied Ecology* 40: 2-16.

Florou-Paneri, P., Christaki, E. and Bonos, E. 2013. Lactic acid bacteria as source of functional ingredients (*In* Kongo, J. M. *ed*. Lactic Acid Bacteria - R and D for Food, Health and Livestock Purposes. Pp. 589-614).

Frank, J. A., Reich, C. I., Sharma, S., Weisbaum, J. S., Wilson, B. A. and Olsen, G. J. 2008. Critical evaluation of two primers commonly used for amplification of bacterial 16S rRNA genes. *Applied and Environmental Microbiology* 74(8): 2461-2470.

Fukatsu, T. and Nikoh, N. 2000. Endosymbiotic microbiota of the bamboo pseudococcid *Antonina crawii* (Insecta, Homoptera). *Applied and Environmental Microbiology* 66(2): 643-650.
Geiger, A., Fardeau, M. L., Grebaut, P., Vatunga, G., Josénando, T., Herder, S., Cuny, G., Truc, P. and Ollivier, B. 2009. *Infection, Genetics and Evolution* 9: 1364-1370.

Gelsomino, R., Vancanneyt, M., Vandekerckhove, T. and Swings, J. 2004. Development of a 16S rRNA primer for the detection of *Brevibacterium* spp. *Letters in Applied Microbiology* 38(6): 532-535.

Genta, F. A., Dillon, R. J., Terra, W. R. and Ferreira, C. 2006. Potential role for gut microbiota in cell wall digestion and glucoside detoxification in *Tenebrio molitor* larvae. *Journal of Insect Physiology* 52: 593-601.

George, D. M., Rind, C. F., Bendall, M. W., Taylor, M. A. and Gatehouse, A. M. R. 2011. Developmental studies of transgenic maize expressing Cry1Ab on the African stem borer, *Busseola fusca*; effects on midgut cellular structure. *Pest Management Science* 68: 330-339.

Gibson, C. M. and Hunter, M. S. 2010. Extraordinarily widespread and fantastically complex: comparative biology of endosymbiotic bacterial and fungal mutualists of insects. *Ecology Letters* 13: 223-234.

Gimonneau, G., Tchioffo, M. T., Abate, L., Boissiére, A., Awono-Ambéne, P. H., Nsango, S. E., Christen, R. and Morlais, I. 2014. Composition of *Anopheles coluzzii* and *Anopheles gambiae*

microbiota from larval to adult stages. *Infection, Genetics and Evolution.* doi:10.1016/j.meegid.2014.09.029.

Gokce, C., Sevim, A., Demirbag, Z. and Demir, I. 2010. Isolation, characterization and pathogenicity of bacteria from *Rhynchites bacchus* (Coleoptera: Rhynchitidae). *Biocontrol Science and Technology* 20: 973-982.

Gómez, I., Sánchez, J., Muńoz-Garay, C., Matus, V., Gill, S. S., Soberón, M. and Bravo, A. 2014. *Bacillus thuringiensis* Cry1Ab toxins are versatile-proteins with multiple modes of action: Two distinct pre-pores are involved in toxicity. *Biochemical Journal* 459: 383-396.

Goodfellow, M. 2012. Phylum XXVI. Actinobacteria phyl. Nov. (*In* Goodfellow, M., Kämpfer, P., Busse, H. J., Trujillo, M. E., Suzuki, K., Ludwig, W. and Whitman, W. B. *eds.* Volume 5 of Bergey's Manual of Systematic Bacteriology. Springer: London. Pp. 33-34).

Gould, F., Martinez-Ramirez, A., Anderson, A., Ferré, J., Silva, F. J. and Moar, W. J. 1992. Broad-spectrum resistance to *Bacillus thuringiensis* toxins in *Heliothis virescens*. *Proceedings of the National Academy of Sciences of the USA* 89: 7986-7990.

Gounou, S. and Schulthess, F. 2006. Effect of traditional rice/maize intercropping on population densities, crop damage and parasitism of stem-borers in the Ivory coast. *African Plant Protection* 12: 93-102.

Gressel, J., Hanafi, A., Head, G., Marasas, W., Obilana, A. B., Ochanda, J., Souissi, T. and Tzotzos, G. 2004. Major heretofore intractable biotic constraints to African food security that may be amenable to novel biotechnological solutions. *Crop Protection* 23: 661-689.

Gringorten, J. L., Crawford, D. N. and Harvey, W. R. 1993. High pH in the ectoperitrophic space of the larval lepidopteran midgut. *The Journal of Experimental Biology* 183: 353-359.

Groote, H. 2002. Maize yield losses from stemborers in Kenya. *Insect Science and its Application* 22(2): 89-96.

Gu, Y., Ding, Y., Ren, C., Sun, Z., Rodionov, D. A., Zhang, W., Yang, S., Yang, C. and Jiang, W. 2010. Reconstruction of xylose utilization pathway and regulons in Firmicutes. *BMC Genomics* 11: 255-270.

Gullan, P. J. and Cranston, P. S. 2010. The insects: an outline of entomology. 5th ed. John Wiley and Sons: Oxford. Pp. 590.

Gunawan, S., Tufts, D. M. and Bextine, B. R. 2008. Molecular identification of hemolymph-associated symbiotic bacteria in red imported fire ant larvae. *Current Microbiology* 57: 575-579.

Gunning, R. V., Dang, H. T., Kemp, F. C., Nicholson, I. C. and Moores, G. D. 2005. New resistance mechanism in *Helicoverpa armigera* threatens transgenic crops expressing *Bacillus thuringiensis* Cry1Ac toxin. *Applied and Environmental Microbiology* 71(5): 2558-2563.

Gupta, A. K., Nayduch, D., Verma, P., Shah, B., Ghate, H. V., Patole, M. S. and Shouche, Y. S. 2012. Phylogenetic characterization of bacteria in the gut of house flies (*Musca domestica* L.). *FEMS Microbioloy Ecology* 79: 581-593.

Gusmão, D. S., Santos, A. V., Marini, D. C., De Souza Russo, E., Peixoto, A. M. D., Bacci, M., Berbert-Molina, M. A. and Lemos, F. J. A. 2007. First isolation of microorganisms from the gut diverticulum of *Aedes aegypti* (Diptera: Culicidae): new perspectives for an insect-bacteria association. *Memórias do Instituto Oswaldo Cruz* 102(8): 919-924.

Hamdi, C., Essanaa, J., Sansonno, L., Crotti, E., Abdi, K., Barbouche, N., Balloi, A., Gonella, E., Alma, A., Daffonchio, D., Boudabous, A. and Cherif, A. 2013. Genetic and biochemical diversity of *Paenibacillus larvae* isolated from Tunisian infected honey bee broods. *BioMed Research International.* doi:10.1155/2013/479893.

Hammer, T. J., McMillan, W. O. and Fierer, N. 2014. Metamorphosis of a butterfly-associated bacterial community. *PLoS ONE* 9(1). doi:10.1371/ journal.pone.0086995.

Handelsman, J. 2004. Metagenomics: Application of genomics to uncultured microorganisms. *Microbiology and Molecular Biology Reviews* 68(4): 699-685.

Handelsman, J. 2007. Metagenomics and microbial communities. *Encyclopedia of Life Sciences*. doi: 10.1002/9780470015902.a0020367.

Harris, K. M. and Nwanze, K. E. 1992. *Busseola fusca* (Fuller), the African maize stalk borer: A handbook of information. Information Bulletin no. 33. ICRISAT, CAB International: Oxon, UK. Pp. 85.

Harrison, J. F. 2001. Insect acid-base physiology. *Annual Review of Entomology* 46: 211-250.

Hassanali, A., Herren, H., Khan, Z. R., Pickett, J. A. and Woodcock, C. M. 2008. Integrated pest management: the push-pull approach for controlling insect pests and weeds of cereals, and its potential for other agricultural systems including animal husbandry. *Philosophical Transactions of the Royal Society* 363: 611-621.

Heckmann, L. H., Griffiths, B. S., Caul, S., Thompson, J., Pusztai-Carey, M., Moar, W. J., Andersen, M. N. and Krogh, P. H. 2006. Consequences for *Protaphorura armata* (Collembola: Onychiuridae) following exposure to genetically modified *Bacillus thuringiensis* (Bt) maize and non-Bt maize. *Environmental Pollution* 14: 212-216.

Helgason, E., Økstad, O. A., Caugant, D. A., Johansen, H. A., Fouet, A., Mock, M., Hegna, I. and Kolstø, A. B. 2000. *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis* - one species on the basis of genetic evidence. *Applied and Environmental Microbiology* 66(6): 2627-2630.

Hellmich, R. L. and Hellmich, K. A. 2012. Use and impact of Bt maize. *Nature Education Knowledge* 3(5): 4.
Hendriksma, H. P., Küting, M., Härtel, S., Näther, A., Dohrmann, A. B., Steffan-Dewenter, I. and Tebbe, C. C. 2013. Effect of stacked insecticidal Cry proteins from maize pollen on nurse bees (*Apis mellifera carnica*) and their gut bacteria. *PLoS ONE* 8(3). doi:10.1371/journal.pone.0059589.

Hernández-Martínez, P., Navarro-Cerrillo, G., Caccia, S., De Maagd, R. A., Moar, W. J., Ferré, J., Escriche, B. and Herrero, S. 2010. Constitutive activation of the midgut response to *Bacillus thuringiensis* in Bt-resistant *Spodoptera exigua*. *PLoS ONE* 5(9). doi:10.1371/journal.pone.0012795.

Hilbeck, A., Moar, W. J., Pusztai-Carey, M., Filippini, A. and Bigler, F. 1999. Prey-mediated effects of Cry1Ab toxin and protoxin and Cry2A protoxin on the predator *Chrysoperla carnea*. *Entomologia Experimentalis et Applicata* 91: 305-316.

Höfte, H., and Whiteley, H. R. 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiological reviews* 53(2): 242-255.

Hongoh, Y. 2010. Diversity and genomes of uncultured microbial symbionts in the termite gut. *Bioscience, Biotechnology, and Biochemistry* 74(6): 1145-1151.

Hongoh, Y., Deevong, P., Hattori, S., Inoue, T., Noda, S., Noparatnaraporn, N., Kudo, T. and Ohkuma, M. 2006. Phylogenetic diversity, localization, and cell morphologies of members of the candidate phylum TG3 and a subphylum in the phylum *Fibrobacteres*, recently discovered bacterial groups dominant in termite guts. *Applied and Environmental Microbiology* 72(10): 6780-6788.

Horner-Devine, M., Carney, K. and Bohannan, B. 2004. An ecological perspective on bacterial biodiversity. *Proceedings of the Royal Society of London, Series B* 271: 113-122.

Hosokawa, T., Kikuchi, Y., Nikoh, N., Shimada, M. and Fukatsu, T. 2006. Strict host-symbiont cospeciation and reductive genome evolution in insect gut bacteria. *PLoS Biology* 4(10): 1841-1851.

Hufbauer, R. A. 2002. Evidence for nonadaptive evolution in parasitoid virulence following a biological control introduction. *Ecological Applications* 12(1): 66-78.

Indiragandhi, P., Anandham, R., Madhaiyan, M., Poonguzhali, S., Kim, G. H., Saravanan, V. S. and Sa, T. 2007. Cultivable bacteria associated with larval gut of prothiofos-resistant, prothiofos-susceptible and field-caught populations of diamondback moth, *Plutella xylostella* and their potential for, antagonism towards entomopathogenic fungi and host insect nutrition. *Journal of Applied Microbiology* 103: 2664-2675.

Inglis, G. D., Lawrence, A. M. and Davis, F. M. 2000. Pathogens associated with Southwestern corn borers and Southern corn stalk borers (Lepidoptera: Crambidae). *Journal of Economic Entomology* 93: 1619-1626.

Iverson, K. L., Bromel, M. C., Anderson, A. W. and Freeman, T. P. 1984. Bacterial symbionts in the sugar beet root maggot, *Tetanops myopaeformis* (von Röder). *Applied and Environmental Microbiology* 47(1): 22-27.

Ivy, R. A., Ranieri, M. L., Martin, N. H., Den Bakker, H. C., Xavier, B. M., Wiedmann, M. and Boor, K. J. 2012. Identification and characterization of psychrotolerant sporeformers associated with fluid milk production and processing. *Applied and Environmental Microbiology* *78*(6): 1853-1864.

James, C. 2012. ISAAA brief 43-2011: Executive summary. Global Status of commercialized Biotech/GM Crops: 2011. http://www.isaaa.org/resources/publications/briefs/43/executivesummary/default.asp. Date of access: 18 November. 2013.

James, C. 2014. ISAAA brief 46-2013: Executive Summary. Global Status of commercialized Biotech/GM Crops: 2013. http://www.isaaa.org/resources/publications/briefs/46/executivesummary/default.asp. Date of access: 26 June. 2014.

Janda, J. M. and Abbott, S. L. 2007. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. *Journal of Clinical Microbiology* 45(9): 2761-2764.

Jander, G., Rahme, L. G. and Ausubel, F. M. 2000. Positive correlation between virulence of *Pseudomonas aeruginosa* mutants in mice and insects. *Journal of Bacteriology* 182: 3843-3845.

Janmaat, A. F. and Myers, J. H. 2003. Rapid evolution and the cost of resistance to *Bacillus thuringiensis* in greenhouse populations of cabbage loopers, *Trichoplusia ni*. *Proceedings of the Royal Society B: Biological Sciences* 270: 2263-2270.

Janson, E. M., Stireman, J. O., Singer, M. S. and Abbot, P. 2007. Phytophagous insect–microbe mutualisms and adaptive evolutionary diversification. *Evolution* 62(5): 997-1012.

Jensen, P. D., Dively, G. P., Swan, C. M. and Lamp, W. O. 2010. Exposure and nontarget effects of transgenic Bt corn debris in streams. *Environmental Entomology* 39(2): 707-714.

Johnson, K. S. and Barbehenn, R. V. 2000. Oxygen levels in the gut lumens of herbivorous insects. *Journal of Insect Physiology* 46: 897-903.

Jukes, T. H. and Cantor, C. R. 1969. Evolution of protein molecules. (*In* Mammalian Protein Metabolism. Munro, H. N. *ed*. Academic Press: New York.

Jung, H. G. and Sheaffer, C. C. 2004. Influence of Bt transgenes on cell wall lignification and digestibility of maize stover for silage. *Crop Science* 44(5):1781-1789.

Kallimanis, A., Frillingos, S., Drainas, C. and Koukkou, A. 2007. Taxonomic identification, phenanthrene uptake activity, and membrane lipid alterations of the PAH degrading *Arthrobacter* sp. strain Sphe3. *Applied Microbiology and Biotechnology* 76(3): 709-717.

Kat, H., Ince, I. A., Demir, I. and Demirbag, Z. 2010. *Brevibacterium pityocampae* sp. nov., isolated from caterpillars of *Thaumetopoea pityocampa* (Lepidoptera, Thaumetopoeidae). *International Journal of Systematic and Evolutionary Microbiology* 60: 312-316.

Kati, A. and Kati, H. 2013. Isolation and identification of bacteria from *Xylosandrus germanus* (Blandford) (Coleoptera: Curculionidae). *African Journal of Microbiology Research* 7(47): 5288-5299.

Kfir, R. 1995. Parasitoids of the African stem borer, *Busseola fusca* (Lepidoptera: Noctuidae), in South Africa. *Bulletin of Entomological Research* 85: 369-377.

Kfir, R. 2002. Increase in cereal stem borer populations through partial elimination of natural enemies. *Entomologia Experimentalis et Applicata* 104: 299-306.

Kfir, R., Overholt, W. A., Khan, Z. R. and Polaszek, A. 2002. Biology and management of economically important lepidopteran cereal stem borers in Africa. *Annual Review of Entomology* 47: 701-731.

Khan, Z. R., Midega, C. A. O., Njuguna, E. M., Amudavi, D. M., Wanyama, J. M. and Pickett, J. A. 2008. Economic performance of the 'push–pull' technology for stemborer and striga control in smallholder farming systems in western Kenya. *Crop Protection* 27:1084-1097.

Khan, Z. R., Pickett, J. A., Van den Berg, J., Wadhams, L. and Woodcock, C. M. 2000. Exploiting chemical ecology and species diversity: stem borer and striga control for maize and sorghum in Africa. *Pest Management Science* 56: 957-962.

Khyade, V. B. and Marathe, R. M. 2012. Diversity of bacterial flore in the midgut of fifth instar larvae of silk worm, *Bombyx mori* (L) (race: PM x CSR_2). *Global Journal of Bio-Science and Biotechnology* 1(2): 191-200.

Kim, P. S., Shin, N. R., Kim, J. Y., Yun, J. H., Hyun, D. W. and Bae, J. W. 2014. *Acinetobacter apis* sp. nov., isolated from the intestinal tract of a honey bee, *Apis mellifera. Journal of Microbiology* 52(8): 639-645.

Klug, M. J. and Kotarski, S. 1980. Bacteria associated with the gut tract of larval stages of the aquatic cranefly *Tipula abdominalis* (Diptera: Tipulidae). *Applied and Environmental Microbiology* 40(2): 408-416.

Knowles, B. H. 1994. Mechanism of action of *Bacillus thuringiensis* insecticidal d-endotoxins. *Advances in Insect Physiology* 24: 275-308.

Köhler, T., Dietrich, C., Scheffrahn, R. H. and Brune, A. 2012. High resolution analysis of gut environment and bacterial microbiota reveals functional compartmentation of the gut in wood-feeding higher termites (*Nasutitermes* spp.). *Applied and Environmental Microbiology* 78(13): 4691-4701.

Krieger, E. K., Allen, E., Gilbertson, L. A., Roberts, J. K., Hiatt, W. and Sanders, R. A. 2008. The Flavr Savr tomato, an early example of RNAi technology. *Hort Science* 43(3): 962-964.

Krogh, P. H., Griffiths, B., Demšar, D., Bohanec, M., Debeljak, M., Andersen, M. N., Sausse, C., Birch, A. N. E., Caul, S., Holmstrup, M., Heckmann, L. H. and Cortet, J. 2007. Responses by earthworms to reduced tillage in herbicide tolerant maize and Bt maize cropping systems. *Pedobiologia* 51: 219-227.

Kruger, M., Van Rensburg, J. B. J. and Van den Berg, J. 2009. Perspective on the development of stem borer resistance to Bt maize and refuge compliance at the Vaalharts irrigation scheme in South Africa. *Crop Protection* 28: 684-689.

Kruger, M., Van Rensburg, J. B. J. and Van den Berg, J. 2010. Transgenic Bt maize: farmers' perceptions, refuge compliance and reports of stem borer resistance in South Africa. *Journal of Applied Entomology* 136(1-2): 38-50.

Kruger, M., Van Rensburg, J. B. J. and Van den Berg, J. 2011. Resistance to Bt maize in *Busseola fusca* (Lepidoptera: Noctuidae) from Vaalharts, South Africa. *Environmental Entomology* 40: 477-483.

Krüger, W., Van den Berg, J. and Van Hamburg, H. 2008. The relative abundance of maize stem borers and their parasitoids at the Tshiombo irrigation scheme in Venda, South Africa. *South African Journal of Plant and Soil* 25(3): 144-151.

Kumar, P. A. 2003. Insect pest resistant transgenic crops. (*In* Upadhyay, R. K. *ed.* Advances in microbial control of insect pests. Springer: US. Pp. 71-82).

La Duc, M. T., Satomi, M., Agata, N. and Venkateswaran, K. 2004. *gyrB* as a phylogenetic discriminator for members of the *Bacillus anthracis-cereus-thuringiensis* group. *Journal of Microbiological Methods* 56: 383-394.

Lacey, L. A., Unruh, T. R., Simkins, H. and Thomsen-Archer, K. 2007. Gut bacteria associated with the Pacific coast wireworm, *Limonius canus*, inferred from 16S rDNA sequences and their implications for control. *Phytoparasitica* 35(5): 479-489.

Lane, D. J. 1991. 16S / 23S rRNA sequencing. (*In* Stackebrandt, E. and Goodfellow, M. *eds*. Nucleic acid techniques in bacterial systematics. John Wiley and Sons: New York. pp 115-147).

Lang, A. and Vojtech, E. 2006. The effects of pollen consumption of transgenic Bt maize on the common swallowtail, *Papilio machaon* L. (Lepidoptera, Papilionidae). *Basic and Applied Ecology* 7: 296-306.

Lauzon, C. R., Bussert, T. G., Sjogren, R. E. and Prokopy, R. J. 2003. *Serratia marcescens* as a bacterial pathogen of *Rhagoletis pomonella* flies (Diptera:Tephritidae). *European Journal of Entomology* 100: 87-92.

Lauzon, C. R., Sjogren, R. E. and Prokopy, R. J. 2000. Enzymatic capabilities of bacteria associated with apple maggot flies: a postulated role in attraction. *Journal of Chemical Ecology* 26(4): 953-967.

Lazzaro, B. P. and Rolff, J. 2011. Danger, microbes, and homeostasis. *Science* 332: 43-44.

Leadbetter, J. R., Crosby, L. D. and Breznak, J. A. 1998. *Methanobrevibacter filiformis* sp. nov., a filamentous methanogen from termite hindguts. *Archives of Microbiology* 169: 287-292.

Lehman, R. M., Lundgren, J. G. and Petzke, L. M. 2009. Bacterial communities associated with the digestive tract of the predatory ground beetle, *Poecilus chalcites*, and their modification by laboratory rearing and antibiotic treatment. *Microbial Ecology* 57: 349-358.

Lemke, T., Stingl, U., Egert, M., Friedrich, M. W. and Brune, A. 2003. Physicochemical conditions and microbial activities in the highly alkaline gut of the humus-feeding larva of *Pachnoda ephippiata* (Coleoptera: Scarabaeidae). *Applied and Environmental Microbiology* 69(11): 6650-6658.

Lepš, J. and Šmilauer, P. 2003. Multivariate analysis of ecological data using CANOCO. Cambridge University Press: Cambridge. Pp.15.

Li, H., Duan, Y., Ma, G., Lei, L., Zhang, K. Q. and Yang, J. 2011. Isolation and characterization of *Acinetobacter* sp. ND12 capable of degrading nicotine. *African Journal of Microbiology Research* 5(11): 1335-1341.

Li, J., Yang, G., Wu, M., Zhao, Y. and Zhou, S. 2014. *Bacillus huizhouensis* sp. nov., isolated from a paddy field soil. *Antonie van Leeuwenhoek* 106: 357-363.

Lindh, J. M., Terenius, O. and Faye, I. 2005. 16S rRNA gene-based identification of midgut bacteria from field-caught *Anopheles gambiae* Sensu Lato and *A. funestus* mosquitoes reveals new species related to known insect symbionts. *Applied and Environmental Microbiology* 71(11): 7217-7223.

Liu, B., Xu, C., Yan, F. and Gong, R. 2005a. The impacts of the pollen of insect-resistant transgenic cotton on honeybees. *Biodiversity and Conservation* 14: 3487-3496.

Liu, N., Yan, X., Zhang, M., Xie, L., Wang, Q., Huang, Y., Zhou, X., Wang, S. and Zhou, Z. 2011. Microbiome of fungus-growing termites: a new reservoir for lignocellulase genes. *Applied and Environmental Microbiology* 77(1): 48-56.

Liu, X. X., Sun, C. G. and Zhang, Q. W. 2005b. Effects of transgenic Cry1A+CpTI cotton and Cry1Ac toxin on the parasitoid, *Campoketis chlorideae* (Hymenoptera: Ichneumonidae). *Insect Science* 12: 101-107.

Liu, Y. and Tabashnik, B. E. 1997. Inheritance of resistance to the *Bacillus thuringiensis* toxin Cry1C in the diamondback moth. *Applied and Environmental Microbiology* 63(6): 2218-2223.

Liu, Z., Yang, C. and Qiao, C. 2007. Biodegradation of *p*-nitrophenol and 4-chlorophenol by *Stenotrophomonas* sp. *FEMS Microbiology Letters* 277: 150-156.

Logan, N. A. and De Vos, P. 2011. Family I Bacillaceae. (*In* De Vos, P., Garrity, G., Jones, D., Krieg, N. R., Ludwig, W., Rainey, F. A., Schleifer, K. H. Whitman, W. B. *eds*. Volume 3 of Bergey's Manual of Systematic Bacteriology. Springer: London. Pp. 19-1318).

Ludwig, W., Euzéby, J., Schumann, P., Busse, H. J., Trujillo, M. E., Kämpfer, P. and Whitman, W. B. 2012. Phylum XXVI. Actinobacteria phyl. Nov. (*In* Goodfellow, M., Kämpfer, P., Busse, H. J., Trujillo, M. E., Suzuki, K., Ludwig, W. and Whitman, W. B. *eds*. Volume 5 of Bergey's Manual of Systematic Bacteriology. Springer: London. Pp. 1-28).

Ludwig, W., Schleifer, K. H. and Whitman, W. B. 2011a. Taxonomic outline of the phylum Firmicutes. (*In* De Vos, P., Garrity, G., Jones, D., Krieg, N. R., Ludwig, W., Rainey, F. A., Schleifer, K. H. Whitman, W. B. *eds*. Volume 3 of Bergey's Manual of Systematic Bacteriology. Springer: London. Pp. 15-18).

Ludwig, W., Schleifer, K. H. and Whitman, W. B. 2011b. Family IV. *Enterococcaceae* fam. nov. (*In* De Vos, P., Garrity, G., Jones, D., Krieg, N. R., Ludwig, W., Rainey, F. A., Schleifer, K. H. Whitman, W. B. *eds*. Volume 3 of Bergey's Manual of Systematic Bacteriology. Springer: London. Pp. 594-607).

Lundgren, J. G. and Lehman, R. M. 2010. Bacterial gut symbionts contribute to seed digestion in an omnivorous beetle. *PLoS ONE* 5(5). doi:10.1371/ journal.pone.0010831.

Lundgren, J. G., Lehman, R. M. and Chee-Sanford, J. 2007. Bacterial communities within digestive tracts of ground beetles (Coleoptera: Carabidae). *Physiology, Biochemistry, and Toxicology* 100(2): 275-282.

Luxananil, P., Atomi, H., Panyim, S. and Imanaka, T. 2001. Isolation of bacterial strains colonizable in mosquito larval guts as novel host cells for mosquito control. *Journal of Bioscience and Bioengineering* 92(4): 342-345.

Lynch, R. E., Lewis, L. C. and Brindley, T. A. 1976. Bacteria associated with eggs and first-instar larvae of the European corn borer: identification and frequency of occurrence. *Journal of Invertebrate Pathology* 27(2): 229-237.

Ma, G., Roberts, H., Sarjan, M., Featherstone, N., Lahnstein, J., Akhurst, R. and Schmidt, O. 2005. Is the mature endotoxin Cry1Ac from *Bacillus thuringiensis* inactivated by a coagulation reaction in the gut lumen of resistant *Helicoverpa armigera* larvae? *Insect Biochemistry and Molecular Biology* 35: 729-739.

Ma, Y., Zhang, J. and Chen, S. 2007. *Paenibacillus zanthoxyli* sp. nov., a novel nitrogen-fixing species isolated from the rhizosphere of *Zanthoxylum simulans. International Journal of Systematic and Evolutionary Microbiology* 57: 873-877.

Macovei, L. and Zurek, L. 2006. Ecology of antibiotic resistance genes: characterization of enterococci from houseflies collected in food settings. *Applied and Environmental Microbiology* 72(6): 4028-4035.

Magurran, A. E. 2005. Species abundance distributions: pattern or process? *Functional Ecology* 19: 177-181.

Mally, C. W. 1920. The maize stalk borer, *Busseola fusca* (Fuller). *Bulletin of the Department of Agriculture Union of South Africa* 3: 111.

Mannion, A. M. and Morse, S. 2013. GM crops 1996-2012: A review of agronomic, environmental and socio-economic impacts. *University of Reading Geographical Paper* 195: 1-40.

Mano, H. and Morisaki, H. 2008. Endophytic bacteria in the rice plant. *Microbes and Environments* 23(2): 109-117.

Marchini, D., Rosetto, M., Dallai, R. and Marri, L. 2002. Bacteria associated with the oesophageal bulb of the medfly *Ceratitis capitata* (Diptera: Tephritidae). *Current Microbiologyl* 44: 120-124.

Martin, J. D. and Mundt, J. O. 1972. Enterococci in insects. *Applied Microbiology* 24(4): 575-580.

Martínez-Ramírez, A. C., Gould, F. and Ferré, J. 1999. Histopathological effects and growth reduction in a susceptible and a resistant strain of *Heliothis virescens* (Lepidoptera: Noctuidae) caused by sublethal doses of pure Cry1A crystal proteins from *Bacillus thuringiensis. Biocontrol Science and Technology* 9: 239-246.

Martinson, V. G., Moy, J. and Moran, N. A. 2012. Establishment of characteristic gut bacteria during development of the honeybee worker. *Applied and Environmental Microbiology* 78(8): 2830-2840.

Matthews, A. 2014. Global area under biotech crops continues to grow while EU policy struggles. http://capreform.eu/global-area-under-biotech-crops-continues-to-grow-while-eu-policy-struggles/. Date of access: 12 March. 2015.

Mboya, R., Tongoona, P., Derera, J., Mudhara, M. and Langyintuo, A. 2011. The dietary importance of maize in Katumba ward, Rungwe district, Tanzania, and its contribution to household food security. *African Journal of Agricultural Research* 6(11): 2617-2626.

McGaughey, W. H. 1985. Insect resistance to the biological insecticide *Bacillus thuringiensis*. *Science* 229: 193-194.

McLinden, J. H., Sabourin, J. R., Clark, B. D., Gensler, D. R., Workman, W. E. and Dean, D. H. 1985. Cloning and expression of an insecticidal k-73 type crystal protein gene from *Bacillus thuringiensis* var. *kurstaki* into *Escherichia coli*. *Applied and Environmental Microbiology* 50(3): 623-628.

Mead, L. J., Khachatourians, G. G. and Jones, G. A. 1988. Microbial ecology of the gut in laboratory stocks of the migratory grasshopper, *Melanoplus sanguinipes* (Fab.) (Orthoptera: Acrididae). *Applied and Environmental Microbiology* 54(5): 1174-1181.

Meihls, L. N., Higdon, M. L., Siegfried, B. D., Miller, N. J., Sappington, T. W., Ellersieck, M. R., Spencer, T. A. and Hibbard, B. E. 2008. Increased survival of Western corn rootworm on transgenic corn within three generations of on-plant greenhouse selection. *Proceedings of the National Academy of Science* 105(49): 19177-19182.

Minard, G., Mavingui, P. and Moro, C. V. 2013. Diversity and function of bacterial microbiota in the mosquito holobiont. *Parasites and Vectors* 6(146). doi:10.1186/1756-3305-6-146.

Mohr, K. I. and Tebbe, C. C. 2006. Diversity and phylotype consistency of bacteria in the guts of three bee species (*Apoidea*) at an oilseed rape field. *Environmental Microbiology* 8(2): 258-272.

Moran, N. A. and Baumann, P. 2000. Bacterial endosymbionts in animals. *Current Opinion in Microbiology* 3: 270-275.

Moro, C. V., Tran, F. H., Raharimalala, F. N., Ravelonandro, P. and Mavingui, P. 2013. Diversity of culturable bacteria including *Pantoea* in wild mosquito *Aedes albopictus*. *BMC Microbiology* 13(70). doi:10.1186/1471-2180-13-70.

Morohoshi, T., Tominaga, Y., Someya, N. and Ikeda, T. 2012. Complete genome sequence and characterization of the *N*-acylhomoserine lactone-degrading gene of the potato leaf-associated *Solibacillus silvestris*. *Journal of Bioscience and Bioengineering* 113(1): 20-25.

Munteanu, N. V., Danismazoglu, M., Moldovan, A. I., Toderas, I. K., Nalçacıoğlu, R. and Demirbag, Z. 2014. The first study on bacterial flora of pest beetles *Sciaphobus squalidus*, *Tatianaerhynchites aequatus* and *Byctiscus betulae* in the Republic of Moldova. *Biologia* 69(5): 681-690.

Nayduch, D., Honko, A., Noblet, G. P. and Stutzenberger, F. 2001. Detection of *Aeromonas caviae* in the common housefly *Musca domestica* by culture and polymerase chain reaction. *Epidemiology and Infection* 127: 561-566.

Ndemah, R., Schulthess, F., Korie, S., Borgemeister, C. and Cardwell, K. F. 2001. Distribution, relative importance and effect of lepidopterous borers on maize yields in the forest zone and mid-altitude of Cameroon. *Journal of Economic Entomology* 94(6): 1434-1444.

Ohkuma, M. 2008. Symbioses of flagellates and prokaryotes in the gut of lower termites. *TRENDS in Microbiology* 16(7): 345-352.

Ohkuma, M., Noda, S. and Kudo, T. 1999. Phylogenetic diversity of nitrogen fixation genes in the symbiotic microbial community in the gut of diverse termites. *Applied and Environmental Microbiology* 65(11): 4926-4934.

Ong'amo, G. O., Le Rü, B., Dupas, S., Moyal, P., Ngala, L., Muchugu, E., Calatayud, P. A. and Silvain, J. F. 2006. The role of wild host plants in the abundance of lepidopteran stem borers along altitudinal gradient in Kenya. *Annales de la Société entomologique de France (NS)* 42(3-4): 363-370.

Oppert, B. 1999. Protease interactions with *Bacillus Thuringiensis* insecticidal toxins. *Archives of Insect Biochemistry and Physiology* 42:1-12.

Øvreås, L. 2000. Population and community level approaches for analysing microbial diversity in natural environments. *Ecology Letters* 3: 236-251.

Özka- Çakici, F., Sevim, A., Demirbağ, Z. and Demir, L. 2014. Investigating internal bacteria of *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae) larvae and some *Bacillus* strains as biocontrol agents. *Turkish Journal of Agriculture and Forestry* 38(1): 99-110.

Patankar, A .G., Giri, A. P., Harsulkar, A. M., Sainani, M. N., Deshpande, V. V., Ranjekar, P .K. and Gupta, V. S. 2001. Complexity in specificities and expression of *Helicoverpa armigera* gut proteinases explains polyphagous nature of the insect pest. *Insect Biochemistry and Molecular Biology* 31: 453-464.

Perry, J. N., Devos, Y., Arpaia, S., Bartsch, D., Gathmann, A., Hails, R. S., Kiss, J., Lheureux, K., Manachini, B., Mestdagh, S., Neemann, G., Ortego, F., Schiemann, J. and Sweet, J. B. 2010. A mathematical model of exposure of non-target Lepidoptera to Bt-maize pollen expressing Cry1Ab within Europe. *Proceedings of the Royal Society B* 277: 1417-1425.

Peyronnet, O., Vachon, V., Brousseau, R., Baines, D., Schwartz, J. and Laprade, R. 1997. Effect of *Bacillus thuringiensis* toxins on the membrane potential of lepidopteran insect midgut cells. *Applied and Environmental Microbiology* 63(5): 1679-1684.

Pigott, C. R. and Ellar, D. J. 2007. Role of receptors in *Bacillus thuringiensis* crystal toxin activity. *Microbiology and Molecular Biology Reviews* 71(2): 255-281.

Poerschmann, J., Gathmann, A., Augustin, J., Langer, U. and Górecki, T. 2005. Molecular composition of leaves and stems of genetically modified Bt and near-isogenic non-Bt maize - characterisation of lignin patterns. *Journal of Environmental Quality* 34: 1508-1518.

Pontes, D. S., Lima-Bittencourt, C. I., Chartone-Souza, E. and Nascimento, A. M. A. 2007. Molecular approaches: advantages and artifacts in assessing bacterial diversity. *Journal of Industrial Microbiology and Biotechnology* 34: 463-473.

Pordesimo, L. O., Hames, B. R., Sokhansanj, S. and Edens, W. C. 2005. Variation in corn stover composition and energy content with crop maturity. *Biomass and Bioenergy* 28: 366-374.

Potrikus, C. J. and Breznak, J. A. 1980. Uric acid-degrading bacteria in guts of termites [*Reticulitermes flavipes* (Kollar)]. *Applied and Environmental Microbiology* 40(1): 117-124.

Powell, C. M., Hanson, J. D. and Bextine, B. R. 2014. Bacterial community survey of *Solenopsis invicta* Buren (Red imported fire Ant) colonies in the presence and absence of *Solenopsis invicta Virus* (SINV). *Current Microbiology* 69: 580-585.

Prasifka, P. L., Hellmich, R. L., Prasifka, J. R. and Lewis, L. C. 2007. Effects of Cry1Ab-expressing corn anthers on the movement of Monarch butterfly larvae. *Environmental Entomology* 36(1): 228-233.

Prather, T. S., Ditomaso, J. M. and Holt, J. S. 2000. Herbicide resistance: Definition and management strategies. UCANR Publications. http://books.google.co.uk/books?id=kUi52NemEgkC&pg=PA9&lr=&source=gbs_selected_pages&cad=2#v=onepage&q=publisher&f=false. Date of Access: 20 November. 2014.

Priya, N. G., Ojha, A., Kajla, M. K., Raj, A. and Rajagopal, R. 2012. Host plant induced variation in gut bacteria of *Helicoverpa armigera. PLoS ONE* 7(1). doi:10.1371/journal.pone.0030768.

Rajagopal, R. 2009. Beneficial interactions between insects and gut bacteria. *Indian Journal of Microbiology* 49(2): 114-119.

Ramírez-Lepe, M. and Ramírez-Suero, M. 2012. Biological control of mosquito larvae by *Bacillus thuringiensis* subsp. *Israelensis*. (*In* Perveen, F. *ed*. Insecticides - Pest Engineering. Pp. 239-264).

Rani, A., Sharma, A., Rajagopal, R., Adak, T. and Bhatnager, R. K. 2009. Bacterial diversity analysis of larvae and adult midgut microflora using culture-dependent and culture-independent methods in lab-reared and field-collected *Anopheles stephensi*-an Asian malarial vector. *BMC Microbiology* 9(96). doi:10.1186/1471-2180-9-96.

Ratledge, C. and Dover, L. G. 2000. Iron metabolism in pathogenic bacteria. *Annual Review of Microbiology* 54: 881-941.

Reddy, K. V. S. 1985. Relative susceptibility and resistance of some sorghum lines to stem-borers in Western Kenya. *Insect Science and its Application* 6(3): 401-404.

Reeson, A. F., Jankovic, T., Kasper, M. L., Rogers, S. and Austin, A. D. 2003. Application of 16S rDNA-DGGE to examine the microbial ecology associated with a social wasp *Vespula germanica*. *Insect Molecular Biology* 12(1): 85-91.

Ren, C., Webster, P., Finkel, S. E. and Tower, J. 2007. Increased internal and external bacterial load during *Drosophila* aging without life-span trade-off. *Cell Metabolism* 6:144-152.

Rhee, M. S., Wel, L., Sawhney, N., Rice, J. D., John, F. J. S., Hurlbert, J. C. and Preston, J. F. 2014. Engineering the xylan utilization system in *Bacillus subtilis* for production of acidic xylooligosaccharides. *Applied and Environmental Microbiology* 80(3): 917-927.

Rheims, H., Fruhling, A., Schumann, P., Rohde, M. and Stackebrandt, E. 1999. *Bacillus silvestris* sp. nov., a new member of the genus *Bacillus* that contains lysine in its cell wall. *International Journal of Systematic Bacteriology* 49: 795-802.

Robinson, C. J., Schloss, P., Ramos, Y., Raffa, K. and Handelsman, J. 2010. Robustness of the bacterial community in the cabbage white butterfly larval midgut. *Microbial Ecology* 59: 199-211.

Romeis, J., Meissle, M. and Bigler, F. 2006. Transgenic crops expressing *Bacillus thuringiensis* toxins and biological control. *Nature Biotechnology* 24(1): 63-71.

Rose, R., Dively, G. P. and Pettis, J. 2007. Effects of Bt corn pollen on honey bees: emphasis on protocol development. *Apidologie* 38: 368-377.

Rosi-Marshall, E. J., Tank, J. L., Royer, T. V., Whiles, M. R., Evans-White, M., Chambers, C., Griffiths, N. A., Pokelsek, J. and Stephen, M. L. 2007. Toxins in transgenic crop byproducts may affect headwater stream ecosystems. *Proceedigs of the National Academy of Sciences of the USA* 104(41): 16204-16208.

Roy, N., Rana, M. and Salah Uddin, A. T. M. 2003. Isolation and some properties of new xylanase from the intestine of a herbivorous insect (*Samia cynthia pryeri*). *Journal of Biological Sciences* 4(1): 27-23.

Sahoo, N., Pakshirajan, K., Ghosh, P. and Ghosh, A. 2011. Biodegradation of 4-chlorophenol by *Arthrobacter chlorophenolicus A6*: effect of culture conditions and degradation kinetics. *Biodegradation* 22(2): 275-286.

Saitou, N. and Nei, M. 1987. The Neighbor-Joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4: 406-425.

Santella, R. M. 2006. Approaches to DNA/RNA extraction and whole genome amplification. *Cancer Epidemiology Biomarkers prevention* 15(9): 1585-1587.

Santo-Domingo, J. W., Kaufman, M. G., Klug, M. J., Holben, W. E., Harris, D. and Tiedje, J. M. 1998. Influence of diet on the structure and function of the bacterial hindgut community of crickets. *Molecular Ecology* 7: 761-767.

Saxena, D. and Stotzky, G. 2001. *Bacillus thuringiensis* (Bt) toxin released from root exudates and biomass of Bt corn has no apparent effect on earthworms, nematodes, protozoa, bacteria, and fungi in soil. *Soil Biology and Biochemistry* 33: 1225-1230.

Schaaf, O. and Dettner, K. 1997. Microbial diversity of aerobic heterotrophic bacteria inside the foregut of two tyrphophilous water beetle species (Coleoptera: Dytiscidae). *Microbiological Research* 152: 57-64.

Schloss, P. D., Delalibera, I., Handelsman, J. and Raffam K. F. 2006. Bacteria associated with the guts of two wood-boring beetles: *Anoplophora glabripennis* and *Saperda vestita* (Cerambycidae). *Environmental Entomology* 35(3): 625-629.

Schmidt, T. R., Scott, E. J. and Dyer, D. W. 2011. Whole-genome phylogenies of the family *Bacillaceae* and expansion of the sigma factor gene family in the *Bacillus cereus* species-group. *BMC Genomics* 12: 430-446.

Schnepf, H. E. and Whiteley, H. R. 1981. Cloning and expression of the *Bacillus thuringiensis* crystal protein gene in *Escherichia coli*. *Proceedings of the National Academy of Sciences USA* 78(5): 2893-2897.

Schuler, T. H., Potting, R. P. J., Denholm, I., Clark, S. J., Clark, A. J., Stewart, C. N. and Poppy, G. M. 2003. Tritrophic choice experiments with Bt plants, the diamondback moth (*Plutella xylostella*) and the parasitoid *Cotesia plutellae*. *Transgenic Research* 12: 351-361.

Schultz, J. E. and Breznak, J. A. 1978. Heterotrophic bacteria present in hindguts of wood-eating termites [*Reticulitermes flavipes* (Kollar)]. *Applied and Environmental Microbiology* 35(5): 930-936.

Secil, E. S., Sevim, A., Demirbag, Z. and Demir, I. 2012. Isolation, characterization and virulence of bacteria from *Ostrinia nubilalis* (Lepidoptera: Pyralidae). *Biologia* 67(4): 767-776.

Selvakumar, G., Sushil, S. N., Stanley, J., Mohan, M., Deol, A., Rai, D., Ramkewal., Bhatt, J. C. and Gupta, H. S. 2011. *Brevibacterium frigoritolerans* a novel entomopathogen of *Anomala dimidiata* and *Holotrichia longipennis* (Scarabaeidae: Coleoptera). *Biocontrol Science and Technology* 21(7): 821-827.

Sezen, K. and Demirbag, Z. 1999. Isolation and insecticidal activity of some bacteria from the hazelnut beetle (Balaninus nucum L.). *Applied Entomology and Zoology* 34: 85-89.

Sezonlin, M., Dupas, S., Le Rü, B., Le Gall, P., Moyal, P., Calatayud, P. A., Griffard, I., Faure, N. and Silvain, J. F. 2006. Phylogeography and population genetics of the maize stalk borer *Busseola fusca* (Lepidoptera, Noctuidae) in sub-Saharan Africa. *Molecular Ecology* 15: 407-420.

Shao, L., Devenport, M. and Jocobs-Lorena, M. 2001. The peritrophic matrix of hematophagous insects. *Archives of Insect Biochemistry and Physiology* 47:119-125.

Sharma, H. C. 2006. Integrated pest management research at ICRISAT: Present status and future priorities. International crops Rresearch institute for the semi-arid tropics. ICRISAT: India. Pp. 51.

Shelton, A. M., Zhao, J. Z. and Roush, R. T. 2002. Economic, ecological, food safety, and social consequences of the deployment of Bt transgenic plants. *Annual Review of Entomology* 47: 845-881.

Shida, O., Takagi, H., Kadowaki, K. and Komagata, K. 1996. Proposal for two new genera, *Brevibacillus* gen. nov. and *Aneurinibacillus* gen. nov. *International Journal of Systematic Bacteriology* 46(4): 939-946.

Shida, O., Takagi, H., Kadowaki, K., Udaka, S., Nakamura, L. K. and Komagata, K. 1995. Proposal of *Bacillus reuszeri* sp. nov., *Bacillus formosus* sp. nov., nom. rev., and *Bacillus borstelensis* sp. nov., norn. rev. *International Journal of Systematic Bacteriology* 45(1): 93-100.

Shigenobu, S., Watanabe, H., Hattori, M., Sakaki, Y. and Ishikawa, H. 2000. Genome sequence of the endocellular bacterial symbiont of aphids *Buchnerasp*. APS. *Nature* 407: 81-86.

Singh, A., Kumar, R., Jain, P. K. and Mangal, A. K. 2007. Environmental implications of integrated pest management: Farming of paddy and vegetables. (*In* Kumar, P. and Sudhakara, B. *eds*. Ecology and human well-being. India: Sage. Pp. 133-146).

Singh, B. K., Walker, A., Alun., W. M. and Wright, D. J. 2004. Biodegradation of chlorpyrifos by *Enterobacter* strain B-14 and its use in bioremediation of contaminated soils. *Applied and Environmental Microbiology* 70(8): 4855-4863.

Six, D. L. and Wingfield, M. J. 2011. The role of phytopathogenicity in bark beetle-fungus symbioses: a challenge to the classic paradigm. *Annual Review of Entomology* 56: 255-272.

Slabbert, O. and Van den Berg, J. 2009. The effect of the adjuvant, Break-Thru S240, on whorl penetration and efficacy of foliar insecticide applications against *Chilo partellus*. *South African Journal of Plant and Soil* 26(4): 254-258.

Snyman, M., Bezuidenhout, C. C., Claassens, S. and Van den Berg, J. 2012. Assessment of the diversity and occurrence of gut microbes in *Busseola fusca* (Lepidoptera: Noctuidae) from different maize producing regions. Potchefstroom: NWU. (Mini-dissertation-Hons).

Soltis, P. and Soltis, D. E. 2003. Applying the bootstrap in phylogeny reconstruction. *Statistical Science* 18(2): 256-267.

Starmer, W. T., Barker, J. S. F., Phaff, H. J. and Fogleman, J. C. 1986. Adaptations of *Drosophila* and yeasts: their interactions with the volatile 2-propanol in the cactus-micro*organism-Drosophila m*odel system. *Australian Journal of Biological Sciences* 39: 69-77.

StatSoft, Inc. (2013). STATISTICA (data analysis software system), version 12. www.statsoft.com. Date of access: 3 March. 2014.

Steinhaus, E. 2012. Insect Pathology V2: An advanced treatise, volume 2. Academic Press: London Pp. 704.

Stenfors Arnesen, L. P., Fagerlund, A. and Granum, P. E. 2008. From soil to gut: *Bacillus cereus* and its food poisoning toxins. *FEMS Microbiology Reviews* 32: 579-606.

Storer, N. P., Babcock, J. M., Schlenz, M., Meade, T., Thompson, G. D., Bing, J. W. and Huckaba, R. M. 2010. Discovery and characterization of field resistance to Bt Maize: *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in Puerto Rico. *Journal of Economic Entomology* 103: 1031-1038.

Studeny, A. C., Buckland, S. T., Illian, J. B., Johnston, A. and Magurran, A. E. 2011. Goodness-of-fit measures of evenness: a new tool for exploring changes in community structure. *Ecosphere* 2(2). doi.org/10.1890/ES10-00074.1.

Suen, G., Scott., J. J., Aylward, F. O., Adams, S. M., Tringe, S. G., Pinto-Toma´s, A. A., Foster, C. E., Pauly, M., Weimer, P. J., Barry, K. W., Goodwin, L. A., Bouffard, P., Li, L., Osterberger, J., Harkins, T. T., Slater, S. C., Donohue, T. J. and Currie, C. R. 2010. An insect herbivore microbiome with high plant biomass-degrading capacity 6(9). doi:10.1371/journal.pgen.1001129.

Suh, S., Marshall, C. J., McHugh, J. V. and Blackwell, M. 2003. Wood ingestion by passalid beetles in the presence of xylose-fermenting gut yeasts. *Molecular Ecology* 12: 3137-3145.

Suh, S., McHugh, J. V., Pollock, D. D. and Blackwell, M. 2005. The beetle gut: a hyperdiverse source of novel yeasts. *Microbiol Research* 109(3): 261-265.

Suribabu, K., Govardhan, T. L. and Hemalatha, K. P. J. 2014. Optimization of physical parameters of α-amylase producing *Brevibacillus borostelensis* R1 in submerged fermentation. *International Journal of Research in Engineering and Technology* 3(1): 517-525.

Sutton, S. 2006. The Gram Stain. *Pharmaceutical Microbiology Forum Newsletter* 12(2): 4-8.

Suzuki, K. I. and Hamada, M. 2012. Phylum XXVI. Actinobacteria phyl. Nov. (*In* Goodfellow, M., Kämpfer, P., Busse, H. J., Trujillo, M. E., Suzuki, K., Ludwig, W. and Whitman, W. B. *eds*. Volume 5 of Bergey's Manual of Systematic Bacteriology. Springer: London. Pp. 814-852).

Swiecicka, I. and Mahillon, J. 2005. Diversity of commensal *Bacillus cereus sensu lato* isolated from the common sow bug (*Porcellio scaber*, Isopoda). *Microbiology Ecology* 56: 132-140.

Tabashnik, B. E., Carriére, Y., Dennehy, T. J., Morin, S., Sisterson, M. S., Roush, R. T., Shelton, A. M. and Zhao, J. 2003. Insect resistance to transgenic Bt crops: Lessons from the laboratory and field. *Journal of Economic Entomology* 96(4): 1031-1038.

Tabashnik, B. E., Finson, N., Johnson, M. W. and Heckel, D. G. 1994. Cross-resistance to *Bacillus thuringiensis* toxin CryIF in the diamondback moth (*Plutella xylostella*). *Applied and Environmental Micobiology* 60(12): 4627-4629.

Tabashnik, B. E., Van Rensburg, J. B. J. and Carriére, Y. 2009. Field-evolved insect resistance to Bt crops: Definition, theory, and data. *Journal of Economic Entomology* 102(6): 2011-2025.

Tagliavia, M., Messina, E., Manachini, B., Cappello, S. and Quatrini, P. 2014. The gut microbiota of larvae of *Rhynchophorus ferrugineus* Oliver (Coleoptera: Curculionidae). *BMC Microbiology* 14(136). doi: 10.1186/1471-2180-14-136.

Talekar, N. S. and Shelton, A. M. 1993. Biology, ecology, and management of the diamondback moth. *Annual Review of Entomology* 38: 275-301.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution* 30: 2725-2729.

Tang, J. D., Shelton, A. M., Van Rie, J., De Roeck, S., Moar, W. J., Roush, R. T. and Peferoen, M. 1996. Toxicity of *Bacillus thuringiensis* spore and crystal protein to resistant diamondback moth (*Plutella xylostella*). *Applied and Environmental Microbiology* 62(2): 564-569.

Tang, M. and You, M. 2012. Isolation, identification and characterization of a novel triazophos-degrading *Bacillus* sp. (TAP-1). *Microbiological Research* 167: 299-305.

Ter Braak, C. J. F. 1994. Canonical community ordination-1: Basic theory and linear methods. *Ecoscience* 1: 127-140.

Ter Braak, C. J. F. and Verdonschot, P. F. M. 1995. Canonical correspondence analysis and related multivariable methods in aquatic ecology. *Aquatic Sciences* 57: 153-298.

Thomas, M. and Waage, J. 1996. Integration of biological control and host plant resistance breeding. CTA, Wageningen: The Netherlands. Pp. 105.

Thomas, T., Gilbert, J. and Meyer, F. 2012. Metagenomics - a guide from sampling to data analysis. *Microbial Informatics and Experimentation* 2(3). doi:10.1186/2042-5783-2-3.

Tokuda, G., Watanabe, H. and Lo, N. 2007. Does correlation of cellulase gene expression and cellulolytic activity in the gut of termite suggest synergistic collaboration of cellulases? *Gene* 401: 131-134.

Towner, K. J., Talbot, D. C. S., Curran, R. and Webster, C. A., Humphreys, H. 1998. Development and evaluation of a PCR-based immunoassay for the rapid detection of methicillin-resistant *Staphylococcus aureus*. *Journal of Medical Microbiology* 47: 607-613.

Van den Berg, J., Hilbeck, A. and Bøhn, T. 2013. Pest resistance to Cry1Ab Bt maize: Field resistance, contributing factors and lessons from South Africa. *Crop Protection* 54: 154-160.

Van den Berg, J., Nur, A. F. and Polaszek, A. 1998. Chemical control. (*In* A. Polaszek., *ed*. Cereal stem borers in Africa: Economic importance, taxonomy, natural enemies and control. International Institute of Entomology. CAB Internasional. Pp. 319-347).

Van der Hoeven, R., Betrabet, G. and Forst, S. 2008. Characterization of the gut bacterial community in *Manduca sexta* and effect of antibiotics on bacterial diversity and nematode reproduction. *FEMS Microbiology Letters* 286: 249-256.

Van Rensburg, J. B. J. 1999. Evaluation of Bt-Transgenic maize for resistance to the stem borers *Busseola fusca* (Fuller) and *Chilo partellus* (Swinhoe) in South Africa. *South African Journal of Plant and Soil* 16(1): 38-43.

Van Rensburg, J. B. J. 2007. First report of field resistance by the stem borer, *Busseola fusca* (Fuller) to Bt-transgenic maize. *South African Journal of Plant and Soil* 24(3): 147-151.

Van Rensburg, J. B. J. and Flett, B. C. 2008. A review of research achievements on maize stem borer, *Busseola fusca* (Fuller) and Diplodia ear rot caused by *Stenocarpella maydis* (Berk. Sutton). *South African Journal of Plant and Soil* 27(1): 74-80.

Van Wyk, A., Van den Berg, J. and Van Rensburg, J. B. J. 2009. Comparative efficacy of Bt maize events MON810 and Bt11 against *Sesamia calamistis* (Lepidoptera: Noctuidae) in South Africa. *Crop Protection* 28: 113-116.

Vega, F. E. and Dowd, P. F. 2005. The role of yeasts as insect endosymbionts. (*In* Vega, F. E. and Blackwell, M. *eds*. Insect-Fungal Associations: ecology and evolution. Oxford University Press: New York. Pp. 211-243).

Vercesi, M. L., Krogh, P. H. and Holmstrup, M. 2006. Can *Bacillus thuringiensis* (Bt) corn residues and Bt-corn plants affect life-history traits in the earthworm *Aporrectodea caliginosa*? *Applied Soil Ecology* 32: 180-187.

Wang, P., Zhao, J. Z., Rodrigo-Simón, A., Kain, W., Janmaat, A. F., Shelton, A. M., Ferré, J. and Myers, J. 2007. Mechanism of resistance to *Bacillus thuringiensis* toxin Cry1Ac in a greenhouse population of the cabbage looper, *Trichoplusia ni. Applied and Environmental Microbiology* 73(4): 1199-1207.

Wang, W. W., He, C., Cui, J., Wang, H. D. and Li, M. L. 2014. Comparative analysis of the composition of intestinal bacterial communities in *Dastarcus helophoroides* fed different diets. *Journal of Insect Science* 14(111). doi.org/10.1673/031.014.111.

Wang, W. Z., Morohoshi, T., Ikenoya, M., Someya, N. and Ikeda, T. 2010. AiiM, a novel class of *N*-Acylhomoserine Lactonase from the leaf-associated bacterium *Microbacterium testaceum. Applied and Environmental Microbiology* 76(8): 2524-2530.

Wiese, T., Kai, M. and Piechulla, B. 2013. Bacterial ammonia causes significant plant growth inhibition. *PLoS ONE* 8(5). doi:10.1371/journal.pone.0063538.

Willey, J. M., Sherwood, L., Woolverton, C. J. and Prescott, L. M. 2008. Prescott, Harley, and Klein's Microbiology. 7th *ed.* New York: McGraw-Hill Higher Education. Pp. 1081.

Williams, P. 2007. Quorum sensing, communication and cross-kingdom signalling in the bacterial world. *Microbiology* 153: 3923-3938.

Wu, Y., Chi, S., Yun, C., Shen, Y., Tokuda, G. and Ni, J. 2012. Molecular cloning and characterization of an endogenous digestive b-glucosidase from the midgut of the fungus-growing termite *Macrotermes barneyi. Insect Molecular Biology* 21(6): 604-614.

Xiang, H., Wei, G., Jia, S., Huang, J., Miao, X., Zhou, Z., Zhao, L. and Huang, Y. 2006. Microbial communities in the larval midgut of laboratory and field populations of cotton bollworm (*Helicoverpa armigera*). *Canadian Journal of Microbiology* 52: 1085-1092.

Xue, J., Liang, G.,l Crickmore, N., Li, H., He, K., Song, F., Feng, X., Huang, D. and Zhang, J. 2008. Cloning and characterization of a novel Cry1A toxin from *Bacillus thuringiensis* with high toxicity to the Asian corn borer and other lepidopteran insects. *FEMS Microbiology Letters* 280: 95-101.

Yadav, K. K., Shariq, M., Maurya, S. K., Alam, M. D. N. and Ahmad, K. 2012. Molecular characterization of cellulose degrading bacteria on the basis of 16S rRNA. *Journal of Recent Advances in Applied Sciences* 27: 80-92.

Yu, H. L., Li, Y. H. and Wu, K. M. 2011. Risk Assessment and ecological effects of transgenic *Bacillus thuringiensis* crops on non-target organisms. *Journal of Integrative Plant Biology* 53 (7): 520-538.

Zhao, J. Z., Collins, H. L., Li, Y. X., Mau, R. F. L., Thompson, G. D., Hertlein, M., Andaloro, J. T., Boykin, R. and Shelton, A. M. 2006. Monitoring of diamondback moth (Lepidoptera: plutellidae) resistance to spinosad, indoxacarb, and emamectin benzoate. *Journal of Economic Entomology* 99(1): 176-181.

Zou, C., Li, Z. and Yu, D. 2010. *Bacillus megaterium* strain XTBG34 promotes plant growth by producing 2-Pentylfuran. *The Journal of Microbiology* 48(4): 460-466.

Zouache, K., Raharimalala, F. N., Raquin, V., Tran-Van, V., Raveloson, L. H. R., Ravelonandro, P. and Mavingui, P. 2011. Bacterial diversity of field-caught mosquitoes, *Aedes albopictus* and *Aedes aegypti*, from different geographic regions of Madagascar. *FEMS Microbiology Ecology* 75: 377-389.

Zurek, L., Schal, C. and Watson, W. 2000. Diversity and contribution of the intestinal bacterial community to the development of *Musca domestica* (Diptera: Muscidae) larvae. *Journal of Medical Entomology* 37(6): 924-928.

APPENDIX 1

Table A1: Characteristics of morphotypes.

Morphotype	Surface appearance and shape of colony	Elevation of colony	Shape of edges of colony	Colour of colony
1	Pinhead	Flat	Circular	Cream
2	Pinhead	Flat	Circular	Transparent
3	Pinhead	Flat	Circular	Yellow (light)
4	Pinhead	Flat	Circular	Yellow (dark)
5	Pinhead	Flat	Circular	White
6	Pinhead	Flat	Serrated	Cream
7	Pinhead	Raised	Circular	Yellow (light)
8	Pinhead	Raised	Circular	Transparent
9	Pinhead	Raised	Circular	Pink
10	Pinhead	Raised	Circular	White
11	Pinhead	Raised	Circular	Cream
12	Pinhead	Raised	Irregular	Cream
13	Pinhead	Low convex	Circular	Yellow (light)
14	Pinhead	Low convex	Circular	White
15	Pinhead	Low convex	Circular	Cream
16	Pinhead	Medium convex	Circular	Yellow (light)
17	Pinhead	Medium convex	Circular	White
18	Smooth	Flat	Circular	Yellow (light)
19	Smooth	Flat	Circular	Cream
20	Smooth	Flat	Circular	Transparent
21	Smooth	Flat	Circular	Cream/White
22	Smooth	Flat	Circular	Yellow (dark)
23	Smooth	Flat	Circular	White
24	Smooth	Flat	Irregular	Yellow (dark)
25	Smooth	Flat	Irregular	Transparent
26	Smooth	Flat	Irregular	Transparent/Cream
27	Smooth	Flat	Irregular	Cream
28	Smooth	Flat	Irregular	Yellow (light)
29	Smooth	Flat	Myceloid	Transparent/Cream
30	Smooth	Flat	Myceloid	Yellow (light)
31	Smooth	Flat	Myceloid	Transparent/Cream/White
32	Smooth	Flat	Lobed-shaped	Transparent
33	Smooth	Raised	Circular	Cream
34	Smooth	Raised	Circular	White
35	Smooth	Raised	Circular	Yellow (light)
36	Smooth	Raised	Circular	Yellow (dark)

37	Smooth	Raised	Circular	Orange
38	Smooth	Raised	Irregular	Cream
39	Smooth	Raised	Irregular	Yellow (light)
40	Smooth	Raised	Lobed-shaped	Cream
41	Smooth	Raised	Lobed-shaped	Cream/Yellow (light)
42	Smooth	Low convex	Circular	Cream
43	Smooth	Low convex	Circular	Cream/White
44	Smooth	Low convex	Circular	Yellow (light)
45	Smooth	Low convex	Circular	White
46	Smooth	Low convex	Circular	Yellow (dark)
47	Smooth	Low convex	Circular	Orange
48	Smooth	Low convex	Irregular	Cream
49	Smooth	Low convex	Irregular	White
50	Smooth	Low convex	Irregular	Yellow (light)
51	Smooth	Low convex	Lobed-shaped	Cream
52	Smooth	Medium convex	Circular	Cream
53	Smooth	Medium convex	Circular	Yellow (light)
54	Smooth	Medium convex	Circular	Yellow (Dark)
55	Smooth	Medium convex	Circular	White
56	Smooth	High convex	Circular	Yellow (dark)
57	Smooth	High convex	Circular	White
58	Smooth	High convex	Circular	Orange
59	Smooth	High convex	Irregular	Cream
60	Smooth	Umbonate	Circular	Cream
61	Smooth	Umbonate	Lobed-shaped	Cream
62	Smooth	Umbonate	Irregular	Cream
63	Smooth/Finely granulated	Medium convex	Circular	Cream/White
64	Finely granulated	Flat	Circular	Cream
65	Finely granulated	Flat	Irregular	Cream
66	Finely granulated	Flat	Filamented	Cream
67	Finely granulated	Flat	Irregular	Yellow (light)
68	Finely granulated	Flat	Irregular	Transparent/Cream
69	Finely granulated	Flat	Myceloid	Cream
70	Finely granulated	Flat	Myceloid	White
71	Finely granulated	Flat	Myceloid	Yellow (light)
72	Finely granulated	Flat	Lobed-shaped	Cream
73	Finely granulated	Raised	Irregular	Cream
74	Finely granulated	Raised	Circular	Cream
75	Finely granulated	Raised	Radially striated	Yellow (light)
76	Finely granulated	Raised	Lacy	White/Cream
77	Finely granulated	Raised	Lacy	White/Cream/Yellow(light)
78	Finely granulated	Raised	Myceloid	Cream
79	Finely granulated	Low convex	Circular	White

80	Finely granulated	Low convex	Irregular	Cream
81	Finely granulated	Medium convex	Circular	White
82	Finely granulated	High convex	Circular	White
83	Coarsely granulated	Flat	Irregular	Transparent
84	Coarsely granulated	Raised	Circular	Cream
85	Coarsely granulated	Raised	Circular	White
86	Coarsely granulated	Raised	Dentoid	White
87	Coarsely granulated	Medium convex	Irregular	White
88	Coarsely granulated	Umbonate	Irregular	Cream
89	Coarsely granulated	Papilla-shaped	Irregular	White
90	Filament-shaped	Flat	Lacy	Cream
91	Filament-shaped	Raised	Irregular	White
92	Filament-shaped	Raised	Filamented	Cream/Yellow (dark)
93	Filament-shaped	Low convex	Irregular	Cream
94	Filament-shaped	Low convex	Dentoid	Yellow (light)
95	Filament-shaped	Medium convex	Dentoid	White
96	Filament-shaped	Medium convex	Myceloid	White
97	Undulating	Flat	Myceloid	Cream
98	Undulating	Flat	Irregular	Cream
99	Undulating	Raised	Circular	Yellow (dark)
100	Undulating	Low convex	Circular	Yellow (light)
101	Undulating	Umbonate	Circular	Yellow (light)
102	Undulating	Raised	Circular	Cream
103	Undulating	Umbonate	Radially striated	Yellow (light)
104	Undulating	Papilla-shaped	Irregular	Cream
105	Smooth	Medium Convex	Irregular	Yellow (Dark)
106	Pinhead	Raised	Circular	Yellow (Very Light)
107	Smooth	High Convex	Circular	Orange (Light)
108	Smooth	High Convex	Circular	Cream
109	Smooth	Low Convex	Circular	Brown
110	Pinhead	Low Convex	Circular	Orange (Light)
111	Smooth	High Convex	Circular	Yellow (Light)
112	Filament-Shaped	Raised	Serrated	Brown
113	Smooth	High Convex	Circular	Cream
114	Smooth	Medium Convex	Circular	Pink
115	Smooth	Raised	Irregular	Orange (Light)

116	Smooth	High Convex	Irregular	Orange (Light)
117	Smooth	Raised	Irregular	Yellow (Very Light)
118	Undulating	Umbonate	Irregular	Red
119	Smooth	Low Convex	Circular	Orange (Light)
120	Undulating	Raised	Irregular	Cream
121	Pinhead	Low Convex	Circular	Yellow (Dark)
122	Undulating	Papilla-Shaped	Irregular	Orange
123	Smooth	Crater-Shaped	Circular	Yellow (Very Light)
124	Undulating	Raised	Lobed-Shaped	Yellow (Light)
125	Undulating	Crater-Shaped	Irregular	Yellow (Dark)
126	Pinhead	Flat	Circular	Pink
127	Coarsely granulated	Papilla-Shaped	Irregular	Orange (Light)
128	Smooth	Low Convex	Circular	Pink
129	Undulating	Umbonate	Circular	Yellow (Dark)
130	Smooth	Low Convex	Irregular	Orange
131	Smooth	Crater-Shaped	Circular	Yellow (Light)
132	Smooth	Raised	Filament	Cream/Yellow (Light)
133	Filament-Shaped	Raised	Irregular	Cream/Yellow (Light)
134	Smooth	Low Convex	Circular	Pink (Light)
135	Undulating	Crater-Shaped	Irregular	Cream

APPENDIX 2

Table A2: GenBank identifications of amplified samples from selected morphotypes, assigned accession numbers as well as abbreviations for species names.

Morphotype	Query length	Query cover (%)	E Value	Similarity (%)	GenBank ID	Species Abbreviation	Accession Number
1	770	100	0.0	99	*Klebsiella pneumoniae*	*K_pne*	KJ742474
2	422	99	6,00E-136	86	*Bacillus thuringiensis*	*Bac_thu*	KJ742475
3	867	97	0.0	99	*Brevibacterium frigoritolerans*	*Bvbac_fri*	KJ742476
4	577	99	0.0	99	*Arthrobacter creatinolyticus*	*Art_cre*	KJ742477
5	699	99	0.0	99	*Leucobacter iarius*	*Leu_iar*	KJ742455
7	707	100	0.0	99	*Leucobacter chromiiresistens*	*Leu_chs*	KJ742441
8	728	100	0.0	100	*Enterococcus casseliflavus*	*Enc_cas*	KJ742456
9	311	77	6,00E-45	78	*Enterococcus mundtii*	*Enc_mun*	*
10	650	100	0.0	100	*Bacillus mycoides*	*Bac_myc*	KJ742442
11	281	70	1,00E-15	74	*Enterococcus gallinarum*	*Enc_gal*	*
12	861	100	0.0	92	*Enterobacter ludwigii*	*Enb_lud*	KJ742478
13	980	100	0.0	99	*Leucobacter chromiiresistens*	*Leu_chs*	KJ742479
14	634	100	0.0	99	*Enterococcus casseliflavus*	*Enc_cas*	KJ742443
15	915	100	0.0	99	*Enterococcus casseliflavus*	*Enc_cas*	KJ742480
16	1002	99	0.0	99	*Leucobacter chromiiresistens*	*Leu_chs*	KJ742481
17	430	100	0.0	96	*Enterococcus casseliflavus*	*Enc_cas*	KJ742482
18	832	100	0.0	91	*Enterobacter cancerogenus*	*Enb_can*	KJ742483
19	968	100	0.0	99	*Klebsiella oxytoca*	*K_oxy*	KJ742484
20	991	100	0.0	99	*Enterococcus gallinarum*	*Enc_gal*	KJ742485
21	587	100	0.0	99	*Klebsiella oxytoca*	*K_oxy*	KJ742486
22	979	99	0.0	99	*Enterococcus gallinarum*	*Enc_gal*	KJ742487
23	567	100	0.0	87	*Enterobacter ludwigii*	*Enb_lud*	KJ742458
24	805	99	0.0	100	*Stenotrophomonas maltophilia*	*Sten_mal*	KJ742488
25	1076	98	0.0	98	*Enterococcus casseliflavus*	*Enc_cas*	KJ742489

26	999	100	0.0	99	*Klebsiella variicola*	*K_var*	KJ742490
27	782	100	0.0	100	*Klebsiella variicola*	*K_var*	KJ742491
28	1089	100	0.0	99	*Microbacterium paraoxydans*	*Mbac_par*	KJ742492
30	989	98	0.0	99	*Microbacterium arborescens*	*Mbac_arb*	KJ742493
31	723	100	0.0	99	*Arthrobacter creatinolyticus*	*Art_cre*	KJ742494
32	699	100	0.0	100	*Enterococcus casseliflavus*	*Enc_cas*	KJ742445
33	1079	99	0.0	99	*Klebsiella pneumoniae*	*K_pne*	KJ742495
34	489	94	1,00E-170	90	*Ochrobactrum ciceri*	*Och_cic*	KJ742496
35	1040	100	0.0	98	*Microbacterium paraoxydans*	*Mbac_par*	KJ742497
37	492	100	0.0	99	*Bacillus subtilis*	*Bac_sub*	KJ742498
38	1058	99	0.0	99	*Klebsiella pneumoniae*	*K_pne*	KJ742499
39	546	100	0.0	99	*Microbacterium oxydans*	*Mbac_par*	KJ742444
40	438	100	0.0	95	*Enterococcus casseliflavus*	*Enc_cas*	KJ742500
41	702	100	0.0	100	*Microbacterium oxydans*	*Mbac_oxy*	KJ742446
42	997	100	0.0	99	*Enterococcus gallinarum*	*Enc_gal*	KJ742501
43	990	100	0.0	99	*Klebsiella variicola*	*K_var*	KJ742502
44	976	99	0.0	99	*Bacillus subtilis*	*Bac_sub*	KJ742503
45	579	100	0.0	99	*Serratia sp.*	*Serr_sp*	KJ742504
46	719	100	0.0	100	*Enterococcus casseliflavus*	*Enc_cas*	KJ742457
47	1001	99	0.0	99	*Enterococcus gallinarum*	*Enc_gal*	KJ742505
48	895	98	0.0	98	*Leucobacter chromiireducens*	*Leu_chd*	KJ742506
49	964	100	0.0	100	*Leucobacter aridicollis*	*Leu_ari*	KJ742507
50	741	100	0.0	99	*Leucobacter tardus*	*Leu_tar*	KJ742459
51	1035	99	0.0	99	*Klebsiella pneumoniae*	*K_pne*	KJ742508
52	1002	100	0.0	100	*Klebsiella oxytoca*	*K_oxy*	KJ742509
53	835	99	0.0	99	*Leucobacter chromiiresistens*	*Leu_chs*	KJ742510
54	929	99	0.0	98	*Pseudomonas plecoglossicida*	*Psdo_ple*	KJ742511
55	1009	100	0.0	99	*Arthrobacter oxydans*	*Art_oxy*	KJ742512
56	662	100	0.0	100	*Enterobacter cloacae*	*Enb_clo*	KJ742460
57	475	98	2,00E-130	83	*Arthrobacter sp.*	*Art_sp*	KJ742513

58	967	100	0.0	99	*Microbacterium testaceum*	*Mbac_tes*	KJ742514
59	611	99	0.0	92	*Enterobacter ludwigii*	*Enb_lud*	KJ742515
60	495	100	0.0	99	*Klebsiella variicola*	*K_var*	KJ742516
61	807	98	0.0	99	*Klebsiella variicola*	*K_var*	KJ742517
62	374	97	0.0	100	*Klebsiella variicola*	*K_var*	KJ742518
63	1053	99	0.0	99	*Klebsiella pneumoniae*	*K_pne*	KJ742519
64	597	100	0.0	99	*Enterococcus casseliflavus*	*Enc_cas*	KJ742447
65	740	100	0.0	100	*Bacillus mycoides*	*Bac_myc*	KJ742448
66	1081	99	0.0	99	*Bacillus cereus*	*Bac_cer*	KJ742520
67	986	100	0.0	99	*Stenotrophomonas maltophilia*	*Sten_mal*	KJ742521
68	567	100	0.0	100	*Caulobacter sp.*	*Caul_sp*	KJ742522
69	739	100	0.0	100	*Bacillus anthracis*	*Bac_anth*	KJ742523
70	689	100	0.0	100	*Enterobacter cloacae*	*Enb_clo*	KJ742461
71	866	100	0.0	99	*Enterococcus casseliflavus*	*Enc_cas*	KJ742524
72	1003	100	0.0	99	*Bacillus megaterium*	*Bac_meg*	KJ742525
73	742	100	0.0	99	*Staphylococcus haemolyticus*	*Sta_hea*	KJ742526
74	954	99	0.0	99	*Brevibacillus borstelensis*	*Bvbac_bor*	KJ742527
75	761	100	0.0	100	*Enterococcus casseliflavus*	*Enc_cas*	KJ742449
76	1049	100	0.0	98	*Bacillus licheniformis*	*Bac_lich*	KJ742528
77	991	100	0.0	99	*Bacillus licheniformis*	*Bac_lich*	KJ742529
78	683	100	0.0	98	*Enterobacter asburiae*	*Enb_asb*	KJ742530
79	998	100	0.0	99	*Klebsiella variicola*	*K_var*	KJ742531
80	936	100	0.0	99	*Klebsiella oxytoca*	*K_oxy*	KJ742532
81	808	99	0.0	99	*Klebsiella pneumoniae*	*K_pne*	KJ742533
82	396	97	0.0	99	*Klebsiella variicola*	*K_var*	KJ742534
83	660	100	0.0	100	*Enterococcus casseliflavus*	*Enc_cas*	KJ742450
84	919	100	0.0	100	*Klebsiella oxytoca*	*K_oxy*	KJ742535
85	1013	100	0.0	99	*Enterobacter hormaechei*	*Enb_hor*	KJ742536
86	695	100	0.0	100	*Pantoea agglomerans*	*Pant_agg*	KJ742462
87	665	100	0.0	100	*Bacillus methylotrophicus*	*Bac_meth*	KJ742463
88	498	99	0.0	99	*Pseudomonas sp.*	*Psdo_sp*	KJ742464

89	1029	100	0.0	99	*Caulobacter crescentus*	*Caul_cres*	KJ742537
90	561	100	0.0	99	*Bacillus licheniformis*	*Bac_lich*	KJ742538
91	686	100	0.0	100	*Enterococcus casseliflavus*	*Enc_cas*	KJ742465
92	518	100	0.0	100	*Serratia marcescens*	*Serr_mar*	KJ742539
93	712	100	0.0	100	*Bacillus thuringiensis*	*Bac_thu*	KJ742540
94	780	100	0.0	99	*Enterococcus casseliflavus*	*Enc_cas*	KJ742541
95	728	99	0.0	99	*Bacillus thuringiensis*	*Bac_thu*	KJ742542
96	1075	99	0.0	99	*Bacillus megaterium*	*Bac_meg*	KJ742543
97	550	99	0.0	88	*Bacillus licheniformis*	*Bac_lich*	KJ742466
98	692	100	0.0	100	*Bacillus subtilis*	*Bac_sub*	KJ742467
99	708	100	0.0	100	*Bacillus thuringiensis*	*Bac_thu*	KJ742451
100	1018	99	0.0	99	*Brevundimonas diminuta*	*Bvdi_dim*	KJ742544
101	651	100	0.0	100	*Leucobacter alluvii*	*Leu_all*	KJ742468
102	873	100	0.0	99	*Bacillus subtilis*	*Bac_sub*	KJ742545
103	708	100	0.0	99	*Micrococcus luteus*	*Mcoc_lut*	KJ742546
104	915	100	0.0	99	*Klebsiella oxytoca*	*K_oxy*	KJ742547
105	652	97	3,00E-166	81	*Halomonas sp.*	*Hal_sp*	KJ742548
107	753	99	0.0	99	*Planomicrobium chinense*	*Pl_chi*	KJ742549
108	368	89	3,00E-93	85	*Pseudochrobactrum kiredjianiae*	*Psbac_kir*	*
109	967	100	0.0	99	*Enterococcus gallinarum*	*Enc_gal*	KJ742550
110	719	100	0.0	100	*Planomicrobium sp.*	*Pl_sp*	KJ742469
112	205	100	1,00E-96	99	*Pseudomonas protegens*	*Psdo_pro*	KJ742551
113	684	98	0.0	100	*Bacillus megaterium*	*Bac_meg*	KJ742552
114	645	100	0.0	100	*Enterococcus mundtii*	*Enc_mun*	KJ742452
115	683	100	0.0	99	*Pseudomonas protegens*	*Psdo_pro*	KJ742553
116	918	99	0.0	99	*Arthrobacter oxydans*	*Art_oxy*	KJ742554
117	840	99	0.0	100	*Achromobacter spanius*	*Ach_spa*	KJ742555
118	704	100	0.0	100	*Enterobacter cloacae*	*Enb_clo*	KJ742470
119	565	99	0.0	99	*Klebsiella oxytoca*	*K_oxy*	KJ742556
120	943	100	0.0	99	*Klebsiella oxytoca*	*K_oxy*	KJ742557
121	710	99	0.0	97	*Bacillus anthracis*	*Bac_anth*	KJ742558

122	790	100	0.0	99	*Bacillus thuringiensis*	*Bac_thu*	KJ742559
123	695	100	0.0	99	*Enterobacter cloacae*	*Enb_clo*	KJ742471
124	589	100	0.0	92	*Microbacterium sp.*	*Mbac_sp*	KJ742453
125	688	100	0.0	100	*Bacillus thuringiensis*	*Bac_thu*	KJ742454
126	969	100	0.0	99	*Enterobacter asburiae*	*Enb_asb*	KJ742560
127	721	100	0.0	100	*Leucobacter alluvii*	*Leu_all*	KJ742472
128	813	100	0.0	99	*Enterococcus casseliflavus*	*Enc_cas*	KJ742561
130	810	100	0.0	99	*Bacillus cereus*	*Bac_cer*	KJ742562
131	639	100	0.0	99	*Enterococcus casseliflavus*	*Enc_cas*	KJ742473
132	997	100	0.0	99	*Solibacillus silvestris*	*Soli_sil*	KJ742563
133	1036	100	0.0	99	*Enterococcus casseliflavus*	*Enc_cas*	KJ742564
134	650	98	0.0	94	*Klebsiella oxytoca*	*K_oxy*	KJ742565
135	515	97	2,00E-110	79	*Achromobacter piechaudii*	*Ach_pie*	KJ742566

*Accession numbers not assigned

APPENDIX 3

Table A3: Classification of the isolates obtained from the midgut of *B. fusca* larvae.

Phylum	Class	Order	Family	Genus	Species
Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	*Enterococcus*	*casseliflavus*
					gallinarum
					mundtii
		Bacillales	Staphylococcaceae	*Staphylococcus*	*haemolyticus*
			Bacillaceae	*Bacillus*	*anthracis*
					cereus
					licheniformis
					megaterium
					methylotrophicus
					mycoides
					subtilis
					thuringiensis
				Solibacillus	*silvestris*
			Planococcaceae	*Planomicrobium*	*chinense*
			Paenibacillacea	*Brevibacillus*	*borstelensis*
Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	*Micrococcus*	*luteus*
				Arthrobacter	*creatinolyticus*
					oxydans
				Leucobacter	*alluvii*
					aridicollis
					Chromiireducens
					chromiiresistens
					iarius
					tardus
			Microbacteriaceae	*Microbacterium*	*arborescens*
					oxydans
					paraoxydans
					testaceum
			Brevibacteriaceae	*Brevibacterium*	*frigoritolerans*
Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Enterobacter*	*ludwigii*

					cancerogenus
					cloacae
					asburiae
					hormaechei
				Pantoea	*agglomerans*
			Klebsiella	*oxytoca*	
					pneumoniae
					variicola
				Serratia	*marcescens*
		Pseudomonadales	Pseudomonadaceae	*Pseudomonas*	*plecoglossicida*
					protegens
		Xanthomonadales	Xanthomonadaceae	*Stenotrophomonas*	*maltophilia*
		Oceanospirillales	Halomonadaceae	*Halomonas*	
	Alphaproteobacteria	Rhizobiales	Brucellaceae	*Pseudochrobactrum*	*kiredjianiae*
				Ochrobactrum	*ciceri*
		Caulobacterales	Caulobacteraceae	*Caulobacter*	*crescentus*
				Brevundimonas	*diminuta*
	Beta Proteobacteria	Burkholderiales	Alcaligenaceae	*Achromobacter*	*spanius*
					piechaudii

APPENDIX 4

Table A4: Species occurrence at the respective sampling sites situated in the different provinces.

Species	Free State												North-West												Gauteng					North-Cape
	BFN	BLH	BOT	BRF	BTF	BTT	KSP	RTZ	THN	TWL	VRF	WSB	COL	DERB	GERD	GPN	LICH1	LICH2	ODL	POTCH1	POTCH2	VLR	VLF	WIT	DEL	HDB	KPD	MGB	RYN	VLH
A. piechaudii	X	X	X	X	X	X	X	X	X	X	X	X	X	✔	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
A. spanius	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	✔	X	X	X	X	X	X	X	X
A. creatinolyticus	X	X	X	✔	X	X	X	✔	X	X	X	X	X	X	✔	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
A. oxydans	X	X	X	X	X	X	X	✔	X	X	X	X	X	X	X	X	X	X	X	X	X	X	✔	✔	X	X	✔	✔	X	X
Arthrobacter sp.	X	X	X	X	X	X	X	X	X	X	X	X	X	✔	X	X	X	X	X	X	X	X	X	X	✔	X	X	X	X	X
B. anthracis	✔	✔	X	✔	X	✔	X	X	✔	X	✔	X	✔	X	X	X	X	X	X	X	X	X	X	X	X	X	X	✔	X	X
B. cereus	X	X	X	✔	X	X	✔	X	X	X	X	✔	X	X	X	✔	✔	X	X	X	✔	X	X	X	X	X	X	X	X	X
B. licheniformis	X	X	X	X	✔	X	X	X	X	X	X	✔	X	X	X	X	X	✔	X	X	X	X	X	X	X	X	X	X	X	X
B. megaterium	X	X	X	X	X	X	X	✔	X	X	✔	X	X	X	X	X	X	X	X	X	X	X	X	✔	X	✔	X	✔	X	X
B. methylotrophicus	X	X	✔	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
B. mycoides	✔	X	✔	X	✔	X	✔	X	✔	X	X	X	X	X	X	X	X	X	X	X	X	X	X	✔	X	X	X	X	X	X
B. subtilis	✔	✔	X	✔	✔	X	✔	✔	X	X	X	✔	✔	✔	X	✔	✔	X	✔	X	X	✔	X	✔	X	X	✔	X	X	X
B. thuringiensis	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	X	✔	✔	✔	✔	✔	✔	✔
B. borstelensis	X	X	X	X	X	X	✔	X	X	✔	✔	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
B. diminuta	X	X	X	X	X	X	X	X	✔	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	✔	X	X	X	X	X
B. frigoritolerans	✔	✔	X	X	X	✔	X	✔	✔	X	✔	X	✔	X	X	✔	X	X	X	X	✔	X	X	X	✔	X	X	✔	X	X
C. crescentus	X	X	X	X	X	X	X	X	X	X	X	X	X	✔	X	✔	X	X	✔	X	X	X	X	X	X	X	X	X	X	X
Caulobacter sp.	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	✔	X	X	X	X	X	X	X	X
E. asburiae	X	X	✔	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	✔	X	X
E. cancerogenus	X	✔	X	X	✔	X	X	X	X	X	X	X	✔	X	X	X	X	X	X	✔	X	X	X	X	✔	✔	X	X	✔	X
E. cloacae	X	X	X	X	✔	X	X	X	✔	X	✔	X	X	✔	X	X	✔	X	X	X	X	✔	X	X	X	X	X	✔	X	X
E. hormaechei	X	✔	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
E. ludwigii	✔	X	X	✔	X	X	X	X	X	X	X	✔	X	X	X	✔	X	✔	X	X	X	X	X	X	X	X	X	X	X	X
E. casseliflavus	X	X	✔	✔	✔	X	✔	✔	✔	X	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	X	✔	X	X	✔	✔	✔	✔	✔	X
E. gallinarum	✔	✔	✔	X	✔	✔	X	✔	✔	X	✔	✔	✔	✔	✔	✔	X	✔	✔	X	✔	✔	✔	✔	✔	X	✔	✔	✔	✔
E. mundtii	X	X	X	X	X	X	X	✔	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	✔	X	✔	✔	✔	X	X
Halomonas sp.	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	✔
K. oxytoca	✔	✔	X	✔	✔	✔	✔	✔	X	✔	✔	✔	✔	✔	X	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	X	✔	✔	✔	✔
K. pneumoniae	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	X	✔	✔	✔	✔	✔	✔
K. variicola	X	✔	X	X	X	✔	X	X	X	X	✔	X	X	✔	X	✔	✔	✔	X	X	X	✔	✔	X	✔	X	X	✔	✔	X
L. alluvii	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	✔	X	X	X	X	X	✔	X	X
L. aridicollis	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	✔	X	X	X	X	X	X	X	X	X	X	X	X	✔	X
L. chromiireducens	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	✔	X	X	✔	X	✔	X	X	✔	X	✔	X	✔	X
L. chromiiresistens	✔	✔	✔	✔	✔	X	✔	✔	✔	X	X	X	X	✔	X	X	✔	X	✔	X	X	✔	X	✔	✔	✔	X	✔	✔	✔
L. iarius	X	X	X	X	✔	X	X	X	X	X	✔	X	X	✔	✔	✔	X	X	X	X	✔	✔	X	X	✔	X	✔	X	✔	X
L. tardus	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	✔	X	X
M. arborescens	X	X	X	X	X	X	✔	X	X	X	✔	X	X	X	X	X	X	X	X	X	X	X	X	X	✔	X	X	✔	X	X
M. oxydans	X	X	✔	X	✔	X	X	X	X	X	X	X	X	X	✔	X	X	X	X	X	X	X	X	X	✔	X	X	X	X	X
M. paraoxydans	✔	✔	✔	✔	✔	✔	X	X	✔	X	✔	X	✔	X	✔	✔	✔	✔	✔	✔	X	X	X	X	✔	✔	✔	X	✔	X
Microbacterium sp.	X	X	X	X	✔	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	✔	X	X	X	X	X
M. testaceum	X	X	X	X	X	X	X	X	X	X	X	X	✔	X	X	X	X	X	✔	X	X	X	X	✔	X	✔	X	X	✔	X
M. luteus	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	✔	X	X
O. ciceri	X	✔	✔	✔	X	X	X	X	X	X	X	X	X	X	X	✔	X	X	✔	✔	X	X	X	X	X	X	✔	X	X	X
P. agglomerans	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	✔	X	X
P .chinense	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	✔	X	X	X	X	X	X
Planomicrobium sp.	X	✔	X	X	X	X	X	X	X	X	X	X	✔	X	X	✔	X	X	X	X	X	X	X	✔	X	X	X	✔	X	X
P. kiredjianiae	X	X	X	X	X	X	X	✔	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	✔	X	✔	X	X	X	X
P. plecoglossicida	X	X	X	X	X	X	X	X	X	X	✔	X	X	✔	X	X	✔	X	✔	X	X	X	X	X	X	X	X	✔	X	✔
P. protegens	X	X	X	X	X	X	X	✔	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	✔	✔	X	X	X	X
Pseudomonas sp.	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	✔	X	X	X	X	X
S. marcescens	X	X	X	X	X	X	X	X	✔	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Serratia sp.	X	X	X	X	X	✔	X	X	X	X	X	✔	X	✔	X	X	X	X	X	X	X	X	X	X	X	X	✔	X	✔	X
S. silvestris	X	X	X	X	✔	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
S. haemolyticus	X	X	✔	X	X	X	✔	X	X	X	✔	X	X	X	X	✔	X	X	X	X	X	✔	X	X	✔	✔	X	X	X	X
S. maltophilia	X	X	X	X	X	X	X	✔	X	X	X	X	X	X	X	X	X	X	X	X	X	✔	X	X	X	X	X	X	X	X
Unknown	X	X	X	✔	✔	X	✔	X	X	X	✔	X	X	✔	X	✔	✔	✔	X	X	X	X	X	✔	✔	X	✔	✔	✔	✔

APPENDIX 5

Table A5: Direct distances between sampling sites in kilometers. Distances were calculated through means of the coordinates of each site.

Sites	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1	0	268	385	364	351	269	275	266	248	208	244	272	417	430	399	339	319	275	66	163	137	150	169	156	193	204	239	240	225	207
2	268	0	135	197	208	33	37	13	48	62	101	71	154	169	168	73	69	73	209	215	208	181	280	259	197	91	105	86	87	88
3	385	135	0	131	156	200	113	145	138	195	151	115	50	56	46	104	143	190	321	285	290	262	346	330	254	182	234	216	220	223
4	364	197	131	0	30	165	160	210	164	233	130	131	180	181	97	211	242	270	299	221	238	217	270	259	185	177	300	281	278	271
5	351	208	156	30	0	176	171	221	170	237	129	138	208	209	126	231	259	282	287	202	221	202	246	238	166	174	308	289	285	276
6	269	33	200	165	176	0	7,1	45	24	77	72	38	148	162	145	88	96	106	206	196	193	165	261	241	174	74	135	116	114	111
7	275	37	113	160	171	7,1	0	50	29	84	72	35	142	156	138	85	97	110	212	199	197	169	264	244	176	79	141	121	120	117
8	266	13	145	210	221	45	50	0	59	59	112	84	160	175	178	74	62	61	209	222	213	186	286	264	205	98	93	74	76	79
9	248	48	138	164	170	24	29	59	0	69	54	34	170	183	158	111	116	117	184	172	169	141	237	217	151	50	138	119	115	107
10	208	62	195	233	237	77	84	59	69	0	110	103	216	231	222	132	113	81	153	184	170	146	245	223	176	74	78	63	53	39
11	244	101	151	130	129	72	72	112	54	110	0	42	194	204	156	157	168	170	178	135	139	111	198	180	107	47	186	168	161	150
12	272	71	115	131	138	38	35	84	34	103	42	0	154	165	128	116	132	144	206	176	178	150	240	221	149	68	170	151	147	141
13	417	154	50	180	208	148	142	160	170	216	194	154	0	15	87	98	138	191	354	329	332	304	391	374	299	218	239	223	230	238
14	430	169	56	181	209	162	156	175	183	231	204	165	15	0	85	113	153	207	367	339	343	315	401	384	308	231	255	239	246	253
15	399	168	46	97	126	145	138	178	158	222	156	128	87	85	0	147	186	229	333	282	291	265	340	325	248	195	270	252	254	254
16	339	73	104	211	231	88	85	74	111	132	157	116	98	113	147	0	40	93	282	284	279	251	348	328	262	161	142	127	135	145
17	319	69	143	242	259	96	97	62	116	113	168	132	138	153	186	40	0	56	266	284	275	248	348	326	266	160	105	92	103	116
18	275	73	190	270	282	106	110	61	117	81	170	144	191	207	229	93	56	0	228	265	251	227	326	304	254	147	49	38	51	68
19	66	209	321	299	287	206	212	209	184	153	178	206	354	367	333	282	266	228	0	110	82	88	138	119	135	139	200	196	181	161
20	163	215	285	221	202	196	199	222	172	184	135	176	329	339	282	284	284	265	110	0	28	39	65	45	36	124	256	245	232	212
21	137	208	290	238	221	193	197	213	169	170	139	178	332	343	291	279	275	251	82	28	0	28	76	53	57	118	238	228	214	195
22	150	181	262	217	202	165	169	186	141	146	111	150	304	315	265	251	248	227	88	39	28	0	100	78	48	91	218	206	193	174
23	169	280	346	270	246	261	264	286	237	245	198	240	391	401	340	348	348	326	138	65	76	100	0	22	92	189	314	304	290	270
24	156	259	330	259	238	241	244	264	217	223	180	221	374	384	325	328	326	304	119	45	53	78	22	0	77	168	291	281	268	248
25	193	197	254	185	166	174	176	205	151	176	107	149	299	308	248	262	266	254	135	36	57	48	92	77	0	107	252	238	227	209
26	204	91	182	177	174	74	79	98	50	74	47	68	218	231	195	161	160	147	139	124	118	91	189	168	107	0	152	136	127	112
27	239	105	234	300	308	135	141	93	138	78	186	170	239	255	270	142	105	49	200	256	238	218	314	291	252	152	0	19	26	44
28	240	86	216	281	289	116	121	74	119	63	168	151	223	239	252	127	92	38	196	245	228	206	304	281	238	136	19	0	15	35
29	225	87	220	278	285	114	120	76	115	53	161	147	230	246	254	135	103	51	181	232	214	193	290	268	227	127	26	15	0	20
30	207	88	223	271	276	111	117	79	107	39	150	141	238	253	254	145	116	68	161	212	195	174	270	248	209	112	44	35	20	0