The role of gut microbes on the efficacy of Bt maize against lepidopteran stem borers

M van Staden

21141002

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:	Dr S Claassens
Co-supervisor:	Prof CC Bezuidenhout
Assistant Supervisor:	Prof J van den Berg

May 2015

Look to the earth and it shall teach thee

– Job 12:8

Table of contents

Acknowledgements

I would like to express gratitude towards the following individuals at the North-West University, Potchefstroom Campus for the opportunity to carry out this project, and for all their guidance, knowledge and support they granted me to complete this study:

Dr. S. Claassens, Prof. C.C. Bezuidenhout and Prof. J. van den Berg for awarding me the opportunity to take part in this study.
Dr. A. van Wyk and staff at the ARC-GCI, Potchefstroom, for their contribution of larvae diet and larval eggs.
Prof. H. du Plessis, Dr. J.J. Bezuidenthout and Clarissa Willers for assistance with the statistical aspects of the study.
Moses Phetoe for the cultivation and maintenance of maize plants used in this study.
Maxi Snyman for the time spent sampling and assistance in the laboratory.
Abram Mahlatsi, Ina van Niekerk and Lee Julies for technical assistance.
Stephan Barnard for the construction of the sampling site map.
My deepest and most sincere appreciations to all the teachers and lecturers I encountered during my education. Thank you for the meaningful impact which only a teacher can have, and the time spent on my education.

Furthermore I would also like to thank my friends and family for the emotional support and encouragement they generously provided during my studies:

Mr. J.P. Viljoen
Ms. H.C. Jansen and Mr. M.J. Jansen (love you to the moon and back)
Mr S.P. van Staden and Ms L.A. van Staden
Mr. S. van Staden
Ms. D. Zaayman
Ms. S. Booyens
The Fermentation Lab brew team
Colleagues at the subject group Microbiology

For financial assistance of the National Research Foundation (South Africa) and GenØk, Center for Biosafety (Norway).

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 full-time during the period of January 2013 - November 2014, under the supervision of Dr. Sarina Claassens, Prof. Carlos Bezuidenhout 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.

The opinions, findings, conclusions and recommendations expressed in this dissertation are those of the author and therefore the NRF does not accept any liability in regard thereto.

Megan van Staden
November 2014

Summary

The evolution of pest resistance to Cry proteins threatens the long-term use of Bt crops. *Busseola fusca* developed resistance to Bt maize in South Africa but the mechanism of resistance is not well understood. According to the gut microbiota theory, extensive cell lysis caused by Cry proteins provide gut microbes access to the more favourable environment of the hemocoel where they germinate and reproduce, causing septicemia and death of the host. This theory brought on questions about the role of gut microbes in the efficacy of Bt maize against target lepidopteran pests. The aim of this study was to determine whether microbes present in the mid-gut of *B. fusca* influence the efficacy of Cry 1Ab proteins. Larvae were collected from 30 different geographical locations, dissected to excise the mid-gut and mid-gut content which was separated according to morphological types. The morphological types were used to test the antibiotic susceptibility of the bacteria and proved that ciprofloxacin, ampicillin and doxycycline were the most effective bacteriostatic and bactericidal antibiotics. These three antibiotics were exposed to the morphological types at different concentrations to visualise the possible deleterious effects of the antibiotics on the bacteria. This visualisation was performed by observing the growth curve of the bacteria in the presence of the combination of antibiotics. The antibiotics concentration of 500 µg/ml showed the highest efficacy compared to the other concentrations tested. An antibiotic concentration of 500 µg/ml of ciprofloxacin, ampicillin and doxycycline was incorporated into an artificial diet for the larvae to feed on for 7 days. This method was used to rid the larvae of gut microbes before allowing them to feed on Bt maize (MON810) plant material expressing Cry proteins. The results suggests that by placing antibiotic reared larvae on a Bt plant, the absence of the mid-gut microbes contributed to larvae survival on Bt maize. This observation will contribute to understanding the role of gut microbes on the efficacy of Cry proteins.

Key words:

Bt maize, *Busseola fusca*, mid-gut, gut microbes, ciprofloxacin, ampicillin, doxycycline

List of Figures

List of Tables

List of Abbreviations

[500]: Artificial *B. fusca* diet infused with ciprofloxacin, ampicillin and doxycycline at equal volumes and a concentration of 500 µg/ml each
ATP: Adenosine triphosphate
BF 1: *Busseola fusca* larval group 1
BF 2: *Busseola fusca* larval group 2
Bt (feeding study): Bt maize
Bt [500]: Larvae fed on antibiotic diet for 7 days and transferred to Bt maize on day 8 of feeding study and remained on Bt maize until day 21
Bt: *Bacillus thuringiensis*
CP 1: *Chilo partellus* larval group 1
CP 2: *Chilo partellus* larval group 2
Cry: Delta-endotoxins produced by *Bacillus thuringiensis*
cry: Genes are located on plasmids of many *Bacillus thuringiensis* which may contribute to the high production of toxins in the different *B. thuringiensis* strains
Cyt: Parasporal inclusion proteins from *Bacillus thuringiensis* that exhibit hemolytic activity
DGGE: Denaturing gradient gel electrophoresis
GM: Genetically modified
HSD: Honest Significant Difference
ICP: Insecticidal crystal proteins
IPM: Integrated pest management
LPS: Lipopolysaccharide
MIC: Minimum inhibitory concentration
NBt [500]: Larvae fed on antibiotic diet for 7 days and transferred to non-Bt maize on day 8 of feeding study and remained on non-Bt maize until day 21
NBt: Non-Bt maize
OD: Optical density
PBP: Penicillin-binding proteins
PCA: Principal component analysis
PFT: Pore-forming toxins
SD Bt: Larvae fed on sterile diet for 7 days and transferred to Bt maize on day 8 of feeding study and remained on Bt maize until day 21
SD NBt: Larvae fed on sterile diet for 7 days and transferred to non-Bt maize on day 8 of feeding study and remained on non-Bt maize until day 21
SD: Sterile, artificial *B. fusca* diet

Chapter 1: Introduction

1.1 Stem borer mid-gut microbes and Bt maize

Maize is a major staple food crop in many regions of the world. The African stem borer (*Busseola fusca*) (Lepidoptera: Noctuidae) is one of the most damaging lepidopteran pests of maize in Africa. Because of its distinctive and destructive feeding behaviour, *B. fusca* is very difficult to control with pesticides (George *et al*. 2011). George *et al*. (2011) established the potential for insect resistant genetically modified (GM) maize (referred to as Bt maize), when used as part of an integrated pest management (IPM) programme, for control of *B. fusca*. Results demonstrated that Bt expression in Bt maize not only extensively decreased larval survival but also reduced pupal and adult weight.

The use of crystalline proteins, also referred to as Cry toxins, from *Bacillus thuringiensis* (Bt) to control insect pests was a radical advancement in crop production (Jurat-Fuentes and Adang, 2006). The large crystal protein inclusions have a narrow range of toxicity. The high specificity, potency, and environmental safety of Bt toxins has led to the wide use of these toxins as insect control proteins in GM crops and biopesticides (Jurat-Fuentes and Adang, 2006). A purely toxin-based hypothesis of Bt suggests that the action of Cry toxins paralyses the mid-gut and leads to concluding death of the insect by starvation (Raymond *et al.* 2009). Alternatively, the Cry toxins paralyse the gut of the insect and cause extensive cell lysis (pore formation) in the intestinal membrane of the mid-gut, allowing bacteria access to the hemolymph (Raymond *et al.* 2009). The bacteria present in the gut of an insect, for example *B. fusca* larvae, are provided access by the action of Bt to the more favourable environment of the hemocoel where they germinate and reproduce, causing septicemia and death of the insect host (Broderick *et al.* 2006). The term microbiota is given to the microbial community in the gut. The mid-gut of Lepidoptera larvae is an extreme but unique environment for mid-gut microbiota to survive in, which makes their bacteria of particular interest (Anand *et al.* 2009). Microorganisms play important and often essential roles in the growth and development of many insect species. Endosymbionts contribute to insect reproduction, nutrition, and pheromone production (Anand *et al.* 2009). The simplicity or diversity of an insect microbial community is particularly apparent compared to other gut environments (Broderick *et al.* 2004).

1.2 Problem statement

The development of resistance of *B. fusca* to Bt maize is a threat to agricultural economics when this pest contributes to a loss in yield. The mechanism of resistance is not well understood. Investigations into the possible effect of mid-gut bacteria on the efficacy of Bt maize against the target pests has yielded interesting and contradicting results. Recently, a pathogenic mechanism was suggested by the claim that *B. thuringiensis* and its toxins are unable of killing aseptically reared gypsy moth larvae, *Lymantria dispar* (Lepidoptera: Erebidae), but that pathogenicity can be reestablished by inoculating hosts with a gut-associated strain of *Enterobacter* (Broderick *et al.* 2004). Other work has extended this claim to a wide range of insect hosts (Broderick *et al*. 2006). An obligate association with the gut microbiota challenges preceding models of the pathogenicity of Bt toxins and has substantial implications for the ecology of Bt and the evolution of resistance mechanisms in invertebrate pests. Results from studies with antibiotic fed larvae suggested that the microbial community in the mid-gut contributed to larval death due to Bt treatment, and therefore concluded that death is unlikely when the mid-gut microbial community is absent (Broderick *et al.* 2006). This theory is directly contradicted by a study by Raymond *et al*. (2009) which state that both Bt toxins and spore/toxin mixtures are pathogenic in the absence of mid-gut bacteria when carry-over effects of antibiotic are excluded by using aseptically reared hosts. The gut microbiota hypothesis remains highly controversial, and is also investigated in the present study.

Busseola fusca and the spotted stem borer, *Chilo partellus* (Lepidoptera: Crambidae), are the most important lepidopteran pests of maize in South Africa (George *et al*. 2011). Damage caused by *B. fusca* and *C. partellus* can cause serious yield losses. The larvae of these pests are difficult to control with contact pesticides and for that reason GM crops are used (George *et al*. 2011). Since the introduction of Bt maize in South Africa, the possibility of resistance development by the target pests was seen as a big threat to the long term use of Bt maize. The development of resistance of *B. fusca* to Bt maize became a reality in 2006, and since then multiple studies focused on the spread of the resistance, the reasons for resistance development and the mechanism of resistance (Carrière *et al.* 2010; Ferré and van Rie, 2002; Gould, 1998; Heckel, 2012; Huang *et al.* 2010; Kruger *et al*. 2012; Pardo-Lopez *et al.* 2013; Tabashnik, 2008; Tabashnik *et al.* 2009, 2013; van Rensburg 2007). However, these reasons for and mechanisms of resistance remain elusive and highly debated.

An insect's response to toxin exposure is often controlled by genetic variation. Ferré and van Rie (2002) noted that the ability of resistant larvae to repair damaged mid-gut cells are more prominent when compared to the ability of susceptible larvae. This observation can be investigated by removing the gut bacteria with the use of antibiotics before placing them on Bt maize. The survival of larvae that only fed Bt can be compared to the larval survival of antibiotic reared larvae. In the present study, the mechanisms of resistance is not directly addressed or examined, but factors that cause septicaemia in the gut microbiota theory are investigated. An investigation into the influence of gut microbes on the efficacy of Bt in lepidopteran target pests was the main focus point.

1.3 Aim and objectives

Aim:

To determine the role of gut microbes on the efficacy of Bt maize against lepidopteran stemborer larvae

Objectives:

- To test the antibiotic susceptibility of the mid-gut bacteria of *B. fusca*
- To develop a method to rid *B. fusca* larvae of mid-gut microbes using a combination of antibiotics
- To determine the concentration of the antibiotics that will rid larvae of the gut microbes
- To determine the role of gut microbes on the efficacy of Bt maize against resistant *B. fusca* and susceptible *C. partellus*

1.4 Outline of dissertation chapters

Chapter 1: Includes an introduction to stem borer mid-gut microbes and Bt maize, the problem statement and the aim and objectives of the present study.

Chapter 2: Contains an overall literature review of the study. It describes lepidopteran stem borers (*B. fusca* and *C. partellus*) and the damage caused by them, as well as GM crops for the control of stem borers. The development of resistance of stem borers to Bt maize and how Bt works (the gut microbiota hypothesis) are discussed in depth. This chapter also includes an overview of the use of antibiotics and antibiotic resistance.

Chapter 3: Describes the experimental layout as well as the materials and methods applied in the study, which includes collection of *B. fusca* larvae, testing the antibacterial susceptibility of the mid-gut bacteria of *B. fusca*, the impact of antibiotics on the mid-gut

bacteria of *B. fusca* and exposing stem borer larvae (*B. fusca* and *C. p*artellus) to Bt maize in the absence of their gut microbes.

Chapter 4: Provides the results obtained from the different aspects of the study.

Chapter 5: General discussion of all the results.

Chapter 6: Includes the conclusions and recommendations with regard to further investigations.

References of all the chapters are provided at the end of the dissertation

Chapter 2: Literature review

2.1 Lepidopteran stem borers of maize

2.1.1 Background

Maize (*Zea mays)* is the most economically important crop in sub-Saharan Africa and an important resource in many small-scale farming areas in South Africa (Krattiger, 1997; Kruger *et al*. 2008). In all agronomic practices insect pest management is primarily important. To produce a sustainable yield, a maize plant has to overcome a number of challenges to survive and sustain yield after an insect attack. Crop loss can be considered as quantitative or qualitative loss. Crop losses which lead to a smaller yield per unit area which resulted from reduced productivity are seen as quantitative loss. A lower actual yield than the site-specific attainable yield of a crop can result from the reduction of crop performance caused by biotic and abiotic environmental factors (Oerke, 2006). A loss from pests which results in reduced market quality is seen as qualitative loss. Between 2001 and 2003, the percentage losses of maize attributed to insect pests were around 31% globally (Oerke, 2006).

2.1.2 Stem borer damage to maize

Cereal crops in Africa, and particularly maize, are attacked by three economically important stem borer species namely *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae), *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae) and *Sesamia calamistis* (Hampson) (Lepidoptera: Noctuidae) (Kfir, 1998; Kruger *et al*. 2008). In South Africa, *B. fusca* and *C. partellus* are the only stem borers of importance in maize and sorghum. For the purpose of this study, *B. fusca* and *C. partellus* were used and will be discussed further (Kfir *et al.* 2002). In 1998, the estimated yield losses due to damages caused *B. fusca* were estimated to range between 5% and 75% (Kfir, 1998). In 2012, the estimated losses were reported to range between 20% to 40%, depending on the intensity of infestation, crop cultivars, agronomic practices and agroecological conditions (Anon, 2012a). Pest densities and plant damage varies between regions, within a country or even the same eco-region of neighbouring countries (Anon, 2012a; George *et al.* 2008; Ndemah *et al.* 2003). The spotted stem borer, *C. partellus,* is becoming a more abundant and noticeable pest with an increasing distribution

area which includes the arid northern and north-western parts of South Africa, the Lowveld region and the northern parts of KwaZulu-Natal (Anon, 2012a; Kruger *et al*. 2008).

The biology and behaviour of a stem borer larvae can be seen as intricate, but several factors about the life cycle are known. The seasonal activity pattern is characterised by two to three distinct generations during spring and summer. These generations are followed by a diapause period of approximately six months during autumn and winter in the lower parts of the dry maize stems (Kfir *et al.* 2002; Kruger *et al.* 2009). *Busseola fusca* has an obligatory diapause and *C. partellus* a facultative diapause (Kfir *et al.* 2002). It has been reported that under field conditions, *C. partellus* emerges from diapause from middle August to the beginning of November. *Busseola fusca* on the other hand, only pupates during October and November. The seasonal activity pattern of this pest is characterised by two to three distinct generations during spring and summer (Kruger *et al*., 2012). This difference in emergence patterns causes the overlapping of generations of the two stem borers which occurs every year in South Africa (Kfir, 1991; Kfir *et al.* 2002). The latter generation infestation of *B. fusca* is seen as particularly damaging to maize (Kruger *et al.* 2012).

Feeding and stem tunnelling by stem borer larvae lead to crop losses as a result of destruction of the growing point, early leaf ageing and interference with translocation of metabolites and nutrients. These damage symptoms result in malformation of grain, stem breakage, plant stunting, lodging, and direct damage to ears. Infestations by stem borers also increase the incidence and severity of stalk rots (Kfir, 1998; Kfir *et al.* 2002).

2.1.3 Control methods

Various control methods have been researched in the past 20 years but no singular method has provided a complete solution to the pest problem (Kipkoech *et al.* 2010). Most of the attacks of stem borers on cereal crops result from infestation by more than one species and, since there are important differences in biology and ecology that limit the effectiveness of some control measures, IPM programmes must be devised to meet local conditions (Anon, 2012b). Pests such as stem borers are especially difficult to control by means of insecticide treatments because older larvae enter the stems and reside there for the duration of their life cycle which provides protection from contact pesticides (George *et al.* 2008).

2.1.4 Genetically modified crops

Genetic modification is the modern scientific tool for developing improved crop varieties to enhance agricultural productivity, increase food production and reduce the use of chemicals (Prakash, 2010; Reddy and Zehr, 2004). Genetically modified (GM) (also called transgenic, genetically engineered, or bioengineered) crops are becoming an increasingly universal feature of agricultural landscapes and represent the fastest-adopted technology in the history of agriculture (Garcia and Altieri, 2005; Murnaghan, 2014)**.** In Figure 2.1, the exponential increase of Biotech crops cultivated throughout the world from 1996 to 2012 can be seen. Soybeans, maize, cotton and canola account for most of these crops, and they have been modified for insect resistance, herbicide tolerance, and disease resistance (Murnaghan, 2014). In Figure 2.2, an illustration of the global map of biotech crop countries present in the world in 2012 is given.

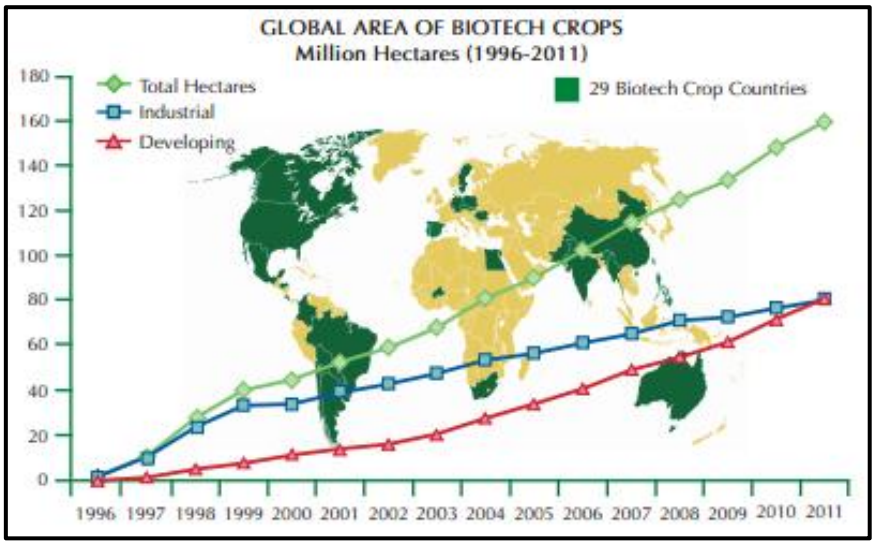

Figure 2.1: An illustration of the exponential increase of biotech crops cultivated over the world from 1996 to 2012. A record 16.7 million farmers, in 29 countries, planted 160 million hectares in 2012, a sustained increase of 8% or 12 million hectares since 2010 (James, 2012)

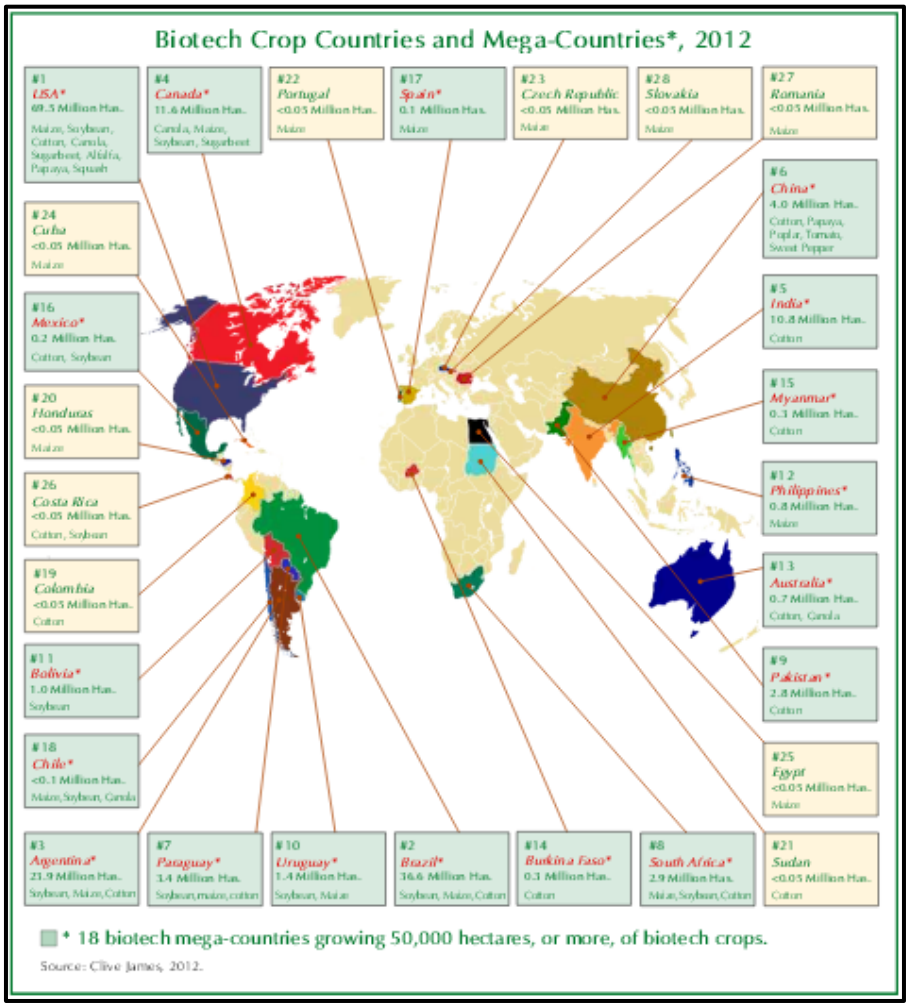

Figure 2.2: Global map of biotech crop countries and mega-countries in 2012 (James, 2012)

The genetic modification of crops refer to plants that are produced by inserting specific pieces of nucleic acids into the plant's DNA using recombinant DNA technology (i.e. *Agrobacterium*-mediated transformation or direct gene transfer methods). This insertion ultimately alters the genetic make-up of the cells (Murnaghan, 2014; Schwember, 2008). Using the gene gun technique (also called particle bombardment or the Biolistic® approach), microscopic particles coated with the desired gene DNA fragment are shot into a plant cell. A small proportion of the entering DNA becomes incorporated into the chromosomes of the plant cell (Murnaghan, 2014). GM technology enables scientists to utilise useful traits to develop new crop varieties with desired traits. GM crops have a variety of traits which are useful in agriculture, but in this section, only the trait for insect resistance will be discussed.

One of these GM control measures is the toxins produced by the soil bacterium *Bacillus thuringiensis* (Bt). This bacterium is widely used in pest management as a microbial insecticide. Its toxins are effective in controlling a number of insect pests in spray form as well as in GM plants into which the insecticidal Bt trait has been incorporated. GM

technology has not only enhanced crop protection but also resulted in a reduction in number of insecticide treatment application required for non-GM crops. GM maize and cotton containing Bt toxin genes are cultivated in South Africa (Peyronnet *et al*. 1997). Numerous researchers have acclaimed GM insecticidal crops as the most important technological advancement in insect pest management since the development of synthetic insecticides (Obrycki *et al*. 2001; Stotzky, 2004).

Although limited, the knowledge about the gut chemistry and mid-gut environment of lepidopteran pests aided in the development of GM crops which offer large benefits to agriculture. Thanks to the increasing availability of information and understanding on this damaging pest, new strategies are achieved through using a combination of plant breeding and biotechnology tools to improve crop protection (Reddy and Zehr, 2004).

An advantage of Bt crops is that they provide reduced input costs to the farmer, season-long protection independent of weather conditions, effective control of burrowing insects difficult to reach with sprays and control at all insect development stages (Kumar, 2003). Despite the numerous advantages, the primary threat to the long-term efficacy of Bt toxins is the evolution of resistance by the target pests (Soberton *et al*. 2007).

2.2 How Bt works

2.2.1 History and background of Bt

The bacterium *Bacillus thuringiensis* was originally discovered by a Japanese biologist named Shigetane Ishiwatari while doing research on “Sudden Collapse Disease” which plagued silkworms, *Bombyx mori* (Lepidpotera: Bombycidae), from 1901 to 1902 (Jisha *et al*. 2013; Lord, 2005; Milner, 1994). Although this was a ground-breaking discovery for the future of genetically modified crops, it was not researched again until 1911 by Ernst Berliner. His description of the bacteria was based on an isolate derived from diseased Mediterranean flour moth, *Ephestia kuhniella* (Lepidoptera: Pyralidae), found in the state of Thuringia, Germany (Jisha *et al*. 2013; Lord, 2005; Milner, 1994). It is thought that the name of the bacteria is derived from the state where it was studied by Berliner. The first records and applications of Bt used for the control of insects were piloted in Hungary at the end of the 1920s. This was followed by Bt applications to control the European corn borer, *Ostrinia nubilalis* (Lepidoptera: Crambidae), during early 1930 in Yugoslavia (Jisha *et al*. 2013; Lord, 2005). The success of the trials in Hungary and Yugoslavia gave rise to the first production

of Bt as a product to control insect pests. This product, named Sporeine, was used as a spray (of Bt spores) and was produced by Laboratoire Libec in France during 1938, but had a short term use due to World War II (Jisha *et al*. 2013; Kruger *et al*. 2008; Lord, 2005). Even though the popularity of this biorational pesticide was growing, the mechanism behind the mode of action was not well understood. It was initially characterised as an insect pathogen, and its insecticidal activity was largely or completely ascribed to the parasporal crystals (Jisha *et al*. 2013). Berliner discovered the crystal inside the bacteria but the efficacy of the crystal was only described years later.

In 1953, C.L. Hannay observed free diamond-shaped crystals in a number of preparations of sporulating cultures of *B. thuringiensis*. He proposed that the crystals might be connected to the pathogenicity of the bacterium (Alford, 1972). These crystals were subsequently demonstrated to be the source of insecticidal activity and became known as S-endotoxin. The S-endotoxin was found to be of a high molecular weight protein, but no quantitative analytical procedures could be performed at the time (Alford, 1972). During the early 1950s, Dr Edward Arthur Steinhaus started looking into the possibility of the large scale commercial use of Bt. A Bt product with the name of Thuricide was manufactured in 1956 by Robert Fisher, Director of Research and Development at Pacific Yeast Products (later Bioferm) (Lord, 2005). Over the succeeding years, many crop protection companies worldwide took part in the commercial activities related to Bt. The early products had various problems. Such problems included that the standardisation of the product was based on spore count instead of potency, the products often contained the heat-tolerant exotoxin, and most products contained a variety of *B. thuringiensis* strains of low potency (Lord, 2005).

A breakthrough in the field of Bt sprays was the isolation of another variety of Bt from *E. kuhniella* (the host of Berliner's isolate) by Edouard Kurstak in France in 1962. Howard Dulmage obtained a similar isolate, designated HD-1, from diseased pink bollworm (*Pectinophora gossypiella*) (Lepidoptera: Gelechiidae). Kurstak's and Dulmage's isolates were serotyped by de Barjac and Lemille (1970) and assigned the variety of *Bacillus thuringiensis kurstaki* (Lord, 2005). Before long all of the companies that produced Bt products were producing variety *kurstaki* and HD-1 became the basis for products that were competitive with chemical insecticides in performance and cost (Lord, 2005).

By using current molecular techniques, the bacterium *B. thuringiensis* and its entomopathogenic properties can be investigated (Bravo *et al*. 2007). *Bacillus thuringiensis*, which is a part of the *Bacillus cereus* group, is a ubiquitous Gram positive spore-forming microbial pathogen that produces crystalline proteins called delta-endotoxins (δ-endotoxin).

These δ-endotoxins are produced during its stationary phase or senescence of its growth cycle (Bravo *et al*. 2007; Jisha *et al*. 2013; Raymond *et al.* 2008; Raymond *et al.* 2009; Schnepf *et al.* 1998). The entomopathogenic properties of this bacterium are due, at least in part, to the production of δ-endotoxins that make up the crystalline inclusions characteristic of *B. thuringiensis* strains (Agaisse and Lereclus, 1995; Höfte and Whiteley, 1989; Schnepf *et al.* 1998). In 1989, Höfteand and Whiteley (1989) proposed a classification for δ–endotoxins.

The crystal proteins, called Cry proteins, are parasporal inclusion proteins from *B. thuringiensis* that exhibit an experimentally verifiable toxic effect to a target organism. Parasporal inclusion proteins from Bt that exhibit hemolytic (cytolitic) activity are known as Cyt proteins (Bravo *et al*. 2007; Koni and Ellar, 1994). The observation of the ability of a *B. thuringiensis* culture to produce the crystalline protein led to the development of bioinsecticides for the control of insect species among the orders Lepidoptera, Diptera and Coleoptera (de Maagd *et al.* 2001; Jisha *et al*. 2013; Lacey *et al.* 2001). Recently, Bt isolates were also reported to be active against certain nematodes, mites and protozoa (Jisha *et al*. 2013; Marvier *et al.* 2007). Theoretically, these toxins are highly specific to their target insect, harmless to humans, vertebrates and plants, and are completely biodegradable (Bravo *et al*. 2007).

Numerous approaches have been developed to enhance the production of Bt bioinsecticides due to the ever growing economic interest. The insecticidal activity of Bt is known to depend not only on the activity of the bacterial culture itself, but several other aspects such as abiotic factors, the medium composition and cultivation strategy (Jisha *et al*. 2013).

2.2.2 Ecology of Bt

Bacillus thuringiensis appears to be indigenous to many environments (Bernhard *et al.* 1997; Martin and Travers, 1989; Schnepf *et al.* 1998). Many members of the *B. cereus* group, including *B. thuringiensis*, can frequently and abundantly be found as vegetative cells or as inactive spores in a variety of environments which include the soil and plant rhizosphere (Carozzi *et al.* 1991; Martin and Travers, 1989; Schnepf *et al.* 1998; Smith and Couche, 1991). *Bacillus thuringiensis* can also be isolated from environments which include insect intestines and the phylloplane of certain deciduous and coniferous trees (Carozzi *et al.* 1991;

Meadows *et al.* 1992; Raymond *et al.* 2008; Schnepf *et al.* 1998; Takatsuka and Kunimi, 2000). The isolation of this bacterium involves heat treatment to select for spores, occasionally with antibiotic selection or an acetate enrichment step (Schnepf *et al.* 1998; Travers *et al.* 1987). The diversity of the flagellar antigen present in *B. thuringiensis,* flagellar H-antigen, was catalogued by the Pasteur Institute and revealed that 55 different flagellar serotypes and 8 nonflagellated biotypes are present. The diversity in flagellar H-antigen agglutination reactions is one indication of the massive genetic diversity among *B. thuringiensis* isolates (Schnepf *et al.* 1998).

Bacillus thuringiensis has a high degree of genetic plasticity which is, at least in part, responsible for the diversity of strains and toxins. Most *B. thuringiensis* toxin genes appear to reside on plasmids as parts of composite structures that include mobile genetic elements (Kronstad and Whiteley, 1984; Lereclus *et al.* 1984; Schnepf *et al.* 1998). *Bacillus thuringiensis* has developed an intriguing array of molecular mechanisms to produce large amounts of insecticidal toxins and the co-expression of multiple toxins. This phenomenon increases the ecological value of the bacterium as well as the several *cry* gene expression systems (Agaisse and Lereclus, 1995; Baum and Malvar, 1995; Schnepf *et al.* 1998).

2.2.3 Application of Bt

The application of Cry toxins as a control mechanism has been achieved in three areas. It is used in the control of defoliator pests in forestry, vectors of human diseases such as mosquitoes and in the development of GM insect resistant crops (Bravo *et al*. 2007). In insect resistant crops, the Cry toxin is continuously produced which provides a constant presence of the toxin in plant tissue eaten by chewing and tunnelling insects (Bravo *et al*. 2007). This Cry protein production in plants is achieved by removal of the putative splicing signal sequences and deletion of the carboxy-terminal region of the protoxin (Bravo *et al*. 2007; de Maagd *et al.* 2001). The coding region controls the expression (Perlak *et al.* 1991; Schnepf *et al.* 1998).

The expression level of a gene may also be influenced by the copy number of the gene. As mentioned, the *cry* genes are located on plasmids and many *B. thuringiensis* strains carry different *cry* genes (Agaisse and Lereclus, 1995; Bietlot *et al.* 1993). The natural amplification of the *cry* genes may contribute to the high production of toxins in the different *B. thuringiensis* strains. Because the size of the crystal does not influence the amount of *cry*

genes it harbours, it appears that the production of toxins in *B. thuringiensis* is not strictly proportional to the copy number of the *cry* genes. Taking this into account, it is indicated that the capacity of *B. thuringiensis* strains to produce the crystal proteins is, although at a high level, limited, and reaches a maximum at a certain number of *cry* gene copies in the cell, above which there is no further increase in synthesis (Agaisse and Lereclus, 1995). The bioprocess used by *B. thuringiensis* is the formation of protease-resistant inclusion bodies that are easily solubilised in the gut of insect larvae in order to be toxic. This formation of the crystal structure and its solubility features depend on a variety of factors. These factors include the secondary structure of the Cry proteins, the energy of the disulfide bonds, and the occurrence of additional components (Agaisse and Lereclus, 1995).

2.2.4 Mode of action

Mode of action of δ-endotoxins requires numerous events that must occur after the ingestion of Bt plant material in order to fulfil the ultimate purpose, which is the death of the target insect. After the ingestion of inactive protoxin (located in the plant material), crystals are solubilised by the alkaline conditions in the insect mid-gut and are subsequently proteolytically converted into a toxic core fragment (Höfte and Whiteley, 1989; Jisha *et al*. 2013). This activated toxin binds to receptors located on the apical microvillus membranes of epithelial mid-gut cells. The extracellular domain of cadherin proteins is traversally located in the insect mid-gut membrane (Jisha *et al*. 2013; Soberton *et al*. 2007). Cry toxins interact with specific receptors situated on the mid-gut cell surface and are activated by host proteases, following receptor binding. This results in the formation of a pre-pore oligomeric structure that is insertion competent. In contrast, Cyt toxins directly interact with membrane lipids and insert into the membrane (Bravo *et al*. 2007; Jisha *et al*. 2013). Although different proteins have been proposed as endotoxin receptors, the role of cadherin-like proteins as the primary functional receptors bears the most supporting evidence (Jurat-Fuentes and Adang, 2006).

Two hypotheses exist and are aimed at explaining how these protoxins function in the mid-gut. These hypotheses are the sequential binding model (pore-formation model) and the signalling pathway model (Jisha *et al*. 2013; Soberton *et al*. 2007; Vachon *et al*. 2012). These hypotheses share initial steps which include the ingestion of protoxins, which are then solubilised in the gut and cleaved by mid-gut proteases such as trypsin. This cleavage yields activated monomeric toxins that bind to cadherin with high affinity (Soberton *et al*. 2007).

Differences in the extent of solubilisation sometimes explain differences in the degree of toxicity among Cry proteins (Du *et al.* 1994).

For the purpose of this study, only the aspects involved in the pore-formation model will be discussed. A purely toxin-based theory suggests that the action of Cry toxins is aimed at paralysing the mid-gut which leads to concluding death of the insect by starvation (Raymond *et al.* 2009). Alternatively, the crystal toxins paralyse the gut and cause extensive cell lysis (pore formation) in the intestinal membrane of the mid-gut, allowing bacteria access to the more favourable environment of the hemolymph. This rapid vegetative growth of Bt within the host, which can cause septicaemia, is proposed as an alternative cause of death (Raymond *et al.* 2008; Schnepf *et al.* 1998). Bt Cry and Cyt toxins are classified as part of a class of bacterial toxins known as pore-forming toxins (PFT). These PFT's are secreted as water-soluble proteins which are in the proses of conformational changes in order to insert into, or to translocate across, cell membranes of their host (Bravo *et al*. 2007; Parker and Feil, 2005). Pore-forming toxins are activated, in most cases, by host proteases after receptor binding which induces the formation of an oligomeric structure that is insertion competent. As a final point, membrane insertion is triggered by a decrease in pH that induces a molten globule state of the protein (Bravo *et al*. 2007; Parker and Feil, 2005).
Very little is known about the factors affecting the multiplication of *B. thuringiensis* in susceptible insect hosts (Takatsuka and Kunimi, 2000). Nevertheless, Akiba (1986) was aware of the effects of intestinal bacteria on the growth of *B. thuringiensis* in insect hosts and proposed that reproduction of *B. thuringiensis* in susceptible insect hosts is affected by the activities of intestinal bacteria. To determine whether this proposal was correct, it is necessary to understand the process of *B. thuringiensis* multiplication along with the multiplication of intestinal bacteria (Takatsuka and Kunimi, 2000). The multiplication of *B. thuringiensis includes* that in favourable conditions Bt spores germinate and vegetative cells multiply, whereas in unfavourable conditions Bt sporulates and produces insecticidal crystal proteins.

Recently, a novel pathogenic mechanism was suggested by the claim that *B. thuringiensis* and its toxins are unable to kill aseptically reared gypsy moth larvae, *Lymantria dispar* (Lepidoptera: Erebidae), but that pathogenicity can be re-established by inoculating hosts with a gut-associated strain of *Enterobacter* (Broderick *et al.* 2004). An obligate association with the gut microbiota challenges the models of the pathogenicity of Bt toxins mentioned, and has substantial implications for the ecology of Bt and the evolution of novel resistance mechanisms in invertebrate pests. This gut microbiota hypothesis remains highly controversial (Takatsuka and Kunimi, 2000). It is also noteworthy to mention that *B.*

thuringiensis and *B. cereus* are capable of multiplying in an insect's hemocoel and provoke septicaemia (Heierson *et al.* 1986; Schnepf *et al.* 1998).

2.2.5 Target pests

The large crystal protein inclusions from Bt have, in theory, a narrow range of toxicity. The specificities of the different Cry proteins determine their subsequent toxicity. *Busseola fusca* is one of the target insects of Bt maize (Soberton *et al*. 2007). The main targets are insects in the orders listed in Table 1. These insecticidal crystal proteins (ICPs) have been classified according to their structure, encoding genes, host range and range of toxicity (Stotzky, 2004).

Table 1: The ICPs derived from *B. thuringiensis* and its host range (Suzuki *et al.* 2004; Stotzky, 2004):

Cry proteins	**Host range**
Cry 1 and Cry 2B	Lepidoptera
Cry 2A	Lepidoptera and Diptera
Cry 3	Coleoptera
Cry 4	Diptera

Although different toxins have been found to be active against a wide range of insects, each toxin is generally restricted in action to a few species within one insect order (Raymond *et al.* 2007). The most widely used Bt toxins are crystal toxins in the Cry1A family, predominantly Cry1Ab in Bt maize which are used to control Lepidoptera larvae (Soberton *et al*. 2007).

2.3 Development of resistance of *B. fusca* to Bt

Since 1998, Bt maize that express the insecticidal Cry 1Ab protein (cultivar event MON810) has been cultivated in South Africa to control the threat of stem borers, *B. fusca* and *C. partellus* (Kruger *et al.* 2009; Kruger *et al.* 2011; Kruger *et al.* 2011a; Kruger *et al.* 2012). As soon as the commercial cultivation of Bt maize started, apprehension of possible resistance development by the target pests to the expressed insecticidal proteins raised concern (Gould, 1998; Kruger *et al.* 2011; Tabashnik, 1994). Tabashnik (1994) noted that families such as Noctuidae, Pyralidae and Plutellidae, all belonging to the order Lepidoptera, possess the ability to develop resistance to Bt under laboratory conditions as well as in the field (Tabashnik, 1994). The first occurrence of resistance in *B. fusca* was presented by van Rensburg (2007) after larvae collected from Christiana in the North-West Province survived

on Bt maize (MON810) in a laboratory study. In 2008, van Wyk *et al.* (2008) documented an observed increase in the prevalence of *B. fusca* larvae on Bt maize in the Highveld-region of South Africa. This study also accurately predicted the evolution of resistance within irrigated areas of South Africa (Kruger *et al.* 2012). The ability of insects to develop resistance to insecticides and other control strategies is seen by many as the main threat to the sustainability of Bt crops (Carrière *et al.* 2010; Ferré and van Rie, 2002; Gould, 1998; Heckel, 2012; Huang *et al.* 2010; Pardo-Lopez *et al.* 2013; Tabashnik, 2008; Tabashnik *et al.* 2009; Tabashnik *et al.* 2013).

Although the concept of resistance development is not ground-breaking in the scientific world, it is becoming more and more essential to correctly define the specific aspects being discussed. Field-evolved resistance can be seen as a form of natural selection because it is defined as a heritable and genetic based decrease in a population's susceptibility to a toxin exposure over successive generations in the field (Heckel, 2012; Kruger *et al.* 2012; Tabashnik, 1994; Tabashnik *et al.* 2009). Laboratory-selected resistance on the other hand, follows after the susceptibility of a laboratory strain decreases when exposed to a toxin in the laboratory. The evolution of resistance occurs at population level because both field-evolved and laboratory-selected resistance entail alterations in gene frequency across generations (Heckel *et al.* 2007; Kruger *et al.* 2012; Tabashnik *et al.* 2009).

As stated, resistance is a heritable occurrence which means that susceptibility is also a heritable occurrence. Natural genetic variation influencing the response to toxins, in this case Bt proteins, commonly occurs in insect populations where some alleles confer resistance and other confer susceptibility (Carrière *et al.* 2010; Gould, 1998; Tabashnik *et al.* 2013). In most insects, the frequency of alleles conferring for resistance is rare until a field population is exposed to Bt maize. This increases the frequency of alleles conferring resistance in successive generations (Gould, 1998; Tabashnik *et al.* 2013). Susceptibility is measured when insects are exposed to toxins in bioassays. Susceptibility of a field population is typically assessed by sampling insects from a field, followed by laboratory rearing of their offspring and observing their response to either artificial diet infused with Bt proteins or Bt plant material. The most common and decisive measure of susceptibility is based on the mortality of insects exposed to the toxin. By controlling the surrounding environmental conditions, this method demonstrates the conclusion that susceptibility is heritable (Tabashnik *et al.* 2009). Resistance in the field is tested by comparing pest population densities in corresponding fields of Bt maize and non-Bt maize. Results will be noteworthy when a significant increase of the pest population density over time in the Bt field relative to the non-Bt field occurs. This occurrence can act as a suggestive, but not

conclusive, indication of resistance development. The population density in a field can be influenced by numerous ecological and environmental factors, and for that reason it is wise to observe the survivors on Bt maize in laboratory tests under controlled conditions to possibly demonstrate genetically based resistance (Tabashnik *et al.* 2000; Tabashnik *et al.* 2009; Venette *et al.* 2000).

Even though the definition of field-evolved resistance does not automatically imply loss of economic efficacy in a Bt maize field, field-evolved resistance is expected to cause a decreased susceptibility to Bt maize (Tabashnik *et al.* 2003; Tabashnik *et al.* 2009). In 2005 only one pest was documented to have developed resistance to Bt crops. In 2010 the number of resistant insects increased to five, namely *B. fusca* (South Africa), *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae) (USA), *Spodoptera frugiperda* (Lepidoptera: Noctuidae) (in Puerto Rico; leading to the withdrawal of Cry1F Bt maize), *Helicoverpa zea* (Lepidoptera: Noctuidae) (USA) and *Pectinophora gossypiella* (India). In all five of these insects, field-evolved resistance was identified before the 10th year that Bt crops was commercialised (Campagne *et al.* 2013; Tabashnik *et al.* 2013). *Busseola fusca* developed resistance to Cry 1Ab proteins after 8 years of the cultivation of Bt maize in South Africa (Campagne *et al.* 2013; van Rensburg, 2007). Tabashnik *et al.* (2013) reported in a review article from 24 cases in eight countries over two decades that the increase in documented cases of resistance development likely reflects increases in the total Bt crop cultivated area, the cumulative exposure duration of pests to Bt crops and the number of exposed populations (Tabashnik *et al.* 2013).

Due to the confirmation of *B. fusca* resistance to Bt maize which was published in 2007 by van Rensburg, the investigation of the mechanism of the Bt toxins, as well the possible reasons for the resistance development, became significant. Since an insect's response to toxin exposure is often controlled by genetic variation, the mechanism of insect resistance to Bt maize could be located at any point during the various steps in the mode of action of Cry proteins after ingestion of Bt material by larvae (Ferré and van Rie, 2002).

In 2002, Ferré and van Rie (2002) described three different biochemical mechanisms of resistance to Bt that were observed up to that time. These mechanisms include the proteolytic processing of protoxins, improved repair of damaged mid-gut cells and modification of a Cry protein–binding site. However, Ferré and van Rie (2002) noted that only the link observed between biochemical modification and a decrease in susceptibility was the binding reduction mechanism. The binding site modification mechanism provided evidence that resistance was due to an alteration only in the binding site for Cry 1Ab during

receptor-binding. Strong cross-resistance which extends to Cry proteins sharing binding sites were also observed (Zhang *et al.* 2012).

An alternative mechanism for resistance is the well documented altered proteolytic processing mechanism. This entails the action of the protoxin instead of the toxin and an alteration in the mid-gut proteases in resistant insect larvae. Simply stated, the activation of the protoxin by gut proteinases in resistant insect larvae is slower, which can lead to a reduced quantity of toxin because of the faster degradation of Cry 1Ab (Kumar, 2003; Tabashnik *et al.* 2013).

The ability of resistant larvae to repair damaged mid-gut cells when compared to susceptible larvae was also discussed by Ferré and van Rie (2002). After ingestion of sub-lethal doses of Cry 1Ac proteins, both the resistant and susceptible *Heliothis virescens* (Lepidoptera: Noctuidae) larvae exhibited similar histopathological changes in columnar gut cells. For this reason, it is possible that the efficient repair (or replacement) of damaged mid-gut cells by the resistant population can account for the lower pathogenicity when exposed to Cry proteins (Ferré and van Rie, 2002).

Studies that were done on the biochemical basis of resistance in some resistant insect populations revealed the possibility of more than one resistance mechanism simultaneously, which proposes the presence of more than one gene conferring for resistance. This statement excludes the cases where inheritance of resistance follows a multifactorial pattern (Ferré and van Rie, 2002). Zhang *et al.* (2012) correlated mutations in a 12-cadherin-domain protein with resistance in *Helicoverpa armigera* (Lepidoptera: Noctuidae) to the Cry 1Ac toxin in China. This discovery opened the door for molecular methods, such as DNA sequencing, to be used as a time saving method of identifying resistant target pests as well as provide tools to monitor the development and spread of field resistance (Ferré and van Rie, 2002; Zhang *et al.* 2012). Advantages of a molecular approach compared with bioassays include the ability to detect single resistance alleles in heterozygotes and in groups of individuals at different life stages. One area where DNA screening is lacking is distinguishing between resistance caused by any mechanism because it only detects alleles associated with previously identified mechanisms of resistance (Tabashnik *et al.* 2009).

Results from grower surveys in South Africa suggest that the low abundance of refuges of non-Bt maize contributed to rapid evolution of *B. fusca* resistance to Cry 1Ab (Campagne *et al.* 2013; Huang *et al.* 2010; Kruger *et al.* 2009; Kruger *et al.* 2011a). Refuges are defined as an adjacent non-Bt field in which the target pest is not under selection pressure because of

the presence of the toxin and it therefore provides a sustainable habitat for pest development (Kruger *et al.* 2011). The underlying principle of the "high dose and refuge strategy" is that any resistant insects emerging from the Bt crop are more likely to mate with one of the much larger number of susceptible pest insects emerging from refuge than with each other, thereby decreasing the selection of Bt resistance alleles (Bourguet, 2004).

The importance of monitoring resistance development can only be equal to the strategies employed to control it. Resistance must be detected as early as possible to enable proactive management of resistance (Heckel, 2012; Tabashnik *et al.* 2008). Resistance management strategies are aimed at decreasing the amount of individuals carrying resistance genes and thus keeping the frequency of resistance genes sufficiently low (Jisha *et al*. 2013). Jisha *et al*. (2013) suggested the use of the following strategies for resistance management: stacking or pyramiding multiple toxins, crop rotation, high or ultrahigh dosages, and spatial or temporal refugia (toxin-free areas).

2.4 Lepidoptera mid-gut

2.4.1 Functioning of lepidopteran digestive tract

A brief introduction into the complexities that are present in the digestive tract of insects is provided below. This background information is essential to understand the possible role of bacteria in the mid-gut, as well as the mode of action of Bt maize. The digestive system of any organism is involved in (1) obtaining food, (2) mechanical breakdown of the food into smaller particles to assist digestive enzymes acting on them, (3) enzymatic breakdown of these particles into molecules that can pass through the digestive tract and enter the hemolymph, and (4) produces molecules (e g. endocrines) that coordinate feeding and other activities (Anon, 2004). The first section of the gut, which is close to the mouth of the insect, is called the foregut (Figure 2.3). The foregut is mainly involved in ingestion of food and breakdown into particles, either mechanically or by means of saliva. The following section of the digestive tract is the mid-gut. Very complex digestive processess take place in the mid-gut which consists of four different cell types: digestive cells, regenerative cells, endocrine cells and goblet cells. The third and final section is known as the hind gut. The hind gut is a major refuge dumping area for waste products from the mid-gut. It's also involved in the reabsorption of certain salts and amino-acids which help to maintain the osmotic pressure of the hemolymph (Anon, 2004).

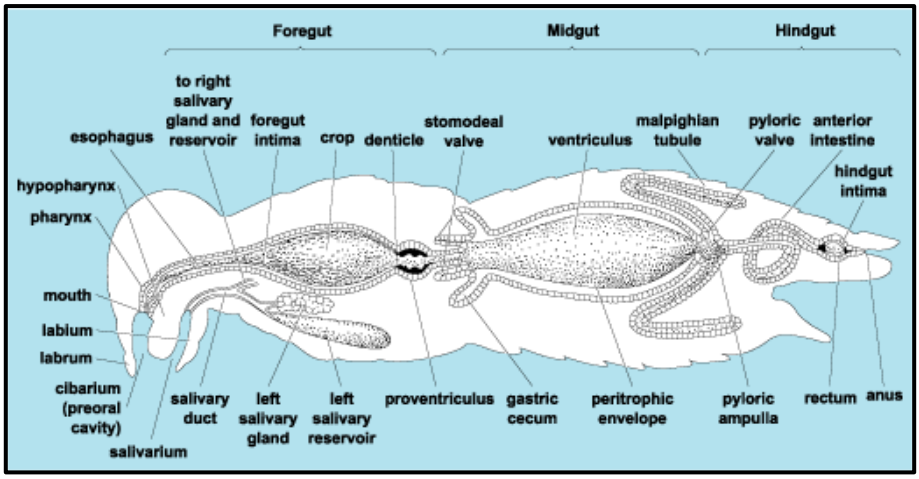

Figure 2.3: Illustration of the generalised digestive tract of insects (Romoser and Stoffolano, 1998)

For the purpose of this study, we focused on the mid-gut of Lepidoptera larvae. The mid-gut of keratinophagous insects is an unusual environment for biotic existence. This is attributed to the pH which varies between 7 and 12, and the redox potential between 100 and 400 mV. These insects have most probably evolved an anaerobic mid-gut with a negative redox potential to assist in the solubilisation of the redox active compounds in their diet (Shannon *et al.* 2001). Individual species have a characteristic mid-gut pH although these values may vary with development stage, feeding time and the buffering characteristics of host foliage (Johnson and Felton, 1996). Serine proteases are present in the gut of *B. fusca* (George *et al.* 2008). The mid-gut of Lepidoptera larvae is a K^+-secreting epithelium composed of two major cell types which appear to be electrically linked, the goblet and columnar cells (Peyronnet *et al.* 2004). The alkaline gut of Lepidoptera is achieved through active secretion of carbonate from epithelial goblet cells and proton pumping at considerable metabolic cost to the insect (Anon, 2004).

Insects that occur in the field are subject to the attack of pathogens and parasitoids (Mostafa *et al.* 2005). In addition to its physiological role in K^+ transport and absorption of nutrients, the mid-gut functions as a barrier to be crossed by numerous microbial pathogens, natural toxins and chemical insecticides that are ingested orally (Johnson and Felton, 1996; Mostafa *et al.* 2005). The following distinct conclusion can therefore be made: mid-gut pH needs to be recognised as an important factor for, not only the optimal activity of digestive enzymes, but also numerous other factors. The alkaline condition of the mid-gut is also an extremely important factor for the sporulation of *B. thuringiensis.*

2.4.2 Microbiota present in the mid-gut of Lepidoptera

The mid-gut of Lepidoptera larvae is an extreme but unique environment, which makes their bacteria of particular interest (Anand *et al.* 2009). The high alkalinity of most lepidopteran mid-guts can be unfavourable to some microbes. Also the diverse chemistry of the mid-gut generated by the unusually broad feeding range of some species, contributes to the challenging nature of this environment for microorganisms (Broderick *et al.* 2004). Insects with rapid food throughput often harbour a large diversity of microorganisms in their gut which can play a role in nutrition by enhancing the digestive efficiency of the insect host. This provides metabolic activities that maximise the extraction of dietary energy (Dillon and Dillon, 2004; Erkosar *et al.* 2013). The term microbiota is given to the microbial community in the gut. The number of microbial cells in the guts of insects outnumbers the quantity of their own cells (Dillon and Dillon; 2004; Erkosar *et al.* 2013). The indigenous gut bacteria also play a role in withstanding the colonisation of the gut by non-indigenous species including pathogens (Dillon and Dillon, 2004; Shannon *et al.* 2001). A successful symbiotic collaboration between gut microbiota and their insect hosts has allowed both entities to take advantage of different niches and a broad range of diets (Amann *et al.* 1995; Dillon and Dillon, 2004; Feldhaar, 2011).

Although studies of the microbial community in the gut of *B. fusca* only started recently, a study done by Broderick *et al.* (2004) showed that the culturable microbial diversity in the mid-gut of *Lymantria dispar,* also a member of the order Lepidoptera, is relatively simple, with 15 phylotypes at its most complex. The simplicity of this community is particularly apparent compared to other gut environments, such as the human intestine and termite hindguts, which contain at least 500 and 50 phylotypes, respectively (Broderick *et al.* 2004).
While Lepidoptera larvae live in a terrestrial macro-environment as external phytophages, the microenvironment which these mid-gut bacteria inhabit is impacted by the host diet. The plants on which this insect feeds are diverse and contain a range of allelochemicals, such as phenolics, tannins, and terpenoids, many of which are toxic to bacteria (Broderick *et al.* 2004; Dillon and Dillon, 2004). A benefit contributed to the host (larvae) from the microbiota comes into play during the ingestion of toxic compounds. Toxic compounds can cause selection pressure on the microbial community in a host. These surviving bacteria are able to degrade and metabolise the compounds that are otherwise toxic to the insect (Broderick *et al.* 2004).

2.4.3 The role of microbiota in the mid-gut of Lepidoptera

Microorganisms play important and often essential roles in the growth and development of many insect species. Endosymbionts contribute to insect reproduction, nutrition, and pheromone production. Evidence has been presented that guts of fifth instar larvae of *Bombyx mori* harbour bacteria that produce digestive enzymes, in a synergic manner, which aid in the digestion of leaf constituents such as cellulose, xylan, pectin and starch and contribute to larval growth (Anand *et al.* 2009). Symbiotic relationships between insects and their microbiota have been studied extensively in several systems, but relatively little is known about microbial associates in insect groups that feed on foliage (Broderick *et al.* 2004). Lepidoptera and its associated bacteria have awoken new interest during the last decade which can be attributed to the mystery of resistance development by these pests.
In a study by Anand *et al.* (2009)**,** they suggest that bacteria present in the gut of insects may secrete enzymes vital in digestion. Most of the digestive enzymes of a Lepidoptera larvae, such as cellulases (β-endoglucanase, cellobiohydrolase/FPcellulase), xylanase and pectinase are produced from microbial origin and enzymes including amylase and β-glucosidase are produced endogenously (Anand *et al.* 2009). Some studies suggest that the microbial-derived enzymes (amylase, cellulase and β-glucosidase) may have a supplementary, non-essential digestive role, which may be of importance during adaptation of the insect hosts to different diets (Visotto *et al.* 2009). Although some facts of the origin of enzymes are known, there is still a knowledge gap regarding the types of microbes present in the mid-gut and of their roles in insect development and function (Broderick *et al.* 2004).

2.4.4 Mode of action of Bt in the mid-gut of Lepidoptera

The influence of mid-gut microbiota on the efficacy of Bt is a research field with many open ends. Previous studies (Broderick *et al.* 2006; Raymond *et al.* 2009) were done about the importance of bacteria in the mid-gut of insects with regards to Bt. These two studies attempted to describe the mode of action of Bt proteins in lepidopterans. These studies came to contradictory conclusions regarding the role of mid-gut microbes on the efficacy of Bt.

Broderick *et al.* (2006) used a method of rearing the larvae of *Lymantria dispar* hatching on either sterile artificial diet or diet amended with a range of concentrations (8 – 500 µg/ml of diet) of an antibiotic mixture that consisted of penicillin, gentamicin, rifampicin and streptomycin. Larvae from each treatment were fed with 0, 1, or 10 units of *B. thuringiensis*

upon molting to the third instar. All treatments were applied in a volume of 1 µl to a standard diet disc (4 mm diameter and 1 mm height) and fed to the larvae on 2 successive days. Mortality was recorded every 24 hours for 7 days (Broderick *et al.* 2006). The *B. thuringiensis* treatment used was a commercial formulation of *B. thuringiensis kurstaki* (DiPel DF, Valent Biosciences, Libertyville, IL) consisting of proteins and spores. They observed that the mortality of insects fed *B. thuringiensis* was inversely proportional to the antibiotic concentration, with no mortality caused by *B. thuringiensis* among larvae fed 10 units and reared on antibiotic mixtures containing 125 µg/ml or more of each antibiotic. This reduction in mortality was accompanied by reduced populations of culturable *Enterococcus* and *Enterobacter* spp. from the mid-guts of larvae (Broderick *et al.* 2006).

To test this possibility more directly, they assayed the effect of *Escherichia coli* strain ECE52, which produces the *B. thuringiensis* toxin, and compared its toxicity to larvae reared on antibiotic-amended opposed to unamended diet. Results showed that *E. coli* ECE52 killed larvae that had been reared on antibiotics as effectively as larvae containing the native gut community (Broderick *et al.* 2006). To further test this theory, *E. coli* cells were heat-killed before feeding. Subsequent feeding studies showed that mortality was equivalent to treatment with live *E. coli* in larvae reared on an unamended diet. However, in larvae reared on antibiotics, there was no mortality due to the toxin alone in the absence of live *E. coli* cells. This research concluded that *E. coli* engineered to produce the *B. thuringiensis* insecticidal toxin killed *L. dispar* larvae regardless of the presence of other bacteria in the mid-gut. However, when the engineered *E. coli* was heat-killed and then fed to the larvae, the larvae did not die in the absence of the indigenous mid-gut bacteria (Broderick *et al.* 2006).

In the hemolymph of the larvae, *E. coli* ECE52 reached a population density of $>10^9$ cfu/ml after 14.5 hours. These results suggested that the *B. thuringiensis* toxin alone was not sufficient to cause larval mortality, because an enteric bacterium (a member of the Enterobacteriaceae family) such as *E. coli* or *Enterobacter* must also be present to induce septicaemia and death. For bacteria to cause septicaemia, they must be able to enter and multiply in the hemolymph. This observation led Broderick *et al.* (2006) to compare the growth of *B. thuringiensis* and an *Enterobacter* sp. *in vitro* in larval hemolymph. The *Enterobacter* sp. reached a population density of 1.3×10^9 cfu/ml after 6 hours of incubation at 25°C, which was equivalent to its growth in tryptic soy broth. In contrast, *B. thuringiensis* populations decreased in numbers to below detectable limits (200 cfu/ml) in hemolymph after 6 hours and remained undetectable throughout the 18.5 hour assay, even though *B. thuringiensis* grew as quickly as the *Enterobacter* sp. in tryptic soy broth. Broderick *et al.*

(2006) concluded that elimination of the gut microbial community with antibiotics abolished *B. thuringiensis* insecticidal activity, and reestablishment of an *Enterobacter* sp. in the mid-gut restored *B. thuringiensis*-mediated killing.

The importance of the presence of mid-gut microbes on the efficacy of Bt was also studied by Raymond *et al.* (2009). *Bacillus thuringiensis kurstaki* HD 1 was isolated from a commercial DiPel stock (DiPel WP, Valent Biosciences Corporation) using *Bacillus cereus* selective agar (BcSA) (Oxoid, UK). Wild-type *B. thuringiensis* isolates were collected from a *Brassica oleracea* (cabbage). The Geneva *Plutella xylostella* (Lepidoptera: Plutellidae) strain was used in all artificial diet experiments where the parents of all aseptically reared larvae were reared on a wheatgerm-based diet containing the antibiotics aureomycin (2.25 g/l) and streptomycin (0.25 g/l). Thereafter, by surface-sterilising eggs and rearing larvae on autoclaved diet in 50 mm Petri dishes, aseptic larvae were prepared (Raymond *et al.* 2009). *Plutella xylostella* larvae were either reared on an antibiotic diet (containing 500 µg/ml rifampicin) or antibiotic-free diet. Inoculation of 2nd to 3rd instar larvae with Bt always took place on ~330 mm^2 antibiotic-free diet). This entailed that the diet was inoculated with 75 µl of pasteurised Bt spore/crystal suspension and three Bt concentrations (200, 400 and 800 spores/µl) were applied with 30 larvae tested per dose (Raymond *et al.* 2009).

In the above mentioned study, Raymond *et al.* (2009) selected for improved reproduction of the bacterial strain within hosts by passaging (DiPel-derived) *Btk* rifR strains in *P. xylostella* larvae in four independent evolutionary lineages. For each lineage they inoculated ten *Brassica pekinensis* leaf discs by dipping the discs in suspensions of 2000 spores/µl. Leaf discs were placed in 50 mm Petri dishes, along with five third-instar larvae that had previously been reared on 500 µg/ml rifampicin containing diet (Raymond *et al.* 2009). Cry 1Ac protoxins were also used expressed as crystals in *E. coli.* The crystals were purified away from disrupted cells and stored at - 80°C. Streaking of purified crystals revealed no contamination with culturable bacteria. Bioassays were conducted with five concentrations and third-instar *P. xylostella* larvae using discs of sterile artificial diet containing rifampicin and streptomycin (100 µg/g diet for each). Each diet disc was immersed in a Cry 1Ac protoxin suspension for 30 s. Raymond *et al.* (2009) concluded that because orally fed antibiotics can persist in the tissues and hemolymph of larvae, it is plausible that the direct effects of antibiotics, and not the absence of gut microbiota, account for the low pathogenicity of Bt in antibiotic fed larvae.

Although both of these studies used *Escherichia coli* engineered to produce the *B. thuringiensis* insecticidal toxin, this step will not be performed in the present study. Both

studies also used discs soaked in Bt toxin suspension to test the pathogenicity. In the present study, the direct effects of maize expressing the Bt insecticidal toxins on the larvae feeding on it were observed.

2.5 Bacteria cell wall and typical growth curve

One of the objectives of the present study was to evaluate the interference of antibiotics on the growth of the mid-gut microbiota of *B. fusca*. For that reason some aspects which are used during the screening of antibiotics is discussed as an overview of the bacterial cell wall and the bacterial growth cycle.

2.5.1 Bacterial cell wall

The synthesis of the bacterial cell wall is the target of several antibiotics, as well as providing a means of separation between bacterial cells. For that reason it is important to understand the structure, design and function. The cell wall is located outside the plasma membrane and aids in maintaining the shape of the cell, protecting the cell from osmotic lysis and damaging toxic substances as well as contributing to the pathogenicity of bacteria (Willey *et al.* 2014). The presence of peptidoglycan in the cell walls is a feature which is common to nearly all bacterial cells and is a signature molecule for bacteria because no other organism has been found to synthesise it (Willey *et al.* 2014). The peptidoglycan forms an enormous mesh like structure referred to as the peptidoglycan sacculus and is composed of many identical subunits. This layer of the cell wall and its mechanical strength is crucial for the bacteria to survive fluctuating environmental conditions. Fluctuating environmental conditions can alter osmotic pressure inside the cell because the degree of peptidoglycan cross-linking correlates with the structural integrity of the cell (Gwynn *et al.* 2010). Variants of peptidoglycan can have taxonomic value because of the different characteristics it lends to particular groups (Willey *et al.* 2014).

Bacterial cell walls can be divided into two groups based on the Gram staining procedure which is a differential staining method. The protocol for this procedure is discussed in the Material and Methods section (Chapter 3). The cell wall of Gram positive bacteria consist of a single 20 to 80 nm thick homogeneous layer of peptidoglycan outside the plasma membrane (Willey *et al.* 2014). Most Gram positive bacteria belong to the phyla *Firmicules* and *Actinobacteria.* In contrast, the cell wall of Gram negative bacteria contain two distinct layers: a 2 to 7 nm thick peptidoglycan layer enclosed by a 7 - to 8 nm thick outer

membrane. The outer membrane has a number of constituents, such as lipopolysaccharide (LPS) that has a number of important functions (Takeuchi *et al.* 1999; Willey *et al.* 2014). These large, complex molecules contain lipids and carbohydrates. One noteworthy function of LPS is that it aids in creating a permeable barrier (Willey *et al.* 2014). This barrier is dependent on the geometry and interactions between the adjacent LPS molecules and is thought to restrict the entry of antibiotics that may be harmful to the bacterium (Willey *et al.* 2014).

2.5.2 Growth cycle of bacteria

The term used for the sequence of the events extending from the formation of a new cell through to the next cell division is known as the cell cycle and is essential for elementary biological processes (Willey *et al.* 2014). Predictive modelling is a field used in many aspects of microbiology, including food microbiology, minimum inhibitory concentration (MIC) calculations and the effect of antibiotics. The growth curve of bacteria is used as a model to describe the behaviour and effects of different physical or chemical conditions on the microorganisms themselves (Lin *et al.* 2000; Zwietering *et al.* 1990). The bacterial cell wall cycle consists of two pathways. One pathway is involved in the replication and partitions the DNA into the progeny cells and the other pathway is involved in the formation of the septum and progeny cells which is known as cytokinesis (Willey *et al.* 2014).

The purpose of the cell cycle is to provide the cell with the ability to change in cell size and to divide. This cell division results in an increase in population size. The term growth is therefore used to refer to the growth in population size (Willey *et al.* 2014). Population growth is often studied by analysing the growth of microbes in a broth (liquid) culture. Population growth of microorganisms reproduced by binary fission in a batch culture (cultivated in a single dosage of broth) can be predicted by plotting it as a logarithm of the number of viable cells against the incubation time (Willey *et al.* 2014). The conditions in this batch culture mimic the conditions of the natural environment of the microbes as closely as possible. The microbial growth can be predicted because of the depletion of nutrients and the increase of the concentration of waste in the closed medium as the population size increases (Willey *et al.* 2014). This curve has four phases which characterise the typical bacterial growth in a batch culture or a liquid nutrient media (Al-Qadiri *et al.* 2008; Willey *et al.* 2014). In the present study, the growth in population was studied by analysing the growth curve of the isolated bacteria (from the mid-gut of *B. fusca*) when they are exposed to different concentrations of antibiotics and observing the effect of the antibiotics on these four

phases (Lin *et al.* 2000; Al-Qadiri *et al.* 2008; Willey *et al.* 2014). These four phases are called the lag phase, exponential/log phase, stationary phase and the senescence/death phase. The phases are characterised by distinct biochemical reactions for synthesis of cellular components essential for cell growth and division (Al-Qadiri *et al.* 2008).

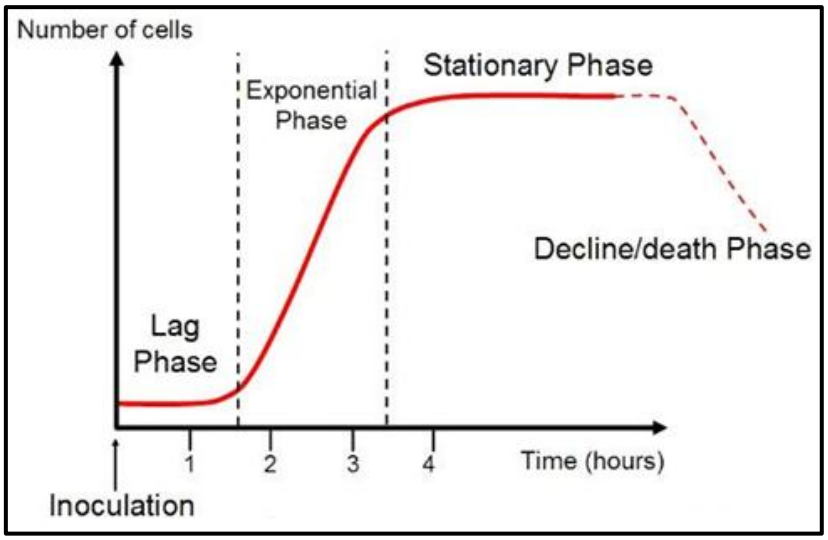

Figure 2.4: Illustration of a typical bacterial sigmoid growth curve without any inhibition predicting the lag, exponential, stationary and death phase (Anon, 2012; Lin *et al.* 2000)

After the inoculation of bacteria into a fresh culture medium, no immediate population size increase occurs, as indicated by the lag phase in Figure 2.4 (Willey *et al.* 2014). During this initial period of cultivation, the cells are synthesising new components for the synthesis of DNA and cellular enzymes although it appears to be inactive (Al-Qadiri *et al.* 2008). Before the growth increase begins, the cells have to overcome the depletion of ATP as well as synthesise new enzymes to utilise different nutrients in a new environment (Lin *et al.* 2000; Willey *et al.* 2014). The cell adaptations are a gradual process which involves many metabolic and physiological changes (Lin *et al.* 1999). Eventually DNA replication starts, the cell mass increases and cell division starts which causes a rise in population size (Lin *et al.* 2000; Willey *et al.* 2014).

During the exponential/log phase the environmental conditions, nature of the medium and genetic potential of the microorganism determine the rate of growth and division (Willey *et al.* 2014). During this phase, it is optimal for the microorganism to obtain the maximum possible rate of growth which includes the completion of the cell cycle, doubling in number and linearly increasing the bacterial biomass with every unit of time (Al-Qadiri *et al.* 2008; Willey *et al.* 2014). These constant intervals cause the population to be at its most uniform in terms of biochemical and physiological properties. Although this is the phase in the growth cycle

where the most growth takes place, it is dependent on the nutrient concentration in the medium (Willey *et al.* 2014). The rate of nutrient uptake by microbial transport proteins are thought to gives rise to the shape of the curve. At adequately high nutrient levels, the growth rate does not increase with increasing nutrient concentrations because the transport systems are saturated (Willey *et al.* 2014).

As soon as the growth curve becomes horizontal (the stationary phase), it means that the population growth has ceased (Willey *et al.* 2014). Microorganisms enter the stationary phase because of severe nutrient depletion during the exponential phase. If a nutrient in the closed system is reduced, the population growth will slow down and ultimately stop (Lin *et al.* 2000; Willey *et al.* 2014). Another reason microorganisms enter the stationary phase can be due to the accumulation of metabolic inhibitory by-products or waste which limits the availability of oxygen for aerobic organisms (Al-Qadiri *et al.* 2008; Lin *et al.* 2000; Willey *et al.* 2014). Some evidence also depicts that microorganisms enter the stationary phase because the population has reached a critical population level (Willey *et al.* 2014). Any of these reasons or a combination thereof can influence the transition into the stationary phase. In the stationary phase the total number of viable microorganisms remains constant because there is no longer a net increase in viable bacterial cells. Cellular metabolic activity is also decreased which causes a balance between cell division and cell death. Even though the cells have stopped dividing, they remain metabolically active (Lin *et al.* 2000; Willey *et al.* 2014). Cellular components are synthesised at uneven rates (unbalanced growth) during the transition from the log to the stationary phase. This causes the biochemical composition of the cells to differ between these two phases (Lin *et al.* 2000).

Cells growing in a batch culture cannot remain in the stationary phase indefinitely and eventually enters the senescence/death phase. As is the trend in this cycle, the behaviour of the cells is at a constant rate (Willey *et al.* 2014). Here, the decline of cells is exponential when the cells die at a constant rate. The reason for this decline in cells can be attributed to nutrient depletion, build-up of inhibitory by-products or toxic waste, depletion of cellular energy and pH changes (Lin *et al.* 2000). These environmental conditions cause irreparable damage to the cells and no additional growth can occur (Willey *et al.* 2014). In theory, there is no loss in total cell number because the cells do not lyse.

Throughout this growth cycle, cell mass is measured to determine the population size. This can be performed by spectrophotometry (Willey *et al.* 2014). Spectrophotometry is a rapid and sensitive method that is based on the principle that microbial cells scatter light that strikes them. Willey *et al.* (2014) describes this principle as the following: Because microbial

cells in a population are of roughly constant size, the amount of scattering of light is directly proportional to the biomass of cells present and indirectly related to cell number. An increase in the concentration of cells present in the medium results in the medium appearing turbid. This turbidity results in less light traveling through the medium. The decrease in transmitted light (extent of light scattering) is measured by means of a spectrophotometer and is called the absorbance or optical density of the medium. The absorbance can then be plotted on a graph against time to illustrate the growth curve of a particular microorganism (Willey *et al.* 2014).

The objective of this study was to evaluate the interference of the antibiotics on the growth of the mid-gut microbiota of *B. fusca,* and to relate this interference to the concentration of antibiotics which had the most effective inhibiting and killing effect on the bacteria. The lowest concentration of antibiotics that modifies the growth curve of bacteria most effectively was visualised using the growth curve of the bacteria (Castillo *et al.* 2006).

2.6 The use of antibiotics and antibiotic resistance

2.6.1 Classification of antibiotics

The death of a cell mediated by the introduction of antibiotics starts with a physical interaction between the antibiotic molecule and a specific target in the bacteria. This interaction is a complex process with many different mechanisms involving interactions at a biochemical, molecular and ultrastructural level of the bacterial cell function (Kohanski *et al.* 2010). The term antibiotic can only be used to describe a class of organic molecules that inhibits the growth or kills microbes by specific interactions. Antibiotic may be derived from a microorganism or synthetically produced. The term antibiotic, however, does not define a class of compounds or the natural function, only the application (Davies and Davies, 2010). Antibiotics can have a bactericidal or bacteriostatic effect on bacteria (Bush, 2010). A bactericidal effect is achieved when bacterial cell death is induced by the antibiotic, while bacteriostatic agents only inhibit bacterial cell growth (Kohanski *et al.* 2007, 2010). The term antibiotic has recently also been used to describe bacteriostatic antibacterials and antifungal agents derived from natural products as well as synthetic chemical methods (Bush, 2010).

The modes of action of antibiotics in different groups are based upon the drug-target interactions and their individual direct effects. The subsequent bacterial response to the effects of antibiotic exposure involves complicated genetic and biochemical pathways

(Kohanski *et al.* 2010). Antibiotics are most commonly classified according to their functional/structural class. The mode of action of antibiotics is based upon the bactericidal effect on bacterial physiology. These effects include the inhibition of DNA synthesis, cell wall synthesis or protein synthesis (Gwynn *et al.* 2010; Kohanski *et al.* 2010; Willey *et al.* 2014). A brief description of the different antibiotic classes is provided in Table 2. In a review article by Kohanski *et al.* (2010), they stated that recent evidence points towards a common mechanism of cell death involving unfavourable cell responses to drug induced stresses that is shared by all classes of bactericidal antibiotics (Kohanski *et al.* 2010).

Table 2: Antibiotic classes and mode of action (antibiotics used in the present study indicated in bold) (Willey *et al.* 2014; Kohanski *et al.* 2007; Kohanski *et al.* 2010)

Cell wall synthesis inhibitors: The most selective antibiotics; have a high therapeutic index because it targets structures and functions that are not found in eukaryotic cells.			
Primary effect	Examples	Mode of action	Members and Spectrum
Bactericidal	Penicillins	• Inhibit transpeptidation • Activate cell wall lytic enzymes • Result in changes to cell shape and size, induction of cell stress responses and ultimately cell lysis Most crucial feature of the penicillin molecule that is essential for bioactivity is the β-lactam ring. The terminal D-alanyl-D-alanine on the peptide side chain of the peptidoglycan subunit resembles the structure of penicillin. This structural similarity blocks the enzyme catalysing the transpeptidation reaction that forms the peptidoglycan cross-links, which causes the formation of an incomplete cell wall, leading to osmotic lysis. Penicillin is limited to the ability of only acting on growing bacteria that are in the process of synthesising new peptidoglycan. Pennicillin also binds to numerous periplasmic proteins named penicillin-binding proteins (PBPs), activating the bacteria's own autolytic enzymes. Membrane leakage and cell death can occur when bacterial holins are stimulated by penicillins. Bacterial holins form holes or lesions in the plasma membrane. Bacteria lacking peptidoglycan hydrolase activity, such as *Streptococcus pneumoniae,* can also be killed by β-lactams, but at a slower rate. This indicates that there is a lysis-independent mode of action by β-lactams.	Penicillin G, penicillin V, methicillin: Narrow (G+) **Ampicillin**, carbenicillin: Broad (G+, some G-)
Bactericidal	Cephalosporins	• Inhibit transpeptidation • Activate cell wall lytic enzymes Structure similar to penicillin; also contain a β-lactam ring and inhibits transpeptidation reaction during peptigoglycan synthesis.	Cephalothin, cefoxitin, cefaperazone, cefriaxone: Broad (G+, some G-)
Bactericidal	Vancomycin and Teicoplanin	• Prevents transpeptidation Composed of a peptide portion which blocks transpeptidation by binding specifically to the D-alanyl-D-alanine terminal sequence on the pentapeptide portion of peptidoglycan. Vancomycin has a different	Vancomycin: Narrow (G+)

		binding site than penicillin.	
Bactericidal	Carbapenems	• Peptidoglycan biosynthesis	**Ertapenem**
<u>Protein synthesis inhibitors:</u> Therapeutic index not quite as high as that of cell wall synthesis inhibitors. Numerous steps during protein synthesis affected. Main mechanism is inhibition of protein synthesis by binding with the bacterial ribosomes and other components during protein synthesis.			
Primary effect	Examples	Mode of action	Members and Spectrum
Bactericidal	Aminoglycosides	• Alteration of protein synthesis • Increase of radical oxygen production • Causes misreading of mRNA Variations in structure but contain cyclohexane ring and amino acids. Antibiotics interfere with protein synthesis by binding to the 30S ribosomal subunit causing an incorrect amino acid to be brought the ribosome by transfer RNA (during translation). Results in a protein with an altered sequence of amino acids. Abnormal proteins are inserted into the plasma membrane of the cell where they induce changes in metabolic pathways that result in hydroxyl radical formation.	Neomycin, **kanamycin**, gentamycin, **streptomycin:** Broad (G+, mycobacteria) Narrow (aerobic G-) Streptomycin: (Bacteriocidal: G+)
Bacteriostatic	Tetracyclines	• Inhibit protein synthesis Antibiotics have a common four-ring structure to which side chains are attached. Bacteriostatic even though their action is similar to aminoglycosides in combining with 30s subunits of ribosomes to inhibit protein synthesis by directly inhibiting synthesis, causing misreading of messenger RNA.	Oxytetracycline, chlortetracycline, **doxycycline:** Broad (G+ and G-, rickettsia and chlamydia)
Bacteriostatic	Macrolides	• Inhibit protein synthesis Contain lactone ring which is linked to one or more sugars. Acts by binding to the 23S ribosomal RNA of large ribosomal subunit (50S) to inhibit peptide chain elongation during protein synthesis.	Erythromycin, **clindamycin:** Broad (aerobic and anaerobic G+, some G-) Bacteriostatic

Bacteriostatic	Chloramphenicol	• Inhibit peptide chain elongation The same mechanism as macrolides; binds to 23S ribosomal RNA of large ribosomal subunit (50S) to inhibit peptide chain elongation during protein synthesis.	Chloramphenicol: Broad (G+ and G-, rickettsia and Chlamydia)
Bacteriostatic	Oxazolidinones	Translation	**Linezolid**
Nucleic acid synthesis inhibition Nucleic acid synthesis inhibitors are not as selectively toxic. Antibiotics function by inhibiting DNA polymerase and topoisomerases or RNA polymerase to block replication or transcription.			
Primary effect	Examples	Mode of action	Members and Spectrum
Bactericidal	Quinolones and Fluoroquinolones	• Blocks DNA replication and transcription DNA synthesis, messenger RNA transcription and cell division require the modulation of chromosomal supercoiling through topoisomerase-catalysed DNA cleavage stage and preventing strand rejoining. Act by inhibiting bacterial topoisomerase II and topoisomerases DNA gyrase which introduces negative twist in DNA and separate its strands. The inhibition of DNA gyrase disrupts DNA replication and repair which causes bacterial chromosome separation during division. Topiomerase II is also inhibited by Fluoroquinolones which is a necessary enzyme for the untangling of DNA during replication.	Norfloxacin, **ciprofloxacin:** Narrow (G- better than G+) Levofloxacin: Broad
Bactericidal	Rifampin	• Inhibits bacterial DNA-dependant RNA polymerase Associated with the formation of double-stranded DNA which breaks down because of the inhibition of topoisomerase II. This inhibits bacterial DNA-dependant RNA polymerase, similarly to the inhibition of DNA replication by quinolones.	R-Cin, rifacilin, rifamycin, rimactane, rimpin, siticox: *Mycobacterium* and some G-

Metabolic antagonists:

Drugs antagonise or block the metabolic pathways. Structurally similar to the substrates of key enzymes and compete with metabolites for binding site of enzymes. Similar to the substrates, once bound to the enzyme, they are dissimilar enough to block enzyme activity and further progression of the pathway.

Primary effect	Examples	Mode of action	Members and Spectrum
Bacteriostatic	Sulfonamides	• Inhibits folic acid synthesis by competing with ρ- aminobenzoic acid Structurally related to sulfanilamide, an analogue of *p*-aminobenzoic acid. *p*-aminobenzoic acid is needed for the synthesis of folic acid which is a precursor of purines and pyrimidines. After sulfanilamide enters the bacterial cell it causes a decline in folate concentration because *p*-aminobenzoic acid competes with the sulfonamide for the active bonding sites. Results in an inhibition of purines and pyrimidines synthesis leading to termination of protein synthesis and DNA replication.	Silver sulfadiazine, sodium sulfacetamine, sulfamethoxazole, sulfanilamide, sulfasalazine, sulfisoxazole : Broad
Bacteriostatic	Trimethoprim	• Block folic acid synthesis by inhibiting the enzyme tetrahydrofolate reductase Interferes with the production of folic acid by binding to dihydrololate reductase. Converts dihydrofolic acid to tetrahydrofolic acid. Trimethoprim used in combination with sulfa drugs to increase the efficacy of antibiotic treatment by blocking two key steps in the folic acid pathway.	Trimethoprim: Broad
Bacteriostatic	Dapsone	• Interfere with folic acid synthesis	Dapsone: Narrow (mycobacterial infections)
Bactericidal (actively growing bacteria); bacteriostatic (dormant bacteria)	Isoniazid	Exact mechanism is unclear, thought to inhibit lipid synthesis (especially mycolic acid); putative enoyl-reductase inhibitor.	Isoniazid : Narrow (mycobacterial infections)

2.6.2 Mechanisms of antibiotic resistance

Bacteria have developed a number of mechanisms to overcome the lethal effects of antibiotics. The concept of the antibiotic resistome, which consist of all antibiotic resistance genes, has been advanced to serve as a framework for understanding the ecology of resistance. These resistance genes include those circulating in pathogenic bacteria, non-pathogenic organisms and antibiotic producers (Wright, 2010). The molecular mechanisms of resistance to antibiotics involve the genetic and biochemical features of different bacterial cell functions (Wright, 2010). Although these mechanisms are all different, they contribute to resistance of bacteria to antibiotics (Willey *et al.* 2014). For the purpose of this study the general mechanisms of antibiotic resistance will be discussed briefly.

Mechanism 1: Altered antibiotic target

The target site of the antibiotics on bacteria can be altered by mutating the gene that synthesises the binding target to reduce the binding capacity. This alteration is possible because chemotherapeutic agents act on specific cellular structures or target enzymes (Schmieder and Edwards, 2012; Willey *et al.* 2014). When the number of target gene copies for an antibiotic increases, it can lead to a reduction in the intracellular concentration of the inhibitor (Davies and Davies, 2010). In microbes that present resistance to vancomycin it appears that the bacteria has altered the target of the antibiotic by changing the terminal D-alanine in the pentapeptide of peptidoglycan into either D-lactate or D-serine (Willey *et al.* 2014).

Mechanism 2: Antibiotic degrading enzymes

This mechanism is also known as drug inactivation. Enzymes are produced that degrade or hydrolyse specific structures in the antibiotic (Schmieder and Edwards, 2012; Willey *et al.* 2014). Antibiotics can also be inactivated by the addition of chemical groups. This mechanism involves acquiring a new function, most often gaining of a gene or genes by horizontal gene transfer (Willey *et al.* 2014). Many bacteria that are classified as penicillin resistant, produce an enzyme called penicillinase (β-lactamases). The genes for β-lactamase enzymes are probably the most abundantly distributed (Torres-Cortés *et al.* 2011). This enzyme hydrolyses a bond in the β-lactam ring which inactivates the antibiotic (Willey *et al.* 2014).

Mechanism 3: Antibiotic-altering enzymes

This mechanism relies on the fact that resistant bacteria either use an alternate pathway to bypass the antibiotic effect, or increase the production of the target metabolite. These changes are most often mediated by horizontal gene transfer (Willey *et al.* 2014; Schmieder and Edwards, 2012). In several cases, this resistance mechanism can be attributed to specific enzymatic modifications of the antibiotics (Davies and Davies, 2010).

Mechanism 4: Efflux pump

Some bacteria force antibiotics out of the cell after entry by using translocases, also called efflux pumps. Efflux pumps confer multidrug-resistance because it is relatively nonspecific and removes many different drugs (Willey *et al.* 2014). The accumulation of intracellular antibiotics can also be reduced by a decrease in permeability of the cell (Schmieder and Edwards, 2012). Even without genetic resistance to antibiotics, bacteria can overcome the effects by severely slowing down their metabolism, decreasing nutrient uptake and thus antibiotic uptake (Willey *et al.* 2014).

Highly selectable phenotypes for antibiotic resistance can be detected by using growth inhibition assays. These assays can be performed by means of studies in agar disc diffusions or in broth. In a dilution-based assay, the results are interpreted as the bacteria being resistant or susceptible to an antibiotic according to the minimum inhibitory concentration (MIC) which is calculated for each isolate. The degree of resistance can vary and for that reason some classification schemes include one or more intermediate levels (Schmieder and Edwards, 2012).

Antibiotics are one of the marvels of the 20th century and are most commonly used for the treatment of bacterial infections (Davies and Davies, 2010). Although the gut bacteria of insects are, more often than not, seen as beneficial or symbiotic, the infestation of microbes were treated as an infection and bombarded them with antibiotics in the present study. Allen *et al.* (2009) did a study on the resident microbiota of *L. dispar* mid-gut which harbours antibiotic resistance determinants, and described the gut of an insect as a mixing ground for bacteria genes. Bacterial communities from wild and laboratory reared *L. dispar* mid-guts were found to have similar compositions and both were used. The MICs for 12 different antibiotics (carbenicillin, erythromycin, ciprofloxacin, rifampin, ceftazidime, gentamicin, kanamycin, streptomycin, vancomycin, chloramphenicol, nalidixic acid and tetracycline) were determined using a 96-well plate with an optical density of 600 nm at 28°C (Allen *et al.* 2009). Among the 44 bacterial isolates from the mid-guts of *L. dispar* larvae, resistance was

detected to 11 of the 12 antibiotics (no resistance to ciprofloxacin). They also studied the antibiotic resistance phenotypes of 44 bacterial isolates from *L. dispar* mid-guts, using metagenomics to assess the genotypes responsible for resistance. Although the genes responsible for antibiotic resistance are diverse, metagenomics lead to the following determinant: efflux pumps, transcriptional regulators and enzymes that degrade antibiotics (Allen *et al.* 2009). These results also suggested three different types of antibiotic tolerance: one that is specific to erythromycin, one that is not specific to antibiotic class, and one that is specific to β-lactam antibiotics (Allen *et al.* 2009). Even though these bacterial communities were never deliberately exposed to antibiotics, the mechanisms of antibiotic resistance were present. In this case, it is safe to assume that antibiotic resistance can be expected in the mid-gut microbiota of *B. fusca.*

Chapter 3: Material and Methods

3.1 Collection and rearing of larvae

Fifth instar *B. fusca* larvae were collected from 30 different geographical areas in South Africa, roughly within the boundaries of the maize triangle. The larvae were stored on maize stems from the same area as where they were collected to rule out possible introduction of different bacteria from another source. These larvae were used for the dissection and isolation of mid-gut bacteria. *Busseola fusca* and *Chilo partellus* eggs were provided by the Agricultural Research Council - Grain Crops Institute (ARC-GCI), Potchefstroom, South Africa from unknown localities. As soon as the eggs hatched, the larvae were placed on non-Bt maize, and were reared on non-Bt maize until the larvae reached a $1^{st}/2^{nd}$ instar.

3.2 Dissection of larvae

The collected larvae were removed from their original maize stems and anesthetised in ethanol. The immersion in ethanol also assured that the dissection was not contaminated by the bacteria present on the surface of the larvae. Three larvae from each geographical area were dissected. The larvae were pinned down in a wax filled petri dish and dissected to reveal the intestinal track. The mid-guts were excised, placed in 1.5 ml Eppendorf tubes with 1 ml distilled water, sonicated (PS-40 Ultrasonic cleaner; Jeken, Guangdong, China) and centrifuged (MiniSpin microfuge; Eppendorf). The supernatant surrounding the mid-guts was extracted from the tube and used in a dilution series (10^{-1} – 10^{-5}). Spread plates on nutrient agar (3 g yeast extract, 5 g NaCl, 5 g peptone power, 15 g bacteriological agar and 1 L distilled water) were made and incubated at 37°C for 24 hours. After 24 hours the growth on the plates was observed and classified morphologically.

3.3 Isolation and morphological classification of bacteria

Colonies from the dilution series were separated and described according to their morphological characteristics. Each distinct colony was ascribed a number (1 – 134). The streak plate technique was performed (Harley, 2011) on nutrient agar for each distinct morphological type and incubated for 24 hours at 37°C. After 24 hours of incubation, Gram stains were performed on the single colonies generated by the streak plate technique to

distinguish between Gram positive and Gram negative bacteria, as well as to visualise the cell shape. The Gram stain procedure according to Harley (2011) is as follows:

- Using a sterilised inoculation loop, place a drop of distilled water on a sterilised microscope plate and suspend the bacteria in the water. Spread the water over the surface of the microscope plate, let it air dry and fixate over the open flame of a Bunsen burner
- Place the smears on a staining rack
- Flood the smear with crystal violet and let it stand for 30 seconds; rinse with water for 5 seconds
- Cover the smear with Gram's iodine mordant and let it stand for 1 minute; rinse with water for 5 seconds
- Decolorise with acetone alcohol for 15 to 30 seconds; rinse with water for 5 seconds
- Counterstain with safranin for 60 to 80 seconds; rinse with water for 5 seconds
- Blot dry with tissue paper, add immersion oil to stained area and use the 100x microscope lens to visualise the bacteria. Gram positive organisms will stain purple, Gram negative organisms stain pink to red and the shape of the bacteria is also noted as bacilli, round or oval

The pure colonies (consisting of only one type of bacteria) were transferred to nutrient agar slants, incubated at 37°C for 24 hours and stored at 4°C. Streak plates were made of the mixed cultures on nutrient agar and incubated at 37°C for 24 hours. The fresh cultures were Gram stained to ensure pure colonies of all the morphological types.

3.4 Antibiotic testing

3.4.1 Antibiotic susceptibility

The Kirby-Bauer method (Harley, 2011) was adapted to suit the needs of this study. The incubation time was extended to 48 hours instead of the standard 24 hour incubation because an extended time is used during the rest of the experiments. The pure isolated colonies were individually placed in nutrient broth (3 g yeast extract, 5 g NaCl, 5 g peptone power and 1 L distilled water) and incubated at 37°C for 48 hours. After 48 hours, individual bacterial lawns were prepared on Meuller-Hinton agar (38 g Meuller-Hinton agar powder and 1 L distilled water) by spreading 100 µl of the cultures using sterile techniques (Harley, 2011). Eight different antibiotic discs were placed on the bacterial lawn of each morphological type and incubated for 48 hours. These antibiotics included kanamycin (10

µg/ml), ciprofloxacin (10 µg/ml), ampicillin (10 µg/ml), clindamycin (10 µg/ml), streptomycin (25 µg/ml), linezolid (30 µg/ml), doxycycline (30 µg/ml) and ertapenem (10 µg/ml). All the antibiotic discs used were provided by Oxoid, United Kingdom. After the 48 hour incubation at 37°C, the inhibition zone of each antibiotic disc was measured. A sterile inoculation loop was used to collect potential bacterial growth from the inhibition zone and to streak on a fresh Meuller-Hinton agar plate. These plates were incubated at 37°C for 48 hours. The presence or absence of growth on this plate determined the efficacy of the antibiotic disc and this data was used to classify the action of the antibiotic as bactericidal or bacteristatic. After the susceptibility of all the morphological types were tested, three antibiotics were chosen form the eight to continue with for the duration of the experiments. These antibiotics are ciprofloxanin (Clearsynth, India), ampicillin (Melford, United Kindom) and doxycycline (Clearsynth, India) and were chosen because of their efficacy toward the bacteria, as well as their solubility in water.

3.4.2 Growth curve analysis

The bacterial isolates were individually inoculated in McCartney bottles containing 10 ml autoclaved Mueller-Hinton broth (21 g Meuller-Hinton broth powder and 1 L distilled water) and incubated at 37°C for 48 hours. Eight isolates were used at a time and the treatments included a control (no antibiotics) and three different concentrations of the combination of antibiotics (ampicillin, ciprofloxacin and doxycycline). This was performed in triplicate each time. The antibiotic concentrations tested included a wide range of concentrations in order to investigate the most effective antibiotic concentration for the individual bacterial isolate. The antibiotic concentrations ranged from 25 µg/ml to 700 µg/ml. Using a 96-microwell F-bottom cell culture plate (Greiner bio-one, Cellstar, Germany), 200 µl cultured broth and 20 µl antibiotics were placed in each well. The 96-microwell plate was placed in the Micro-well plate reader (Powerwave X, Micro-well plate reader) for 48 hours at 28°C and the absorbance (560 nm) of each well measured every hour. Bacterial growth was assessed by observing the turbidity of the medium. The growth cycle of the bacterial cells was illustrated by a graph of absorbance over time by the KF 40 computer program. After 48 hours, the 96-microwell plate was removed from the plate reader and the content of each well was spread onto a Meuller-Hinton agar plate to observe the presence or absence of growth. The presence or absence of growth on the agar plates determined the efficacy of the antibiotic concentration tested. This observation, as well as the influence of the antibiotics on the growth curve of the bacteria, was used to decide on the concentration of antibiotics that was used during the feeding study.

3.5 Sterilisation of maize stems and leaves

Bt maize (MON810) and the non-Bt maize (isoline of MON810) were sterilised prior to being given to the larvae that fed on the antibiotic infused diet to ensure that the larvae were not exposed to bacteria which occur naturally on the maize stems and leaf tissue. Four percent JIK® (sodium hypochlorite) was used because of its antibacterial properties. Pieces of maize stem and maize leaf were submerged for 5 minutes in JIK® and rinsed with distilled water. These pieces were placed on nutrient agar and incubated at 37˚C for 24 hours to observe the antibacterial properties of the JIK®.

3.6 Feeding study

During the feeding study, there were four treatments in stage 1 (Figure 3.1). The larvae fed on: 1) a sterile artificial diet (ingredients not published), 2) artificial diet infused with antibiotics, 3) Bt maize stems or 4) non-Bt maize stems respectively. A concentration of 500 µg/ml of the combined ciprofloxacin, ampicillin and doxycycline was incorporated into the antibiotic *B. fusca* diet (provided by the ARC-GCI) in equal volumes. All the treatments were performed in sterile falcon tubes with the same number of falcon tubes per treatment (repeats) and the same number of larvae exposed to each treatment. The sterile diet (no antibiotics added) and the antibiotic diet was divided into 20 ml bottles and left to solidify. Once solidified, $1^{st}/2^{nd}$ instar *B. fusca* larvae were placed in each bottle and allowed to feed for 7 days. Each stage in Figure 3.1 represents treatments for 7 days.

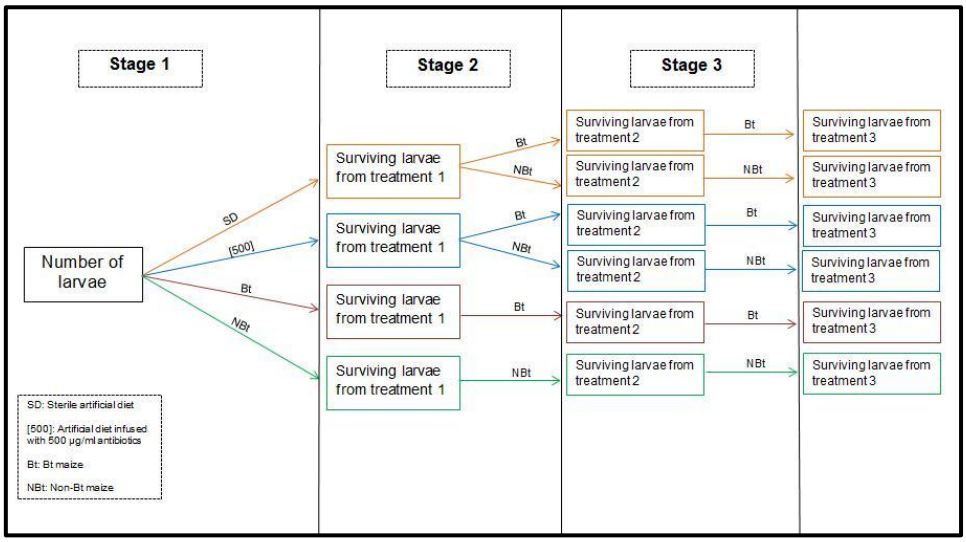

Figure 3.1: Flow chart illustrating the different feeding treatments used in the feeding study. Stage one represents day 1-7, stage 2 represents day 8-14 and stage 3 represents day 15-21. Different colours indicated the different treatments

After the first 7 days (stage 1), the surviving larvae from the sterile diet treatment (SD) and the antibiotic diet treatment ([500]) were divided into two groups, and placed on either Bt maize (Bt) or non-Bt maize (NBt) and remained there for 7 days (stage 2). The larvae that fed on Bt maize (Bt) and non-Bt maize (NBt) in stage 1 were moved to fresh Bt maize and non-Bt maize stems and remained there for 7 days (stage 2). During stage 3, the larvae that fed on Bt maize during stage 2 were placed on fresh Bt maize stems, and the larvae that fed on non-Bt maize were placed on fresh non-Bt maize stems for 7 days. The survival of the larvae were observed each time the larvae were provided with fresh maize stems. The falcon tubes were kept in a laminar flow cabinet to avoid contamination. The method was followed for 21 days. The survival of the larvae were observed and noted. This procedure was also performed with *C. partellus* larvae.

3.7 Statistical analyses of data

Microsoft Office Excel software (Microsoft Office 2010) was used for the organisation and primary analysis of data, as well to calculate the averages and standard errors of the data. To evaluate the influence of different antibiotic discs on the mid-gut bacteria of *B. fusca,* a Principal Component Analysis was performed using CANOCO 4.5 (Ter Braak and Šmilauer, 1998; Ter Braak and Šmilauer, 2002). STATISTICA version 12 was used to perform one-way ANOVA's to compare 4 feeding treatments as well as Tukey's Honest Significant Difference (HSD) test (StatSoft, 2013).

Chapter 4: Results

The purpose of all the methods performed was to rid *B. fusca* and *C. partellus* larvae of mid-gut microbes. This was done to ultimately test the possible influence of gut microbes on the efficacy of Bt maize. To achieve this task, it was necessary to start at the beginning, which included isolating the bacteria from the mid-guts of collected *B. fusca* larvae. After the isolation of 134 morphological types, the antibiotics and the concentration of the antibiotics that will be used to rid the larvae of mid-gut microbes was evaluated. The antibiotic tests started by choosing antibiotics from different antibiotic classes which were also soluble in water. The solubility of the antibiotics in water is important because the antibiotics were incorporated into an artificial diet for the larvae to feed on before being exposed to Bt maize. Antibiotic discs (8) were used to determine the antibiotics with the highest bacteristatic (inhibiting) and bactericidal (killing) effect on the 134 morphological types. Three antibiotics were chosen to use in combination at different concentrations to test the effects of antibiotics on each individual morphological type's growth curve. The data from the growth curve analysis was used to determine the antibiotic concentration that was incorporated into the antibiotic diet. The feeding study, which included feeding the larvae the antibiotic diet, also entailed other feeding treatments that were evaluated against each other. This comparison was used to reveal the influence of gut microbes on the efficacy of Bt maize against *B. fusca* and *C. partellus*.

4.1 Collection of *Busseola fusca* larvae

Figure 4.1 is a geographical illustration of the 30 sampling points across the theoretical maize triangle of South Africa. The stars and numbers indicate the farms where the larvae were collected. The larvae were collected from a wide variety of different maize crops which include a wide variety of agricultural practices.

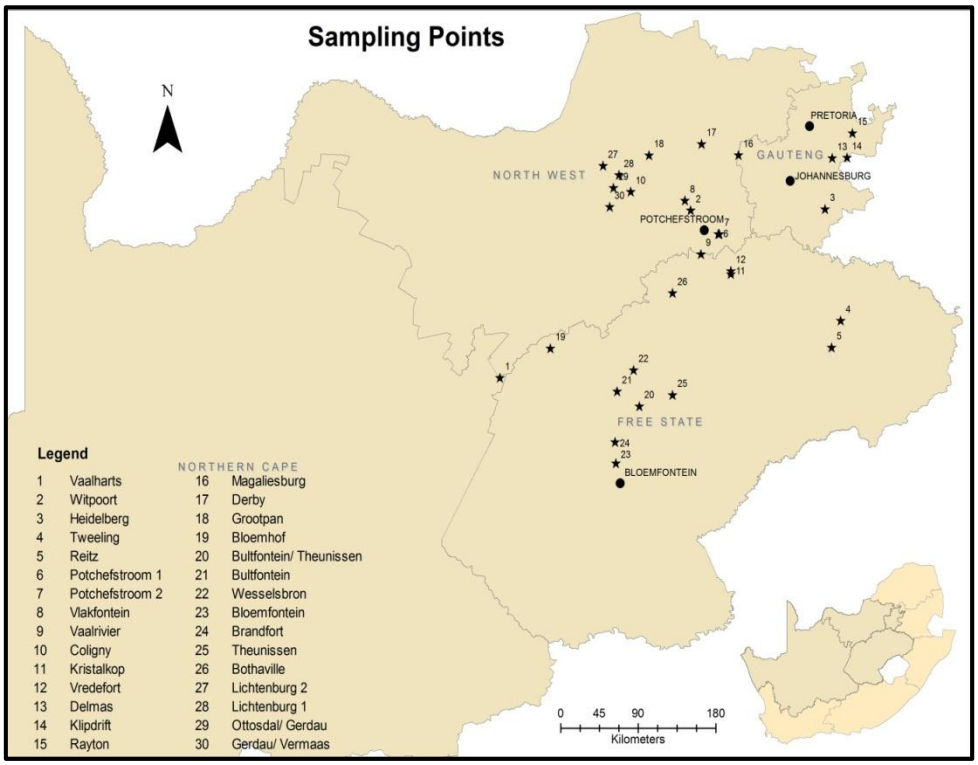

Figure 4.1: The geographical sampling points where *B. fusca* larvae were collected

The collected larvae resided either in the bottom part of the maize stem (Figure 4.2), or the maize ear. Collected larvae were stored in plastic containers accompanied by plant material from which it was collected to avoid contamination of bacteria from the different sampling points. These 5th instar larvae were used in the dissection.

Figure 4.2: *B. fusca* larva residing in the bottom part of maize stem

4.2 Dissection of larvae and morphological classification of mid-gut bacteria

During the dissection of *B. fusca*, the different sections of the gut are clearly visible (Figure 4.3 A). This makes the excision of the mid-gut region more accurate. Three mid-guts of larvae from the same sampling point were pooled in a 1.5 ml Eppendorf tube (Figure 4.3 B) before being sonicated. This ensured that the bacterial sample was representative of the specific sampling point and not just of one individual.

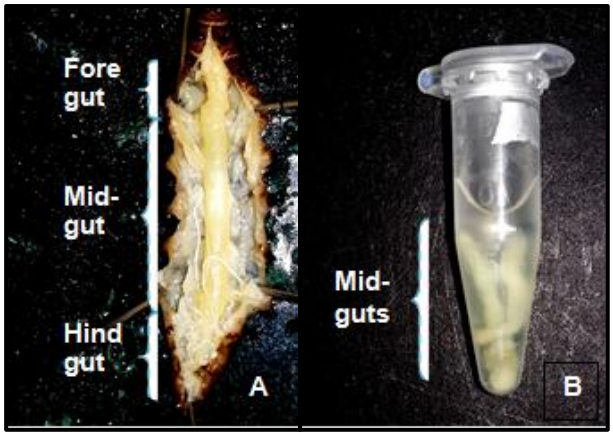

Figure 4.3: The dissection of a *B. fusca* larva to reveal the fore gut, mid-gut and hind gut (A). The mid-guts of 3 *B. fusca* larvae (B)

Figure 4.4 represents some examples of the different colonies observed after the dilutions series made from the mid-gut content was spread onto nutrient agar plates and incubated at 37°C for 24 hours. Different morphological types were used as a means of differentiation between the bacteria isolated. Although the morphological types were used as individuals, it was not presumed that there were no similarities between the colonies that appeared different on media.

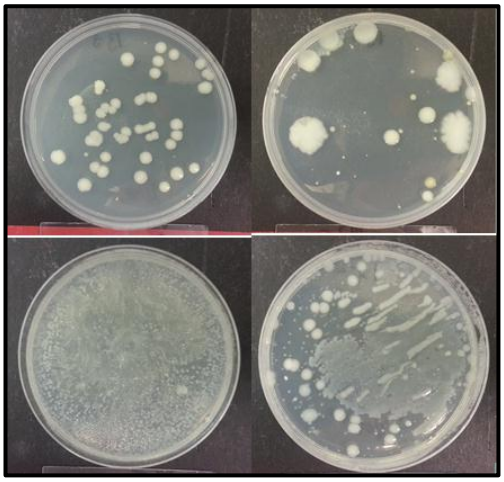

Figure 4.4: Examples of the different morphological types (colonies) derived from the mid-gut of *B. fusca*

The morphological types were further divided according to their Gram stain results. Figure 4.5 shows that there was only a slight difference in the percentage of Gram positive (53%) and Gram negative (47%) bacteria isolated from the mid-guts of the dissected larvae. These percentages represent all the bacteria isolated and selected for further analysis.

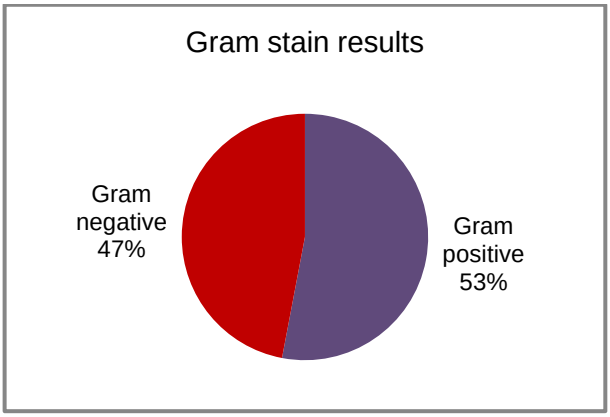

Figure 4.5: A comparison of percentage of Gram positive and Gram negative isolates found in the mid-gut of *B. fusca*

The Gram stain results also revealed the cell shape of the bacteria. The cell types that make up the composition of the bacterial content of the mid-gut include round, oval and rod shaped. The bacterial composition consisted of 21% round bacterial cells (cocci), 24% oval shaped cells and 55% rod shaped cells (bacilli) (Figure 4.6).

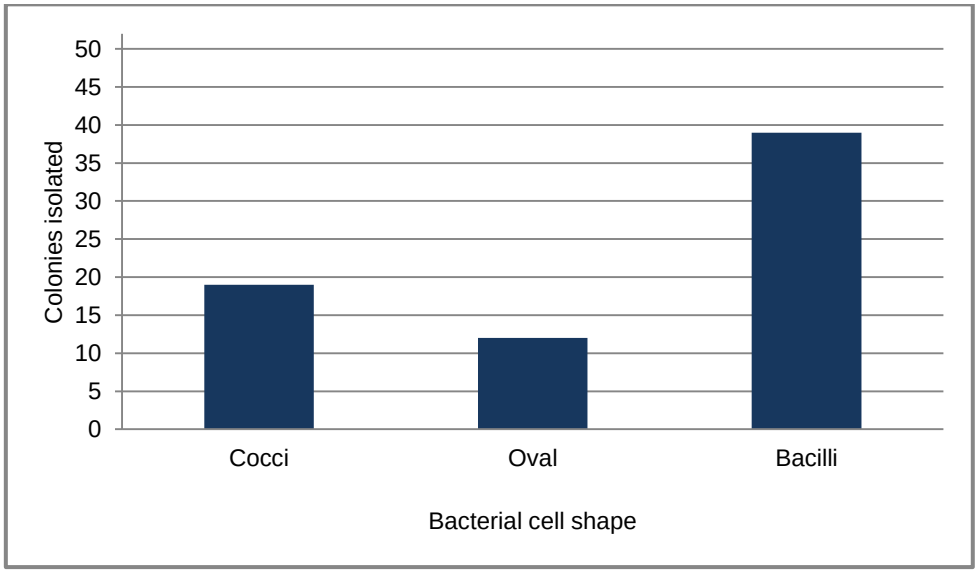

Figure 4.6: A comparison of the percentage of different bacterial cell types found in the mid-gut of *B. fusca*

Of the 70 Gram positive bacteria isolated from the mid-guts of the larvae (Figure 4.5), 19 were found to be cocci, 12 oval and 39 bacilli shaped. Of the 64 of Gram negative bacteria isolated from the mid-guts of the larvae (Figure 4.5), 9 were found to be cocci, 20 oval and 35 bacilli shaped. This comparison between Gram positive and Gram negative cell shapes is depicted in Figure 4.7.

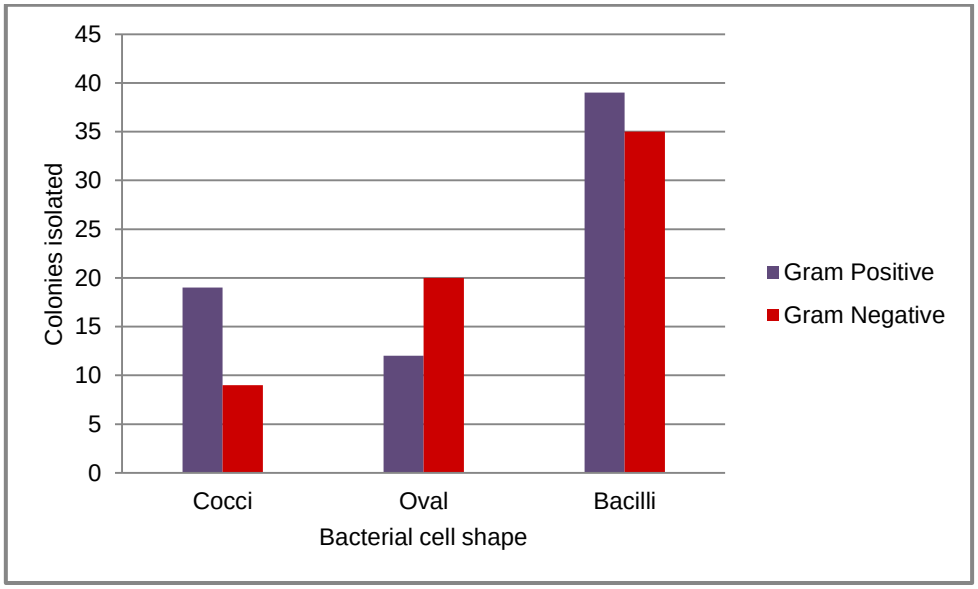

Figure 4.7: Bacterial cell shapes of the Gram positive and Gram negative bacteria isolated

As soon as pure cultures were obtained from all the 134 morphological types isolated, bacterial lawns were made to test the antibiotic susceptibility of each morphological type.

4.3 Antibiotic testing

4.3.1 Antibiotic susceptibility

The size of inhibition zones (Figure 4.8) determine the effectiveness of the antibiotic discs on the bacterial growth on the Mueller-Hinton agar plates. The resulting inhibition zones were measured and used as an inoculation on a fresh Mueller-Hinton agar plate to determine if the antibiotic disc had a bacteriostatic or bactericidal effect on the bacteria. Analysis of this data is illustrated in a PCA (Figure 4.9) and a bar graph (Figure 4.10).

Figure 4.8: The inhibiting effect of antibiotics on bacterial growth which result in clear inhibition zones

The PCA (Figure 4.9) illustrates the distribution of the isolates based on their suscetibility to the different antibiotics. Based on the results obtained from the PCA, it is evident that there is clustering of the bacterial isolates in reaction to the antibiotics and that a differentiation is visible based on the Gram reaction of the bacteria. The distribution of the antibiotics tested (arrows) is seen in the upper and lower right quadrants with mostly Gram positive bacterial isolates scattered among them. This distribution means that the antibiotics are more positively associated with the bacterial isolates adjacent to them (noteworthy inhibition took place) than the Gram negative isolates located in the lower left quadrant or the bacterial isolates located in the upper left quadrant. The clustering of Gram negative bacterial isolates in the lower left quadrant share a high similarity in terms of their reaction to the antibiotics. The distance between the Gram negative bacterial cluster in the lower left quadrant and the Gram positive cluster distributed in the upper left and right quadrant sheds light on the dissimilarity between these two clusters. The cluster of bacterial isolates which are found among the vectors (right upper and lower quadrant)are in closer proximity to each other which means that they share a higher similarity in terms of their reaction to the antibiotics when compared to the bacterial isolates in the other quadrants.

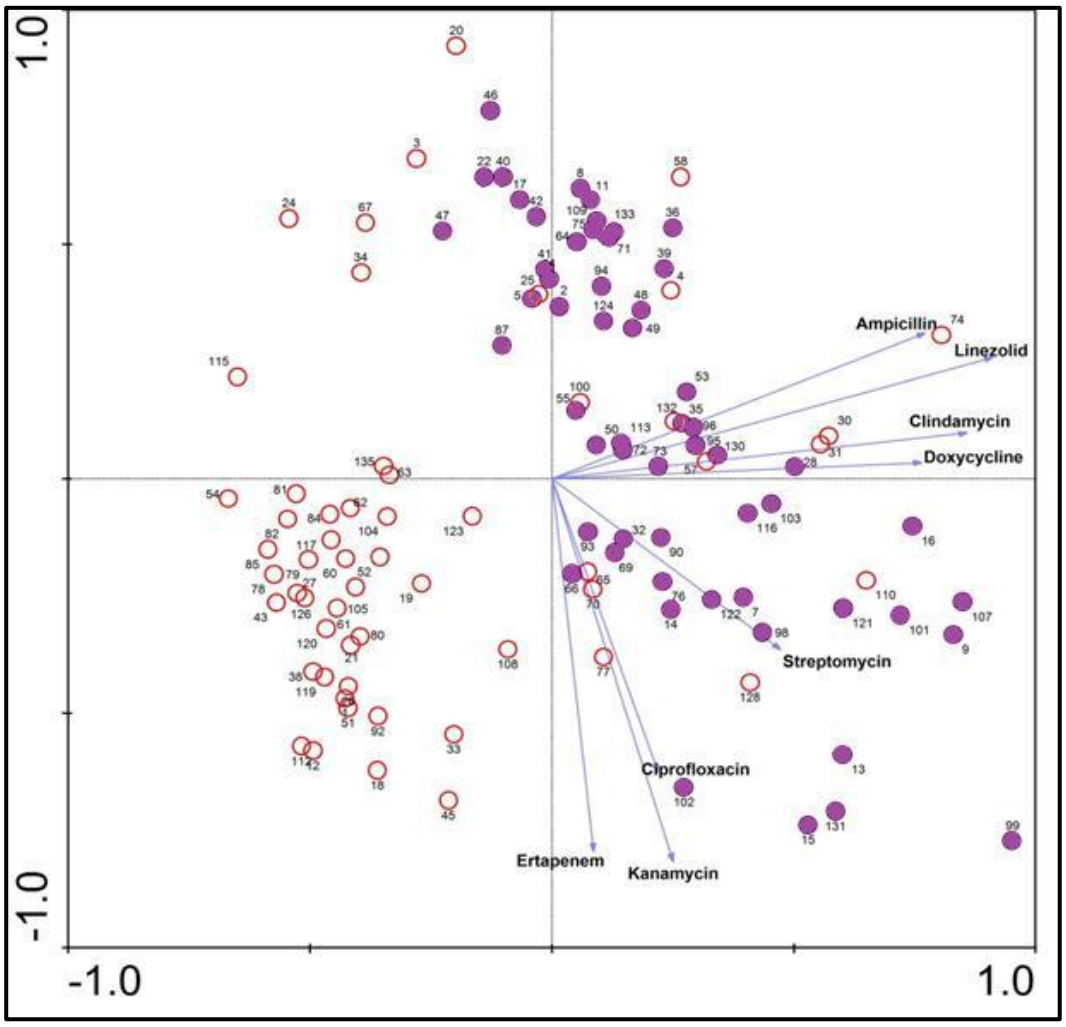

Figure 4.9: Principal Component Analysis (PCA) ordination diagram illustrating the relationship between the inhibition effects of antibiotics on bacterial isolates from the mid-gut of *B. fusca*. The eigenvalues for the first two ordination axes were 0.386 and 0.250, respectively. These two axes accounted for 63.6% of the total observed variance. Arrows indicate different antibiotic species used and coloured circles represent Gram positive (purple) and Gram negative (red) bacteria. Numbers indicate morphological types

Inhibition zones created by the inhibiting effects of the antibiotics were measured (in mm) and evaluated for all 134 morthoplogical types. The total inhibition zone diameter (Figure 4.10) represents the sum of all the inhibition zones measured for each antibiotic disc tested against all 134 morphological types. Ciprofloxacin had the highest inhibiting effect overall against all the morphological types with a total inhibition zone diameter of 3271 mm. Ertapenem was the second most effective antibiotic disc with a total inhibition zone diameter of 2837 mm, followed by doxycycline (2492 mm), linezolid (2491 mm) and ampicillin (2277 mm).

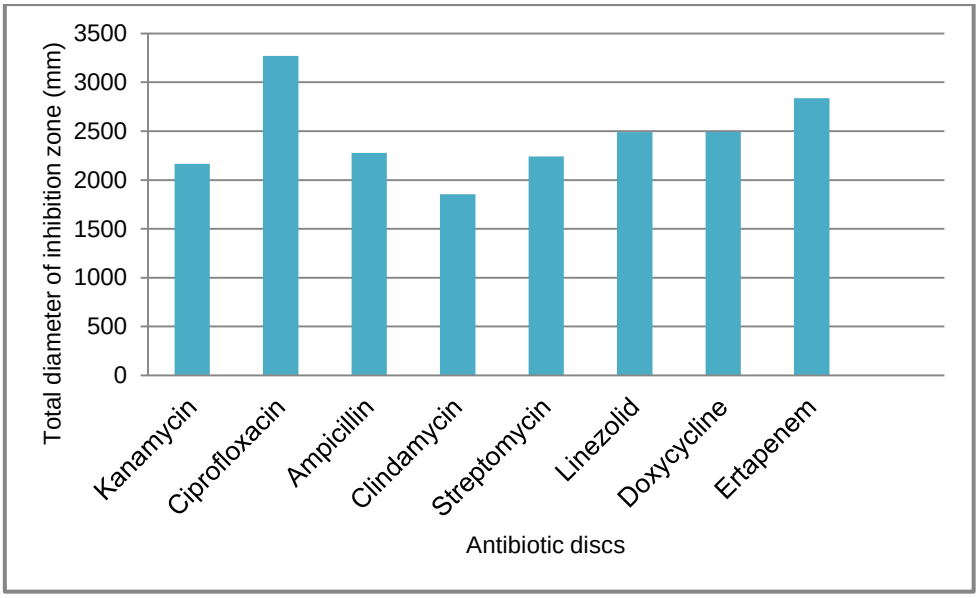

Figure 4.10: The total inhibition zone diameter of all the bacterial isolates from the mid-gut of *B. fusca* when exposed to the antibiotic discs tested

After measuring the inhibition zones greated by the antibiotics, the potential bacterial growth in the inhibition zone (as seen in figure 4.8) was collected and used to inoculate a new Mueller-Hinton agar plate to determine the efficacy of the antibiotic. A loop was used to inoculate a fresh Mueller-Hinton agar plate to determine if the antibiotics had a bacteristatic or bactericidal effect on the bacteria. As in Figure 4.11, ciprofloxacin had the most noteworthy effect on the isolates it was exposed to. It caused the highest averaged percentage of isolates killed (60.2%). Streptomycin was the second most effective antibiotic in killing the isolates (52.4%), followed by kanamycin (46.6%), ertapenem (35.2%) and ampicillin (29.8%) (Figure 4.11).

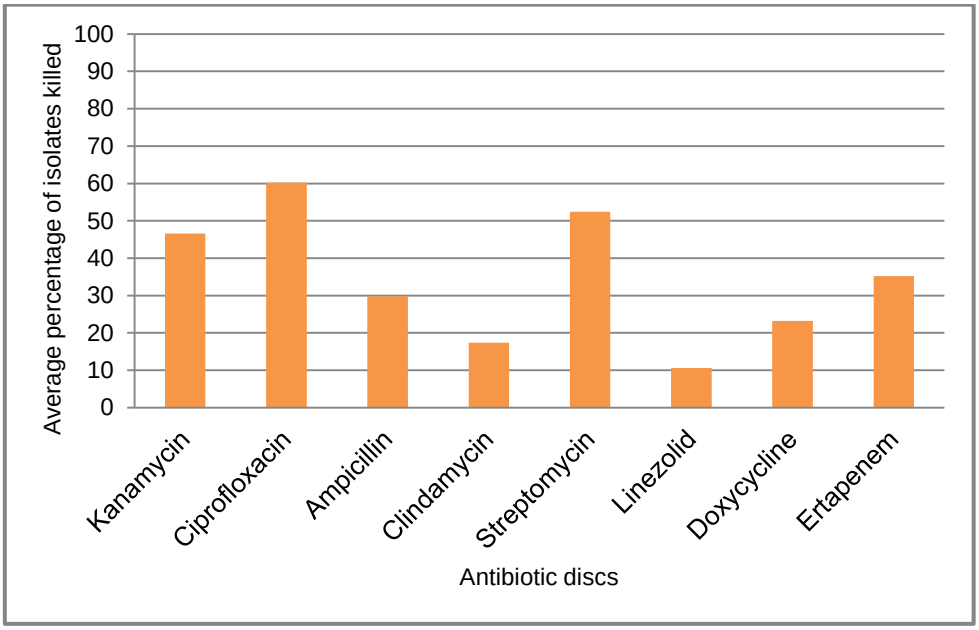

Figure 4.11: The average percentage isolates from the mid-gut of *B. fusca* killed when exposed to the antibiotic discs tested

4.3.2 Growth curve analysis

After 8 antibiotics were screened for their effectiveness against the mid-gut bacteria of *B. fusca* by using antibiotic discs, 3 antibiotics were chosen to continue with for the growth curve analysis and the feeding study. These antibiotics were ciprofloxacin, ampicillin and doxycycline which were used as a combined antibiotic treatment and tested at various concentrations. During the growth curve analysis, the influence of the antibiotics on the bacteria exposed to it was tested and visualised in the form of a growth curve graph (Figure 4.12).

It is clear from the growth curve graph that 200 µg/ml was the most effective antibiotic concentration (of the combined antibiotics) in maintaining a low optical density at a wavelength of 560 nm (Figure 4.12). This means that the turbidity of the wells that contained bacteria and an antibiotic concentration of 200 µg/ml (green line) were constantly lower than the control (blue line) as well as the other two antibiotic concentrations (red and green line). The green line is also the line that resembles the theoretical growth curve slope the most. Error bars added to the line graph represent the overall distribution of the data while the line represents the mean value of all the bacteria and repeats tested with that concentration of antibiotics.

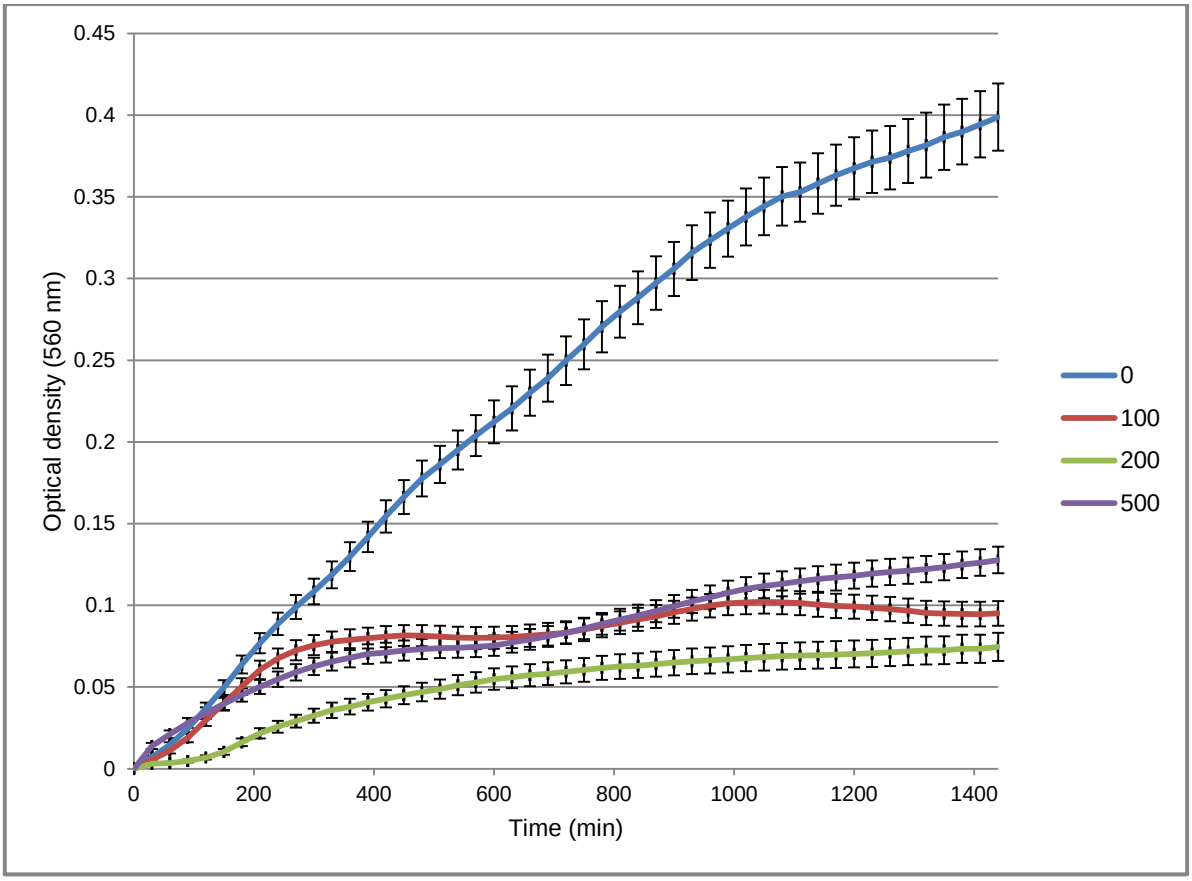

Figure 4.12: The optical density of bacterial isolates tested under the influence of three concentrations of antibiotics (red, green and purple line) as well as a control group where no antibiotics were present (blue line), over time (48 hours)

Although the growth curve data (Figure 4.12) shows that the antibiotic concentration of 200 µg/ml had the most deleterious effect on the bacteria it was exposed to, the present study was aimed to find the most effective concentration of the combination of antibiotics to inhibit or kill the bacteria. In Figure 4.13, it is seen that the concentration of 500 µg/ml had the greatest efficacy out of the three antibiotic concentrations. Although the inhibiting ability of the other two concentrations outweighed that of 500 µg/ml, the latter concentration was chosen because of the high bactericidal (killing) ability.

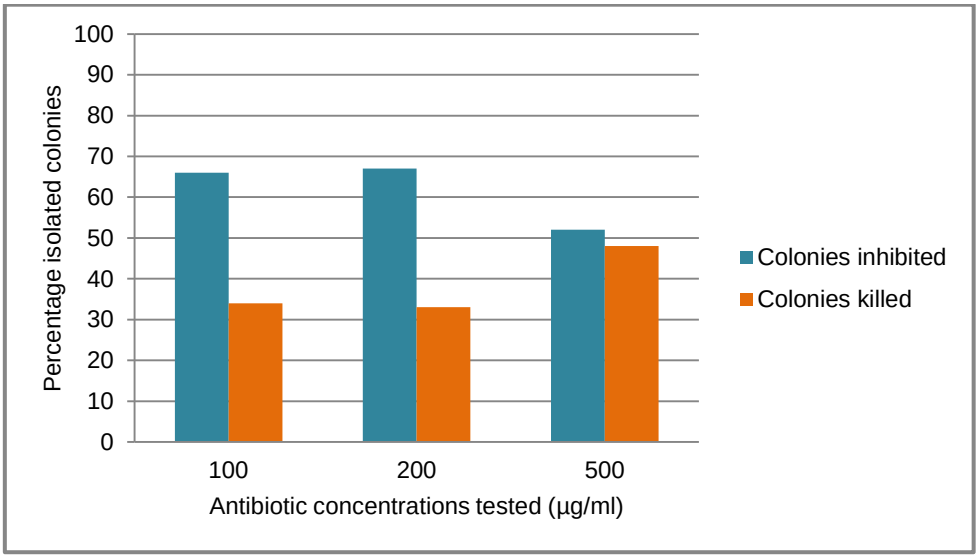

Figure 4.13: The percentage of bacterial isolates inhibited and killed by three different concentrations of antibiotics (100, 200 and 500 µg/ml)

After the exposure to antibiotics in the 96-well plate, the content of each well was used as an inoculation on a fresh Mueller-Hinton plate. After 48 hour incubation at 37°C, these plates were observed and the presence of bacterial growth on the plate was noted. On plate (A), Figure 4.14, the bacterial growth is very prominent because the media was not supplemented with antibiotics (control). On plate (B), the growth decreased drastically after being exposed to antibiotics. Although some growth of the different morphological types (for example morphological types 41, 42, 44, 46 and 47) are present, there are visible changes in the colony morphology when compared to the same colony in plate (A). This change in morphology can be attributed to the effects of the antibiotics. The presence of growth on plate B means that the effects of the antibiotics can be seen as bacteristatic. The absence of growth on plate (B) (43, 45 and 48) shows that the antibiotics had a bactericidal effect.

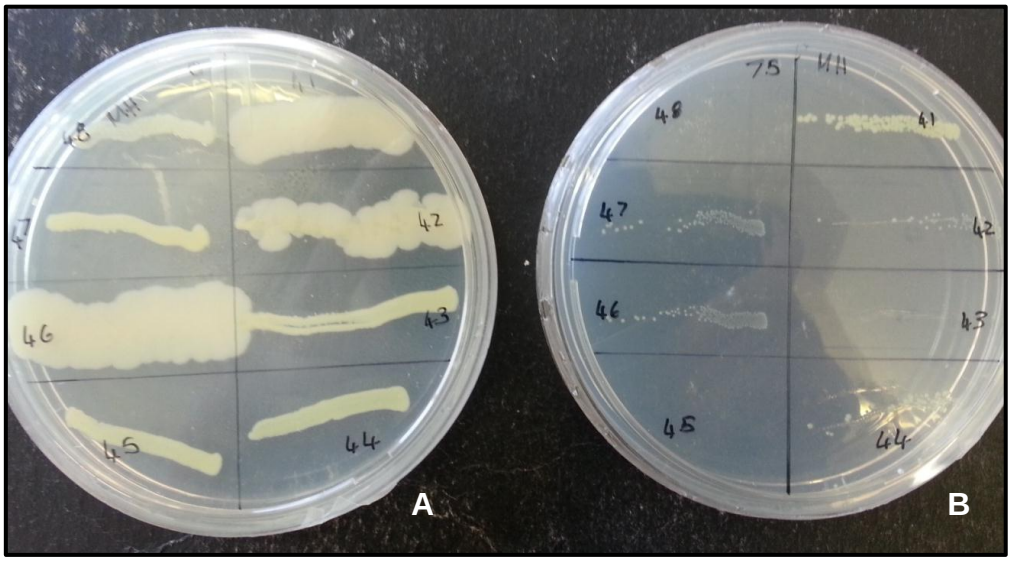

Figure 4.14: The decline in bacterial growth after being exposed to the combined antibiotics. (A) is a Mueller-Hinton agar plate with isolates 41 to 48 which were not exposed to antibiotics in the 96-microwell plate. The Mueller-Hinton plate (B) resembles the same colonies exposed to an antibiotic concentration of 75 µg/ml for 48 hours

Although numerous antibiotic concentrations were tested (25 µg/ml to 700 µg/ml), the antibiotic concentrations of 100, 200 and 500 µg/ml were tested extensively in the present study to establish the effect of antibiotics on the growth curve of the isolated bacteria. In Figure 4.15, plate (A) depicts the bacterial growth of all 8 isolates which were not exposed to antibiotics. Plate (B) represents the growth of 6 isolates that were exposed to an antibiotic concentration of 100 µg/ml, plate (C) represents the growth of 6 isolates that were exposed to an antibiotic concentration of 200 µg/ml and plate (D) represents the growth of 5 isolates that were exposed to an antibiotic concentration of 500 µg/ml. A clear decline in bacterial growth is visible with a rise in antibiotic concentration.

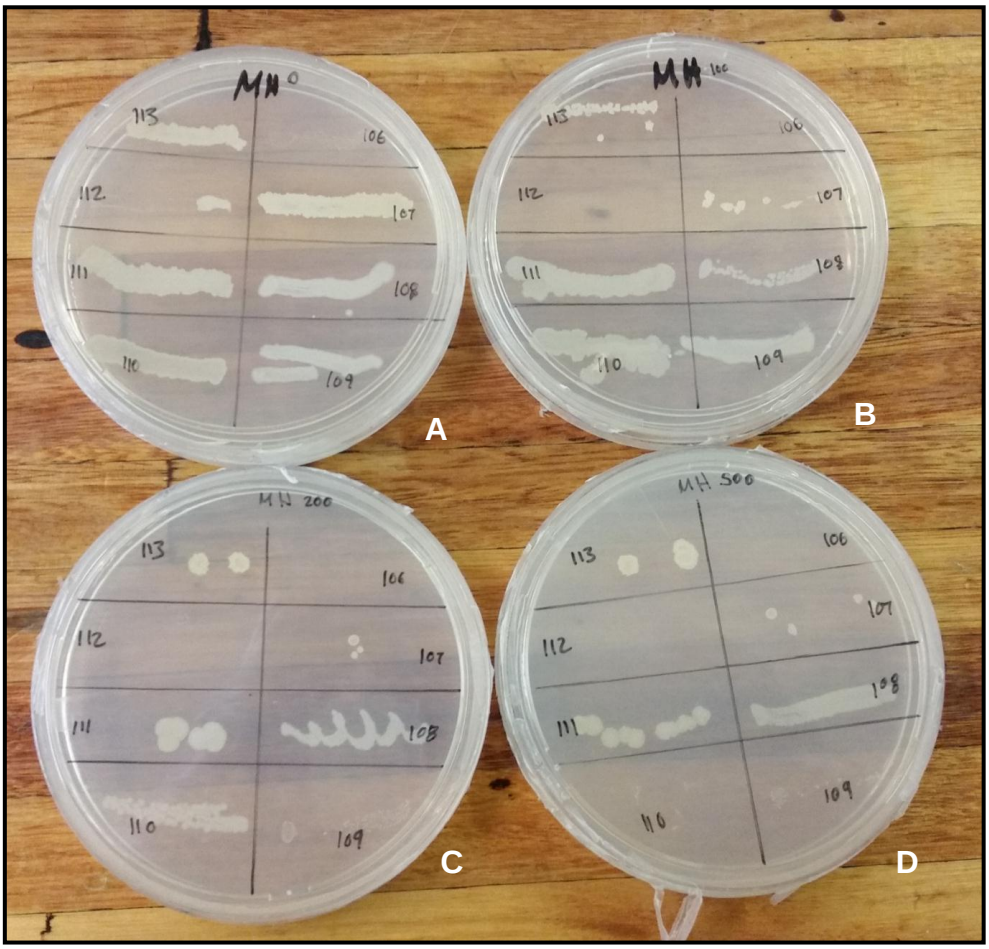

Figure 4.15: The decline in bacterial growth after being exposed to different antibiotic concentration. (A) is a Mueller-Hinton agar plate with isolates 106 to 113 which were not exposed to antibiotics in the 96-microwell plate. The Mueller-Hinton plates (B)-(D) resembles the same colonies that were exposed to three different antibiotic concentrations for 48 hours (B: 100 µ/ml, C: 200 µ/ml and D: 500 µg/ml)

Antibiotic concentrations higher than 500 µg/ml were also tested. In Figure 4.16, (A) is a Mueller-Hinton agar plate with morphological types numbers 25 to 32 which were exposed to an antibiotic concentration of 500 µg/ml in the 96-microwell plate. (B) Depicts the bacterial growth after a 48 hour exposure to 600 µg/ml and (C) depicts the bacterial growth after 48 hour exposure to 700 µg/ml. It is shown in Figure 4.16 that the higher concentrations had no effect on the bacterial growth even though they were exposed for 48 hours.

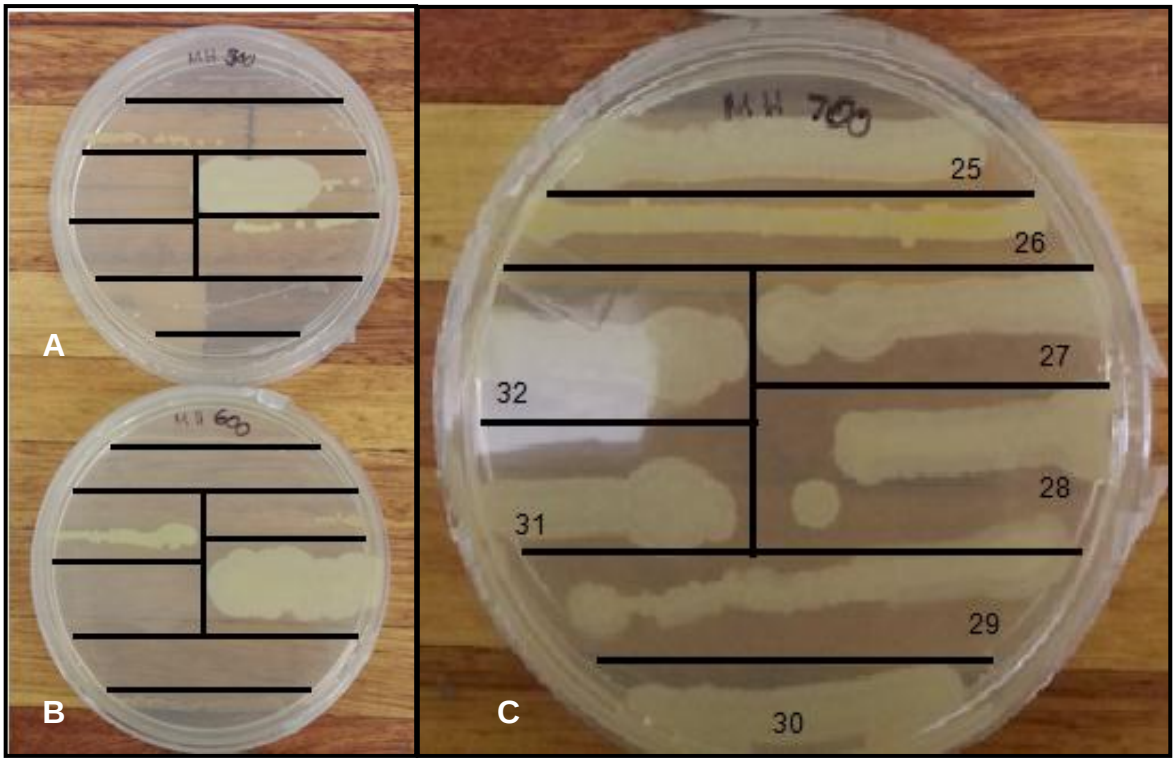

Figure 4.16: The increase in bacterial growth after being exposed to different antibiotic concentrations. The Mueller-Hinton plates (A)-(C) resembles colony numbers 25 to 32 that were exposed to three different antibiotic concentrations (500, 600 and 700 µg/ml) for 48 hours (A: 500 µg/ml, B: 600 µg/ml, C: 700 µg/ml)

4.4 Sterilisation of maize stems

The purpose of this demonstration was to show the presence of naturally occurring bacteria or fungi on maize leaves. Even after being rinsed in distilled water, the bacterial or fungal growth is still present and can be seen in the white microbial growth under and around the maize leaves (Figure 4.17).

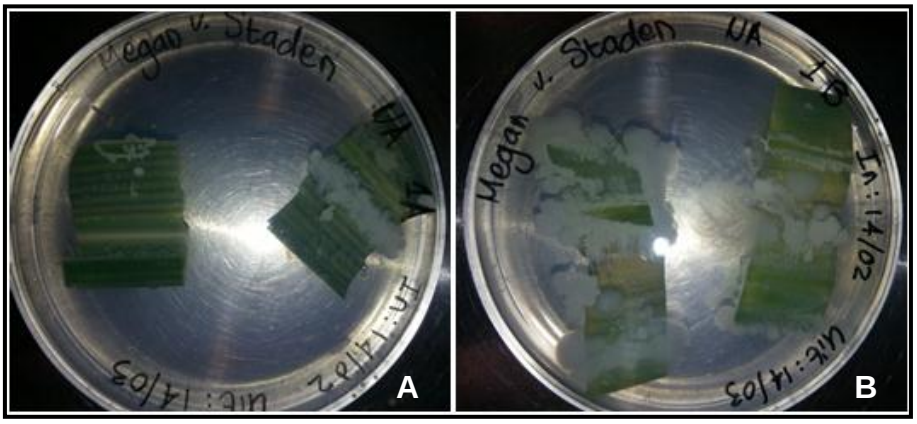

Figure 4.17: Maize leaves that were rinsed in distilled water, placed on nutrient agar plates and incubated at 37°C for 24 hours. (A) is a non-Bt maize leaf and (B) is a Bt maize leaf

The purpose of the submersion of the leaf tissue in 4% JIK® was to decrease the presence of bacteria or fungi on the surface of the leaf tissue as illustrated by Figure 4.17. The result from this experiment encouraged the use of 4% JIK® to submerge the maize plant material and sterilise the surface of the maize leaves because of the drastic decrease in white microbial growth present under and around the maize leaves on the agar plates (Figure 4.18). However, the submersion time in 4% JIK® was extended to 5 minutes to further aid in the sterilisation process.

Figure 4.18: Maize leaves that were submerged in JIK® for 2 minutes, rinsed in distilled water and placed on a nutrient agar plate and incubated at 37°C for 24 hours

4.5 Feeding study

After the combination of the most effective antibiotics (ciprofloxacin, ampicillin and doxcycline) and the most effective antibiotic concentration (500 µg/ml) were established, the feeding study was performed. For the first seven days after egg hatch, larvae fed on either sterile diet, antibiotic diet (antibiotic concentration of 500 µg/ml of each antibiotic infused into the diet at equal volumes), non-Bt maize or Bt maize. Surviving larvae that fed on the sterile diet treatment were divided into two groups and placed on non-Bt maize and Bt maize respectively on day 8. Those surviving larvae that fed on the antibiotic treatment were also divided into two groups and placed on non-Bt maize and Bt maize respectively on day 8. Larvae that fed on non-Bt maize for the first 7 days were transferred to fresh non-Bt maize stems on day 8. Larvae that fed on Bt maize for the first 7 days were transferred to fresh Bt maize stems on day 8. On day 14, all the treatment groups were provided with fresh non-Bt or Bt maize plant material and the larval mortality was recorded for every treatment. On day

21, the larval mortality was recorded for the last time. Two larval groups of *B. fusca* (BF 1 and BF 2), and two larval groups of *C. partellus* (CP 1 and CP 2) was used

4.5.1 *Busseola fusca*:

Figure 4.19 represents the survival of *B. fusca* larval group 1 (BF 1) from day 8 to day 21 of the experiment. The number of surviving larvae on the non-Bt treatment dropped 16% between day 8 and day 21. The decline in number of larvae on the Bt treatment between day 8 and day 21 was 19%. This decrease in larval survival was also visible when observing the survival of the larvae that fed on the SD NBt (10%) and the survival of larvae that fed on the SD Bt treatment (8%) at day 21. This difference in larval survival can be attributed to the deleterious effects of Bt maize on the larvae. An interesting observation was seen when comparing the larval survival between the Bt treatment group (1%) and those feeding on the antibiotic diet before feeding on Bt, indicated by 500 Bt, (20%) at day 21.

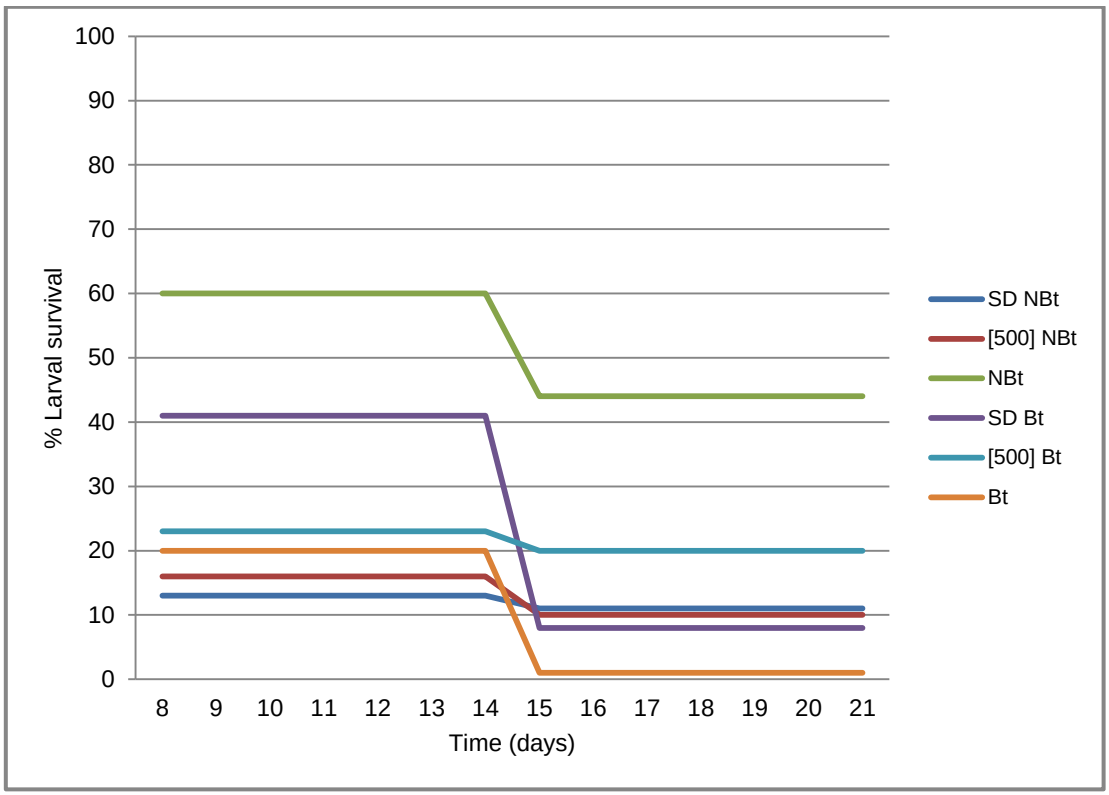

Figure 4.19: The larval survival of BF 1 after exposure to different treatments over time (day 8 – 21). (NBt: non-Bt maize; Bt: Bt maize; SD NBt: larvae fed on sterile diet for 7 days and transferred to non-Bt maize on day 8; SD Bt: larvae fed on sterile diet for 7 days and transferred to Bt maize on day 8; [500] NBt: larvae fed on antibiotic diet for 7 days and transferred to non-Bt maize on day 8; [500] Bt: larvae fed on antibiotic diet for 7 days and transferred to Bt maize on day 8)

After the results illustrated in Figure 4.19 were observed, further statistical analysis of the data was performed in the form of a one way Anova. This was done for all larval groups. Each larval group's survival line graph is followed by a bar graph depicting the mean larval survival.

The percentage of surviving larvae (Figure 4.20) on the Bt treatment differed significantly from the other treatments evaluated. There was, however, no significant difference between the percentage of surviving larvae on the 500 Bt, 500 NBt and NBt treatments. The percentage surviving larvae on these treatments were significantly higher than that on the Bt treatment. Each bar represents the mean percentage larval survival on each treatment and the error bars added to each bar represents the overall distribution of the data (repeats).

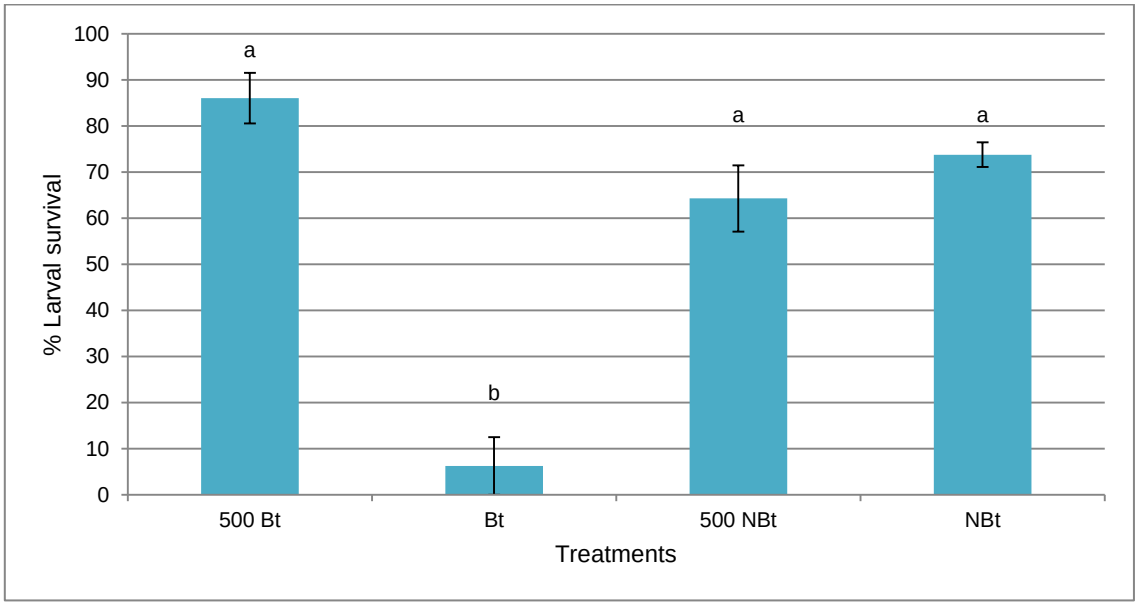

Figure 4.20: The mean percentage larval survival of larval group BF 1 across four different treatments. Different letters indicate statistically significant differences ($p<0.000002$). (NBt: non-Bt maize; Bt: Bt maize; 500 NBt: larvae fed on antibiotic diet for 7 days and transferred to non-Bt maize on day 8; 500 Bt: larvae fed on ntibiotic diet for 7 days and transferred to Bt maize on day 8)

Figure 4.21 represents the larval survival of *B. fusca* larval group 2 (BF 2) from day 8 to day 21 of the experiment. The number of surviving larvae on the non-Bt treatment decreased 23% between day 8 and day 21, whereas the decline of larval survival on the Bt treatment between day 8 and day 21 was only 12%. This higher larval survival on Bt maize is also observed at the survival of larvae that fed on the SD NBt treatment (9%) and the survival of larvae that fed on the SD Bt treatment (11%). This difference in larval survival between the non-Bt and Bt treatments can be attributed to the resistance of this *B. fusca* larval group to Bt maize. An interesting observation is the difference in larval survival when comparing the

survival of larvae that fed on Bt (12%) and the survival of larvae that fed on the antibiotic diet before feeding on Bt (2%) at day 21.

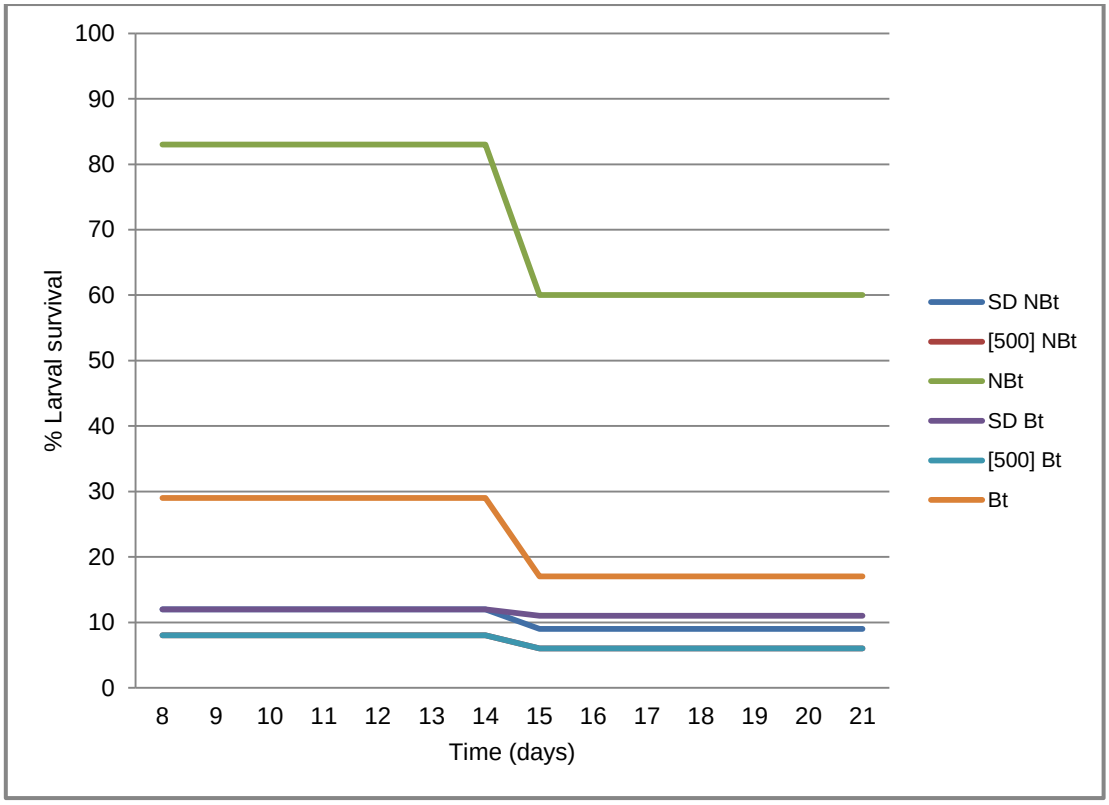

Figure 4.21: The larval survival of BF 2 after exposure to different treatments over time (day 8 – 21). (NBt: non-Bt maize; Bt: Bt maize; SD NBt: larvae fed on sterile diet for 7 days and transferred to non-Bt maize on day 8; SD Bt: larvae fed on sterile diet for 7 days and transferred to Bt maize on day 8; [500] NBt: larvae fed on antibiotic diet for 7 days and transferred to non-Bt maize on day 8; [500] Bt: larvae fed on antibiotic diet for 7 days and transferred to Bt maize on day 8)

The percentage of surviving larvae (Figure 4.22) that fed on different treatments did not differ significantly between treatments. Each bar represents the mean percentage larval survival on each treatment and the error bars added to each bar represents the overall distribution of the data (repeats).

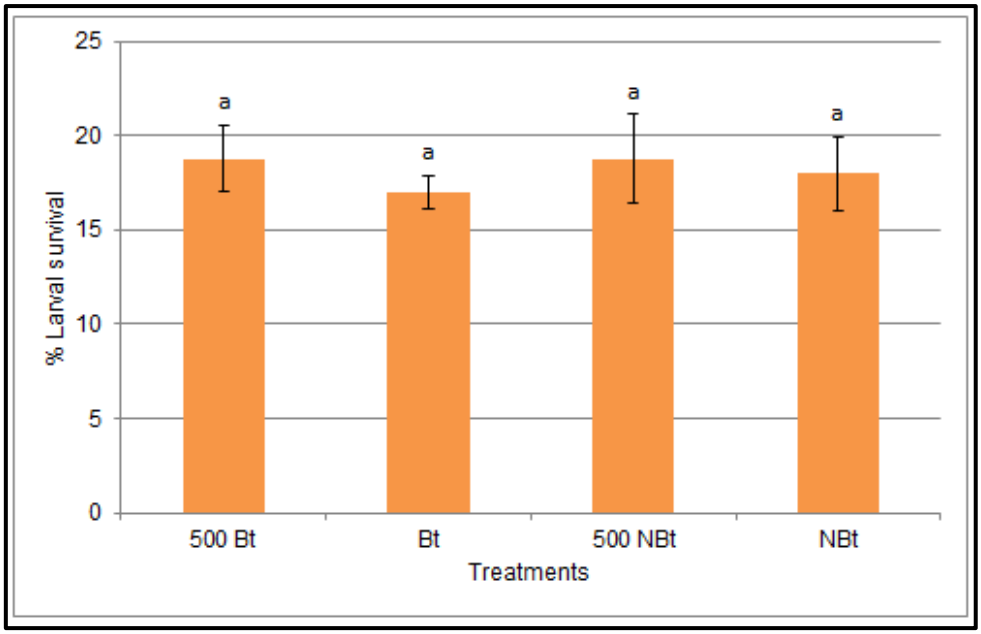

Figure 4.22: The mean percentage larval survival of larval group BF 2 across four different treatments. Different letters indicate statistically significant differences ($p<0.255013$). (NBt: non-Bt maize; Bt: Bt maize; 500 NBt: larvae fed on antibiotic diet for 7 days and transferred to non-Bt maize on day 8; 500 Bt: larvae fed on antibiotic diet for 7 days and transferred to Bt maize on day 8)

The larvae depicted in the photo (Figure 4.23) originated from non-Bt maize (A) and Bt maize (B) treatments on day 21 of the feeding study. These larvae hatched from the eggs at the same time, were placed on the respective treatments at the same time and fed on the respective treatments for the same period. A clear difference in size and mass is visible and can be attributed to the deleterious effects of Cry 1Ab proteins on larvae.

Figure 4.23: *Busseola fusca* larvae removed from non-Bt maize (A) and Bt maize (B) on day 21 of the feeding study

4.5.2 *Chilo partellus*:

Figure 4.24 represents the larval survival of *C. partellus* larval group 1 (CP 1) from day 8 to day 21 of the experiment. All larvae on the non-Bt treatment survived from day 8 and day 21. However, 80% of larvae on the Bt treatment did not survive after day 15. An increase in larval survival on Bt maize was observed with the survival of the larvae on the SD NBT treatment (6%) and the survival of larvae on the SD Bt treatment (12%). An interesting observation is the difference in larval survival when comparing the survival of larvae of the Bt treatment (4%) and the survival of larvae of the 500 Bt treatment (14%) at day 21.

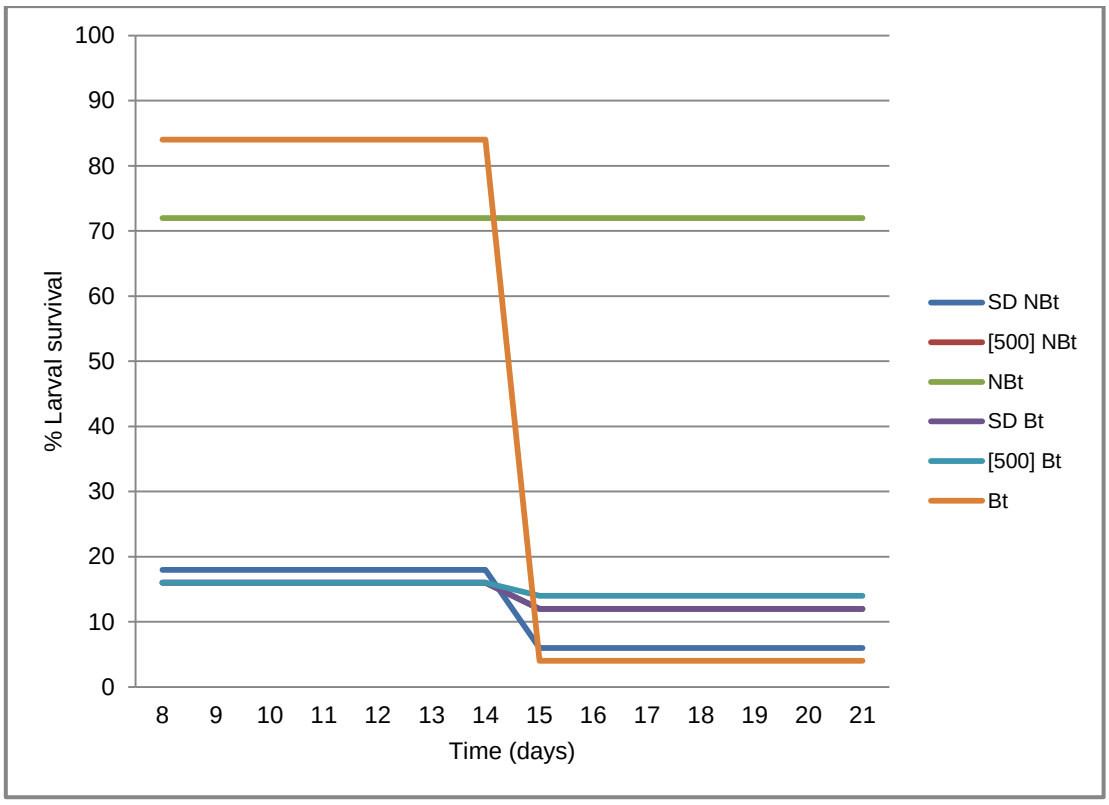

Figure 4.24: The larval survival of CP 1 after exposure to different treatments over time (day 8 – 21). (NBt: non-Bt maize; Bt: Bt maize; SD NBt: larvae fed on sterile diet for 7 days and transferred to non-Bt maize on day 8; SD Bt: larvae fed on sterile diet for 7 days and transferred to Bt maize on day 8; [500] NBt: larvae fed on antibiotic diet for 7 days and transferred to non-Bt maize on day 8; [500] Bt: larvae fed on antibiotic diet for 7 days and transferred to Bt maize on day 8)

The percentage larval survival (Figure 4.25) in the Bt treatment was lower than the other treatments. The percentage of surviving larvae on the 500 Bt and non-Bt treatments were significantly higher than those on the Bt and 500 non-Bt treatments. Each bar represents the

mean percentage larval survival on each treatment and the error bars added to each bar represents the overall distribution of the data (repeats).

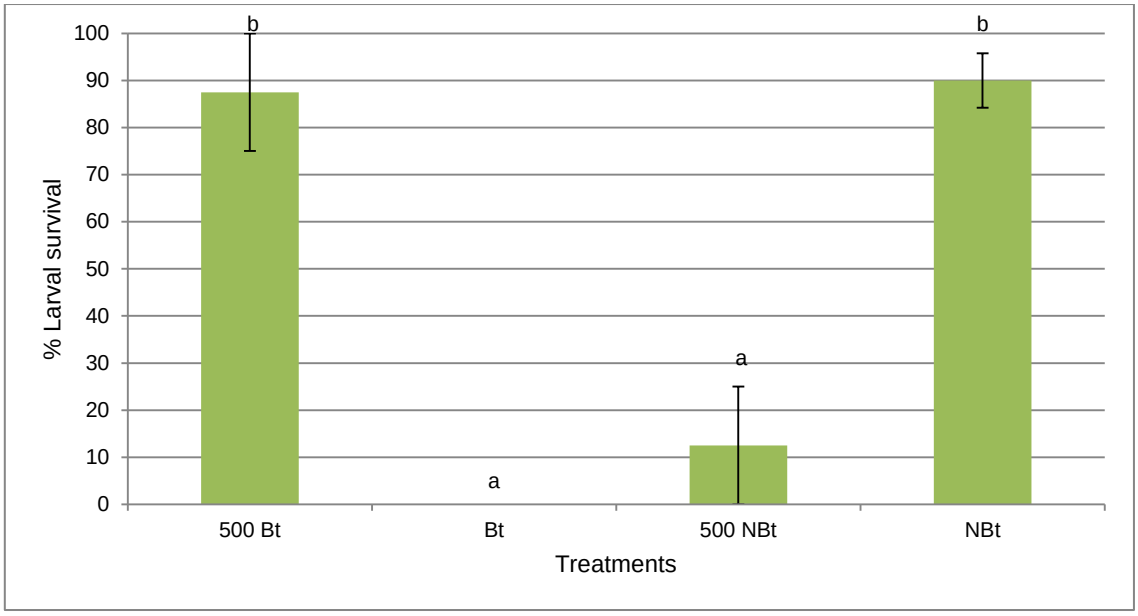

Figure 4.25: The mean percentage larval survival of larval group CP 1 across four different treatments. Different letters indicate statistically significant differences ($p<0.000014$). (NBt: non-Bt maize; Bt: Bt maize; 500 NBt: larvae fed on antibiotic diet for 7 days and transferred to non-Bt maize on day 8; 500 Bt: larvae fed on antibiotic diet for 7 days and transferred to Bt maize on day 8)

Figure 4.26 represents the larval survival of *C. partellus* larval group 2 (CP 2) from day 8 to day 21 of the experiment. Ten percent of larvae that fed on non-Bt maize survived. The larvae that were placed on Bt did not survive past the first 7 days of the treatment. A decrease in larval survival on Bt maize is visible when looking at the survival of the larvae at day 21 on the SD NBt treatment (2%) and the survival of larvae on the SD Bt treatment (0%). An interesting observation is the difference in larval survival when comparing the survival of larvae on the Bt treatment (0%) and the survival of larvae on the 500 Bt treatment (6%) at day 21.

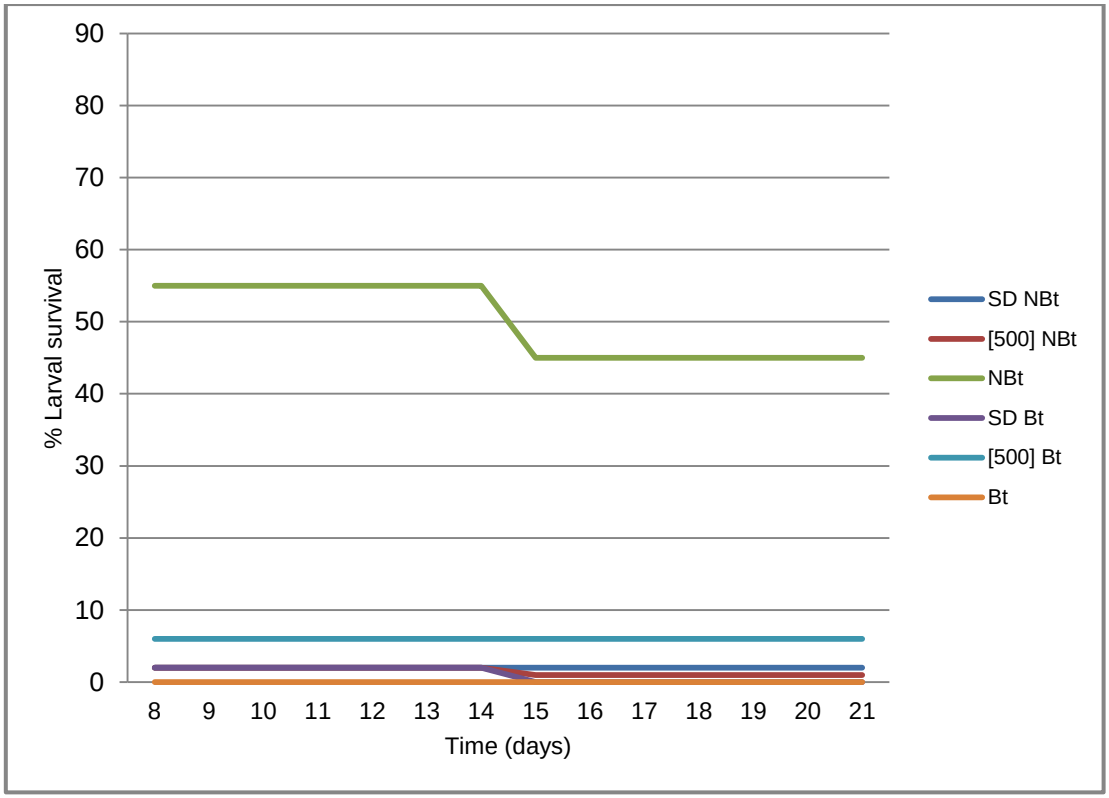

Figure 4.26: The larval survival of CP 2 after exposure to different treatments over time (day 8 – 21). (NBt: non-Bt maize; Bt: Bt maize; SD NBt: larvae fed on sterile diet for 7 days and transferred to non-Bt maize on day 8; SD Bt: larvae fed on sterile diet for 7 days and transferred to Bt maize on day 8; [500] NBt: larvae fed on antibiotic diet for 7 days and transferred to non-Bt maize on day 8; [500] Bt: larvae fed on antibiotic diet for 7 days and transferred to Bt maize on day 8)

The percentage larval survival (Figure 4.27) the Bt treatment and the 500 NBt treatment did not differ significantly from one another, but the survival was significantly lower than that of the 500 Bt and NBt treatments. Each bar represents the mean value of the surviving larvae on each treatment and the error bars added to each bar represent the overall distribution of the data (repeats). All the larvae of the 500 Bt treatment survived the treatment (no error bar generated) and no larvae survived the Bt treatment (lack of bar and error bar).

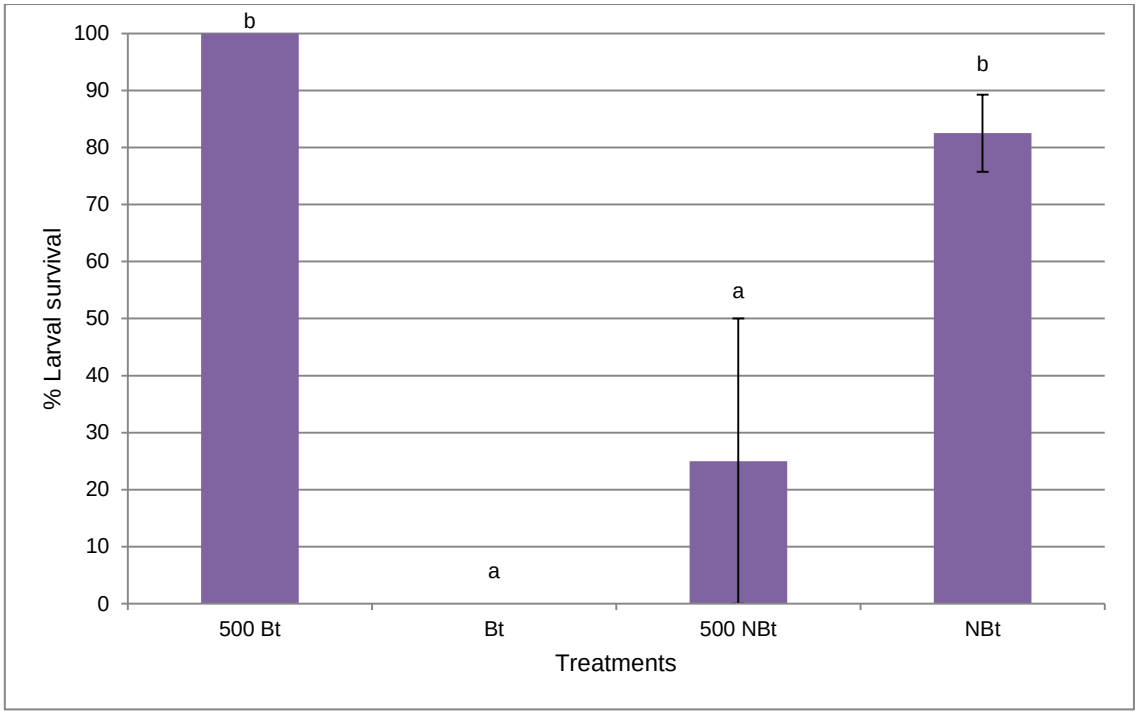

Figure 4.27: The mean percentage larval survival of larval group CP 2 across four different treatments. Different letters indicate statistically significant differences ($p<0.000409$). (NBt: non-Bt maize; Bt: Bt maize; 500 NBt: larvae fed on antibiotic diet for 7 days and transferred to non-Bt maize on day 8; 500 Bt: larvae fed on antibiotic diet for 7 days and transferred to Bt maize on day 8)

Chapter 5: Discussion

5.1 Collection of *B. fusca* larvae

Stem borer pest densities vary between regions, within a country or even the same eco-region of neighbouring countries (Anon, 2012a; George *et al.* 2008; Ndemah *et. al.* 2003). This represents one of the reasons multiple random sampling points throughout a large part of the biggest maize producing areas in South Africa was chosen to collect larvae (Figure 4.1). There was no discrimination between locations, maize cultivar, agricultural practice used on the individual farms or damage levels caused by the potential presence of stem borer larvae. A noticeable similarity between most of the sampling points was the age of the maize plants. The maize plants sampled were all of roughly the same age because all sampling was done within 2 months and the plant dates of the maize were very similar because of the seasonality.

An effort was made to keep the sampling points approximately 2 km away from each other as not to sample the same field more than once and to get an even distribution of sampling sites. Because *B. fusca* only pupates during October and November (Kfir, 1991; Kfir *et al.* 2002), a sampling period was chosen that would decrease the chance of collecting pupae instead of larvae. As seen in Figure 4.2, the larvae were collected from the lower parts of the stalks (Kfir *et al.* 2002; Kruger *et al.* 2009), and in some cases in the maize ears. This could mean that the third generation of the pest was present on the maize plants because the flight activity of the second generation *B. fusca* moth's peak in February and sampling for the present study took place in early March (Ebenebe *et al.* 1999).

5.2 Dissection of larvae and morphological classification of mid-gut bacteria

In a study by Takatsuka and Kunimi (2000) on the effect of intestinal bacteria on the growth of *Bacillus thuringiensis* in larvae of *Homona magnanima* (Lepidoptera: Torticidae), the fifth instar larvae were surface sterilised by 70% ethanol and aseptically dissected to extract their guts. A dissection of this nature was also done in the present study in a laminar flow hood (Figure 4.3). It is safe to assume that the gut of an insect is inhabited by microorganisms as a result of the external environment the insect is continuously exposed to (Visotto *et al.* 2009). In a study done on *Ostrinia nubilalis,* the results suggested a clear association between the environment and the composition of the main bacterial taxa in the mid-gut of this lepidopteran pest (Belda *et al.* 2011). The environment and specific location in the gut

(Figure 2.3) influence the microbiota in the intestinal track of insects. Bacterial communities inside the gut of an insect vary immensely in location in the gut, size, composition and functions within the gut (Engel and Moran, 2013). In the present study only the mid-gut of the larvae were dissected meticulously as far as possible to reduce the possibility of contamination with bacteria present in other areas on the gut. Akiba (1986) observed that the density of intestinal bacteria of both *Bombyx mori* and *Hyphantria cunea* (Lepidoptera: Arctiidae), are seasonally influenced. For instance, in spring the density was found to be lower than in autumn. Although the seasonal density was not tested in the present study, the diversity in morphological types isolated was considered abundant enough in diversity to consider it representative. One area where the seasonality was taken into account was during the sampling of the larvae. All the sampling was done in the same agricultural season within a time span of roughly 2 months.

The collected larvae were kept on the same plant material from which it was collected to avoid bacterial contamination from other sites or sources. Allen *et al.* (2009) used the same approach. As soon as the bacteria were isolated from the mid-guts, they were assigned to a morphological type. Because of the high number of morphological types isolates, it is probable that the larvae collected were not the first generation of that season in the present study. This observation contributes to the statement that insect gut bacteria are acquired from their environment, multiplying with each successive generation (Engel and Moran, 2013). The total number of morphological types isolated from all 30 sampling sites was 134. This number, however, decreased as the study progressed because some bacteria could not be recultured on nutrient agar. During a study by Heckel *et al.* (2007) on the census of the bacterial community of *Lymantria dispar* larval mid-gut, culturing methods yielded 15 distinguishable types of bacteria across cellular and colony morphological traits. This small diversity of colonies, compared to the 134 morphological types isolated in the present study, can be attributed to the diet treatments in Heckel *et al.* (2007) which the larvae fed on prior to dissection. The larvae collected in the present study were collected in maize fields which can attribute to the high number of morphological types.

Various studies show that the composition and populations of gut communities of insects can vary widely within insect species (Broderick *et al.* 2003; Heckel *et al.* 2007). Even though no assumptions were made about the possible diversity or quantity of different bacteria that would be uncovered from the mid-gut of *B. fusca* larvae, it was clear that the environment where they survive will add to the selection pressure and influence the type of bacteria present in the mid-gut. Anticipated factors that can play a role in the selection pressure include the optimal pH for digestive enzymes, anaerobic environment and chemical

composition of the insect gut, as well as the plant material on which this insect feeds. This may contain an array of allelochemicals, such as phenolics, tannins, and terpenoids, many of which can interact with intestinal bacteria or possibly be toxic to microorganisms (Broderick *et al.* 2003; Govenor *et al.* 1997; Heckel *et al.* 2007; Johnson and Felton, 1996). The pH of insect mid-guts are generally neutral to acidic ranging between 4 to 7, whereas lepidopteran mid-guts are typically pH 7 to 12 (Dow, 1992; Heckel *et al.* 2007; Jensen *et al.* 2003;). Bacteria that live in this pH are known as haloalkaliphilic bacteria (Padan *et al.* 2005). Most of the bacteria grew adequately during the isolation of the morphological types.

In a study by Anand *et al.* (2009) on the isolation and characterisation of bacteria from the gut of *Bombyx mori* that degrade several plant constituents, they isolated cultivable facultative anaerobic bacteria and obligate anaerobic bacteria. This distinction and cultivation methods were, however, not possible in the present study because of the growth curve analysis of the mid-gut bacteria that took place in an aerobic plate reader. By only using aerobic conditions to cultivate the isolated bacteria, there is a possibility that not all bacteria that were present in the mid-gut were culturable with the available methods (Anand *et al.* 2009). One key aspect in the culture methods performed was the choice of agar. The use of selective media specific for different types of bacteria imposes *a priori* bias on the types of bacteria that can be enumerated (Xiang *et al.* 2006). Nutrient agar is not a selective or differential agar and promotes the growth of most bacteria. The initial steps followed by Takatsuka and Kunimi (2000) are also similar to the present study. These include separating the colonies isolated on the basis of morphology and colour (Figure 4.4), Gram staining (Figure 4.5) and the shape of the cells (Figure 4.6 and Figure 4.7) (Takatsuka and Kunimi, 2000).

Although it is known that culturable and unculturable bacteria reside in the mid-gut of insects, in the present study only the culturable bacteria were used throughout experimental steps to ultimately rid the larvae of mid-gut microbes. It is well known that bacterial composition indicated by culture-independent methods exhibited greater divergence and diversity than bacteria recovered by culturing (Broderick *et al.* 2004). Although the morphological types isolated were used as individuals, it was not presumed that there were no similarities between the colonies. It was used as a means of differentiation in this study because no identification was performed. The morphological types were divided and isolated on the grounds of the surface appearance and shape of the colony, elevation of the colony, shape of the edges of the colony and colour of the colony (Figure 4.4).

After each morphological type was removed from the original nutrient agar plate derived from the dilution series, streak plates were performed to ensure pure colonies. These pure colonies were then Gram stained and observed under a microscope. Bacteria were classified as Gram positive when all the cells on the slide stained purple and the cell shapes were uniform, and classified as Gram negative when all the cells stained red and unified in shape. Presence of more than one colour cell or cell shape was classified as impure. The streak plate method was employed to purify the impure colonies until all the colonies were divided into groups according to their Gram stain and cell shape. This classification led to the conclusion that 53% of the total bacteria isolated from the mid-gut of *B. fusca* was Gram positive, and 47% was Gram negative (Figure 4.5). This majority of Gram positive bacteria is in agreement with a study done by Broderick *et al.* (2004) which involved the evaluation of the mid-gut bacteria of *L. dispar* larvae where 70% of the bacteria were found to be Gram positive. Raymond *et al.* (2008) identified bacteria which are c-proteobacteria or low G+C Gram positive bacteria and noted that these two bacterial divisions are typically found in gut microbial populations of lepidopteran hosts. Contradicting results were introduced by Anand *et al.* (2009). They noted that among the seven isolated bacterial colonies from the gut of *Bombyx mori*, one isolate belonged to Gram positive bacteria and the other six isolates were found to be Gram negative bacilli shaped bacteria (Anand *et al.* 2009).

The shapes of the cells isolated from the mid-guts of lepidopterans are, however, not as prominently mentioned in the literature. In Shannon *et al.* (2001)**,** no bacilli shaped bacteria were seen under the microscope in mid-gut sections of *Hofomannophila pseudospretella* (Lepidoptera: Oecophoridae). In the present study, bacilli shaped bacteria were the most abundant (Figure 4.6), and were classified as Gram negative and Gram positive. Overall, 55% of the cell shapes isolated and Gram stained were rod shaped cells. Of these, 39 isolates were Gram positive and 35 were Gram negative. The remaining 31 Gram positive bacteria were determined as 19 cocci shaped cells and 12 oval shaped cells (Figure 4.7). The remaining 29 Gram negative bacteria were determined as 9 cocci shaped cells and 20 oval shaped cells (Figure 4.7).

Although culturable methods were adequate for the purpose of this study, molecular techniques have identified much higher levels of bacterial diversity in lepidopteran species (Broderick *et al.* 2004; Raymond *et al.* 2008; Takatsuka and Kunimi, 2000). Broderick *et al.* (2004) evaluated the mid-gut bacteria of *L. dispar* larvae using a combination of 16S rRNA analysis of the cultured bacteria as well as culture-independent PCR amplification of 16S rRNA sequences. This evaluation led to the identification of 23 phylotypes. A phylotype is

described by Willey *et al.* (2014) as a taxon which is characterised only by its nucleic acid sequence generally discovered during metagenomic analysis.

5.3 Antibiotic testing

5.3.1 Antibiotic susceptability

As mentioned, in this study only culture dependent techniques were used. This was also used in numerous other studies when concerned with the microorganism diversity in the intestinal tract of lepidopteran larvae (Broderick *et al.* 2004; Shannon *et al.* 2001; Visotto *et al.* 2009). The exposure to antibiotics can have one of two effects: (i) Bactericidal effects cause cell death and no bacterial growth will be visible after exposure to antibiotics; (ii) Bacteriostatic effects cause the inhibition of cell growth and bacterial growth will be visible after the antibiotic exposure is removed (Kohanski *et al.* 2007; 2010). Figure 4.14 shows a good representation of the difference between bacteriostatic or bactericidal effects of antibiotics.

Determining MICs are considered the gold standard for determining the susceptibility of organisms to antibiotics or to confirm unusual resistance (Andrews, 2001). In the present study determining the MIC of the bacteria isolated from the mid-gut of *B. fusca* was not the goal. Rather, the antibiotics and antibiotic concentrations which had the most prominent efficacy on the bacteria it was exposed to, were under investigation. These findings were later used to rid the larvae of mid-gut bacteria. Even though other microorganisms such as fungi are part of the microbial load of an insect gut, bacteria are the most abundant (Chellaram *et al.* 2012). The presence of fungi was not tested in the present study, nor was there any treatment with a fungicide.

Antibiotic screening can be done in numerous ways. In the present study, the Kirby-Bauer method was used and modified to suit the incubation time. This was increased to 48 hours instead of the standard 24 hours (Figure 4.8). Results of the antibiotics used in the antibiotic screening test can be seen in Figure 4.10. The efficacy of antibiotics in achieving gut sterilisation required preliminary selection of suitable antibiotics for the purpose (Visotto *et al.* 2009). Eight antibiotic discs were chosen representing all the different functional/structural classes of antibiotics, except the class of metabolic antagonists. These include inhibition of DNA synthesis, cell wall synthesis or protein synthesis (Table 2 and Figure 4.10) (Gwynn *et al.* 2010; Kohanski *et al.* 2010; Willey *et al.* 2014). Ciprofloxacin had the highest inhibiting

effect overall against all the isolates. This bactericidal nucleic acid synthesis inhibitor acts by blocking DNA replication and transcription (Willey *et al.* 2014). It was therefore not surprising that it caused the highest overall inhibition zone diameter of all the colonies isolated from the mid-gut of *B. fusca* when exposed to this antibiotic disc. In a study by Allen *et al.* (2009) on the MICs for 44 bacterial isolates from the mid-guts of laboratory reared and wild-caught *Lymantria dispar* larvae they tested 12 antibiotics. Ciprofloxacin was the only antibiotic to which all the isolates were susceptible. In the present study ciprofloxacin also had the highest averaged percentage of isolates killed during the antibiotic disc test (Figure 4.11).

The second class of antibiotics investigated was cell wall synthesis inhibitors (Willey *et al.* 2014). The bactericidal antibiotics tested in this class were ampicillin and ertapenem. Ertapemen is an antibiotic that performed well in both inhibition and killing ability, but the antibiotic was too coslty to continue with for the duration of the experiment. Ampicillin gave average results in both its inhibiting and killing effect (Figure 4.10 and Figure 4.11). In a study by Visotto *et al.* (2009), the bacterial response of the gut bacteria of *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) larvae to being exposed to ampicillin was considered susceptible and for this reason was selected for further use. It was neccesary to include an antibiotic from this class into the group of antibiotics that were used later during the growth curve analysis and feeding study, and for that reason ampicillin was chosen.

The third class of antibiotics, protein synthesis inhibitors, included the bactericidal antibiotics kanamycin and streptomycin which did well in the inhibition analysis as well as killing ability, but proved ineffective in ridding *B. fusca* larvae of mid-gut microbes in an earlier attempt (data not shown). For this reason, we chose to proceed with bacteriostatic doxycycline which produced the third highest total inhibition zone diameter (Figure 4.10). Doxycycline, which is part of the group of tetracycline antibiotics were aslo tested in the Visotto *et al.* (2009) study and exhibited the strongest levels of inhibition of the mid-gut bacteria of *Anticarsia gemmatalis* larvae with clear and broad halos of inhibition.

Principal component analysis (PCA) is a widely used mathematical tool for high dimension data analysis to reveal relationships among data items (Jeong *et al.* 2009). Principal component analysis reduces dimensionality of a data set without losing variation. The variance is illustrated by principal components (PCs) or axes, where the first two components indicate the highest variance (Yeung and Ruzzo, 2000). In the PCA, individual isolated bacteria (samples) are indicated by points whereas the antibiotics tested are represented by vectors (arrows). Dissimilarities are associated with the distance between the samples. The closer the samples are to each other or to a vector, the higher the

similarities between them. The linear correlation coefficients among the antibiotics can be indicated by the angles between the antibiotic vectors. Antibiotic vectors pointing in the same direction have a large positive correlation, whereas vectors pointing in opposite directions indicate large negative correlations. If an antibiotic vector points in the same direction as a sample, the antibiotic is positively correlated to the bacteria.

The PCA illustrated in Figure 4.9 was constructed using the inhibition zone diameter of the antibiotic discs on the bacterial isolates. Based on the results obtained from the PCA, it is evident that there is clustering of the bacterial isolates in reaction to the antibiotics. The distance between the vectors and the Gram negative isolates in the lower left quadrant means that the antibiotics had less of an effect on these isolates than the Gram positive isolates located among the vectors. This result was expected because Gram negative bacteria are typically more resistant to antibiotics than Gram positive bacteria. In a study by Ibrahim *et al.* (2010) on the antibiotic resistance in Gram negative pathogenic bacteria in hospital drains, they also performed the standard disc diffusion tests using Mueller- Hinton medium. The bacterial isolates were tested for their susceptibility to seven antibiotics belonging to different groups, namely: ampicillin, cephalothin, clindamycin, gentamicin, cefoxitin, penicillin G and sulphamethoxazole (Idrahim *et al.* 2010). The results generated from this study suggested that increasing levels of resistance to antimicrobial agents, particularly in Gram negative rods resistant to β-lactam antimicrobial drugs, have become evident. Idrahim *et al.* (2010) also noted that Gram negative bacteria quickly acquired resistance to cephamycins and broad spectrum cephalosporins. Ash *et al.* (2002) examined the antibiotic resistance of Gram negative bacteria in rivers in the United States. This study also used the disc diffusion approach and exposed the isolated bacteria to antibiotic discs of cefotaxime, ceftazidime, amoxicillin plus clavulanic acid, cephalothin, imipenem, kanamycin, streptomycin, chloramphenicol, tetracycline, and ciprofloxacin. The use of selective media (LB agar sublimated with 150 µg/ml ampicillin) resulted in the isolation of Gram negative organisms with high levels of resistance (Ash *et al.* 2002). The high levels of resistance in Gram negative isolates in the studies by Idrahim *et al.* (2010) and Ash *et al.* (2002) are similar to the results generated by the present study. Without the investigation into specific resistance mechanisms of the Gram negative isolates in the present study, the resistance of the Gram negative isolates can be attributed to the presence of the outer membrane permeability barrier in Gram negative bacteria which limits access of the antibiotics to their targets in the bacterial cell (Poole, 2001; Vaara, 1992).

To summarise, the antibiotics that were subjected to additional study included ciprofloxacin, ampicillin and doxycycline. The concentration-dependent inhibitory effect of these antibiotics

on the culturable bacteria isolated from the mid-gut of *B. fusca* was assessed during the growth curve analysis.

5.3.2 Growth curve analysis

One of the objectives of the present study was to evaluate the interference in the growth curves of the mid-gut bacteria isolated from *B. fusca* triggered by different concentrations of a combination of antibiotics (ciprofloxacin, ampicillin and doxycycline). The antibiotic concentrations tested ranged from 25 µg/ml to 700 µg/ml for each of the three antibiotics, and a control was also included in every reading. Antibiotics can be expected to cause stress to the bacteria even at sub-MIC concentrations (Drummond *et al.* 2003). A common effect of antibiotics on the bacterial strains that is visible in Figure 4.12, is the lower growth rate over time in comparison with the control. Neither the control nor the concentrations tested exhibited a typical growth curve with the theoretical four phases (lag, exponential/log, stationary phase and the senescence/death phase). However, there is a clear difference between the optical density (OD) reading of the control group and the groups that were exposed to the antibiotic concentration. In addition to slower growth, the bacteria exposed to antibiotics failed to achieve the OD that the controls reached, even at the lowest concentration of the antibiotics (100 µg/ml). Both of these observations were also seen in a study by Drummond *et al.* (2003). Theoretically, sub-inhibitory concentrations of antibiotics tend to delay the growth of the bacteria by increasing the lag phase (Drummond *et al.* 2003). This is visible when comparing the lag phase produced by the control group (blue line) and the lag phase produced by the bacteria exposed to the 200 µg/ml (purple line). The lag phase is less clear at the 100 µg/ml (red line) antibiotic concentration, but it is indicated by a small upward slope coming out of the concave line. The antibiotic concentration of 500 µg/ml did not produce any increase in lag time.

A typical use for error bars in a line graph such as Figure 4.12, is to show variability in the measures which are plotted in the graph (Anon, 2004a). In Figure 4.12, it is clear that the error bars (which graphically shows the potential error amounts relative to each line) of 100 and 500 µg/ml overlap for the majority of the time. This potentially means that there is a much lower likelihood that the impact of these two antibiotic concentrations differ significantly in terms of the growth curves generated. When observing the error bars for each line individually, it is expected that the error bars of the control group would be wider because only water was added to the wells which had no inhibitory or killing effect and allowed the bacteria to grow unprohibited. The error bars for the individual antibiotic concentrations on

the other hand are smaller than that of the control group and mostly uniform in size which shows the low potential for error or degree of uncertainty relative to each data marker in a series.

The effects of sub-MIC levels of antibiotics on bacterial growth can vary with both antibiotic and bacterial strain (Drummond *et al.* 2003). This observation can be seen in the results of the present study. There is a difference in the way that the individual bacteria react to the antibiotics, which is why we decided to test the effectiveness of the antibiotics in this way (growth curves). Even though a spectrometry reading was taken every hour, the slope of the curves generated is so different from the theoretical growth curve that the four different phases (exponential phase, stationary phase or death phase) cannot be linked to a specific time during the 48 hour reading. This was in contrast to the findings of Drummond *et al.* (2003) who described the observation that each strain was clearly in the exponential phase by 8 h and in the stationary phase by 24 h. A death phase was then apparent from 32 h, with the OD stabilising by approximately 56 h. In a study done by Castillo *et al.* (2006), it was illustrated that the antibacterial activity of the tested antibiotics (penicillin, amoxicillin, cefuroxime, erythromycin, spiramycin, roxithromycin and clindamycin) against oral streptococci is maintained from 4–6 h, not only with sub-MIC concentrations but even with concentrations two, three or more times below the MIC value. They noted that these results point to the *in vitro* efficacy of the tested antibiotics at sub inhibitory concentrations (Castillo *et al.* 2006).

In order to use a growth curve to demonstrate the influence of antibiotics on bacteria and act as an adequate model, it is necessary to reduce measured data to a limited number of parameters (Zwietering *et al.* 1990). In the present study, the number of influential environmental conditions were limited by keeping all the 96-microwell plates under the same conditions. All samples were in the plate reader for 48 hours at a temperature of 28°C. This temperature was chosen to prolong the effects of the antibiotics which tend to denature at high temperatures.

The antibiotic concentration of 200 µg/ml modified the growth curve the most with respect to the control. However, we were interested in finding the antibiotic concentration which had the highest efficacy (killing effect) on the bacteria exposed to it. In Figure 4.13, it is clear that the combined antibiotics (ciprofloxacin, ampicillin and doxycycline) concentration of 500 µg/ml had the greatest bactericidal effect amongst the three antibiotic concentrations tested. Although the inhibiting ability of the other two concentrations was greater than that of 500

µg/ml, this concentration was chosen to use in the feeding study because of the higher overall efficacy.

Whenever attempting a study involving the inhibition or efficacy of antibiotics, one must always consider the possibility of resistance. The selection pressures in the mid-gut of lepidopteran larvae have been discussed in terms of the type of bacteria that can survive in such an environment. Environmental and intestinal microbes are exposed to a chemically diverse environment which may create opportunities for mutations which are necessary for survival, and in this case, the ability to survive the exposure to antibiotics (Wright, 2010). Gut bacterial consortia adapt by the transfer of plasmids and transconjugation between bacterial strains, and some insect species provide ideal conditions for bacterial conjugation. Results like these suggest that the gut is a "hot spot" for gene transfer (Dillon and Dillon, 2004). This can account for the shared resistance to antibiotics between the morphological types. These selection pressures and genetic transfer can also contribute to adaptations in the bacteria that confer specific or nonspecific antibiotic resistance (Allen *et al.* 2009; 2010). Insects, which are highly successful invasive organisms, carry along their resident microbiota when entering a new environment. This invasion can act as a possible and convenient distribution mechanism for antibiotic-resistant bacteria (Allen *et al.* 2009; Vasanthakumar *et al.* 2008). Even though the invasion of insects can act as a means of distribution for antibiotic resistant bacteria, the origin of the resistance is still unknown. It is unlikely that these bacteria have been subjected to human sources of antibiotics (Wright, 2010). In a study by Allen *et al.* (2009), they used *Lymantria dispar* as an example of an invasive species that exhibit such selective pressures. The microbial community of *L. dispar* mid-gut is dominated by *Enterococcus* spp. and members of the Enterobacteriaceae (Broderick *et al.* 2004). Clinical isolates of these taxa showed that some members of these groups are efficient in gene exchange in gut environments and have developed antibiotic resistance (Salyers *et al.* 2004). A possible reason for this can be attributed to the wide range of host plant species, exposing its mid-gut microbial community to diverse plant compounds, some of which may resemble antibiotics (Allen *et al.* 2009). Although *B. fusca* is exposed to GM maize, it is unlikely that this exposure led to the development of antibiotic resistance in the mid-gut bacteria. In a study noted by Demaneche *et al.* (2008), they investigated whether antibiotic resistance genes in transgenic plants were at risk of increasing the resistance gene burden of soil microbes. Demaneche *et al.* (2008) surveyed cultivatable β-lactamase resistant organisms from soils growing transgenic Bt176 maize (for 10 consecutive years) and a non-agricultural control field. Their results showed that β-lactamase producing bacteria were prevalent in soil regardless of whether the soil was not used for agriculture or GM maize was grown there. When taking this study into account it is safe to assume that the cause of the

high antibiotic resistance found in the present study cannot necessarily be attributed to being exposure to the Bt maize.

The process by which bacteria acquire genes from the environment, namely horizontal gene transfer, is responsible for the presence of antibiotic resistance elements in bacteria (Allen *et al.* 2010; Wright, 2010). Many of the known antibiotic resistance genes are found on transposons, integrons or plasmids, which can be mobilised and transferred to other bacteria of the same or different species (Allen *et al.* 2010). Horizontal gene transfer can be responsible for the multi-drug resistance in the mid-gut bacteria of *B. fusca* because of the closed and constant environment they share. The study by Allen *et al.* (2009) demonstrated that the microbial community of *L. dispar* harbours antibiotic-resistant bacteria and antibiotic resistant genes. Allen *et al.* (2009) noted that the 44 bacterial isolates from the mid-gut of laboratory reared and wild-caught *L. dispar* larvae was a source of new genes encoding antibiotic tolerance. Resistance of these isolates were detected to 11 of the 12 antibiotics (carbenicillin, erythromycin, rifampin, ceftazidime, gentamicin, kanamycin, streptomycin, vancomycin, chloramphenicol, nalidixic acid, tetracycline and ciprofloxacin which showed no resistance) tested. They also found that antibiotic resistance profiles were similar among bacteria isolated from laboratory reared and wild-caught *L. dispar* larvae mid-guts (Allen *et al.* 2009). The feeding study performed during the present investigation relies on this observation because mid-gut bacteria from wild caught larvae were used to determine the antimicrobial susceptibility and growth curve. The feeding study was done on laboratory reared larvae. Despite the importance of antibiotic-resistant bacteria in modern medicine or as environmental genetic polluters, only a few insect species have been screened for them (Allen *et al.* 2009).

Although the 12 antibiotics tested in the study of Allen *et al.* (2009) represent a broad range of drug classes and modes of action, they reported that resistance determinants specific for other antibiotics may be present in the microbial community of *L. dispar* (Allen *et al.* 2009). In the present study antibiotics were chosen from different classes and modes of action. Although the mechanisms of antibiotic resistance were not investigated in the present study, the Allen *et al.* (2009) study revealed that the mid-gut community of *L. dispar* larvae possessed a transcriptional regulator that confers tolerance to numerous antibiotics. Furthermore, a resistance gene called ramA which confers multidrug resistance was identified in *E. coli* isolated from the mid-gut of *L. dispar*. The identification of this gene illustrates the diversity of gene families that contribute to this antibiotic resistome, but likely still underestimates the extent of *L. dispar* mid-gut microbial community resistome (Allen *et al.* 2009).

The MIC values were measured at two-fold increases in antibiotic concentration by Allen *et al.* (2009) and they found that apparent differences appear more significant at higher concentrations. Figure 4.14 and Figure 4.15 depicts the lower bacterial growth rates with an increase of antibiotic concentration. This however was not the case when the antibiotic concentration increased to 700 µg/ml. Figure 4.16 is an example of bacteria that potentially possess an efflux pump as a mechanism of resistance. At the lower antibiotic concentrations (500 and 600 µg/ml) there was a visual bacteristatic and bactericidal effect, but at the 700 µg/ml antibiotic concentration no inhibiting or killing effect is visible. The growth of the bacteria that were exposed to 700 µg/ml, for example colony number 26, looks exponential when compared to the growth exposed to a lower antibiotic concentration (colony 26 at 500µg/ml). A reason for this can be that certain classes of efflux pumps have the ability to pump various toxins, such as heavy metals and other toxic molecules, out of cells before any deleterious effects can take place (Allen *et al.* 2010). Allen *et al.* (2010) found that the microbial communities of insect guts that had no known exposure to antibiotics contain efflux pumps that confer resistance to antibiotics. The resistome of *L. dispar* mid-gut microbial community contains genes that encode efflux pump machinery which tend to have broad substrate specificity (Allen *et al.* 2009). This resistance mechanism may explain the growth pattern when isolates were exposed to a concentration of 700 µg/ml antibiotics. This mechanism entails active drug excretion functions to limit drug accumulation, thereby protecting bacteria from the deleterious effects of these agents (Poole, 2001). The morphological types of colonies 25-32 (with the exception of 26) were very similar on the original isolation plate, which could point to the same or very similar identity of these morphological types. If these identities are similar, it is safe to assume that these isolates all possess efflux pumps, which also corroborates the appearance of the isolates after being exposed to the antibiotic concentration of 700 µg/ml.

In the present study, a morphological type was labelled as resistant if it survived 48 hour exposure to a particular antibiotic concentration. Reasons for survival, other than the presence of efflux pumps, may also include the presence of impermeable barriers which make them intrinsically resistant to certain antibiotics. It is also possible that some bacteria lack the target of the antibiotic which restricts drug entry into the cell (Allen *at al.* 2010; Poole, 2001). The work of Allen *et al.* (2009) demonstrates that antibiotic-resistant bacteria and antibiotic resistance genes occur in *L. dispar* mid-gut bacteria, in particular β-lactamase (Allen *et al.* 2009). Extended-spectrum β-lactamases are increasingly common in the Enterobacteriaceae and hydrolyse both penicillins and cephalosporins. Although specific resistance mechanisms were not investigated in this study, the literature provides a large

number of identified mechanisms and examples of mid-gut bacteria of insects possessing the ability to overcome the deleterious effects of antibiotics.

5.4 Sterilisation of maize stems

The sterilisation of the maize stems was performed to ensure that the bacteria that occur naturally in the environment and on the maize stems do not contaminate the larvae that has been rid of mid-gut microbes with the use of antibiotics. Distilled water and several concentrations of JIK® (1%, 2%, 3% and 4%) were tested to find the concentration which showed the best antibacterial results (all data not included). JIK® is the most frequent choice for surface sterilisation (Oyebanji *et al.* 2009). The results in the present study suggested that rinsing the maize plant material in distilled water alone did not produce the desired results (Figure 4.17). Even though no investigation was done on the microbial growth depicted in Figure 4.17, there is a clear difference between the amount of microbial growth present on the Bt maize plant material and the non-Bt maize plant material, and the reason for this is unknown. In Figure 4.18, the decrease in microbial growth after submersion in 4% JIK® is clearly visible. The mechanism of JIK®, sodium hypochlorite, is as follows. The hypochlorite salts, which include sodium hypochlorite (NaOCl) and calcium hypochlorite ($Ca(OCl)_2$), lead to the formation of hypochlorous acid (HOCl) when diluted in water. The concentration of this HOCl is correlated with bactericidal activity (Nakagarwara *et al.* 1998). It was decided to extend the submersion time to 5 minutes and the results showed no detectible microbial growth (data not included). We were confident in the antibacterial properties of JIK® and used a 4% solution to sterilise Bt and non-Bt maize material during treatments in the feeding study.

5.5 Feeding study

The overall aim of the present study was to determine whether the intestinal bacteria of *B. fusca* and *C. partellus* larvae play any role in the larvae's susceptibility to Bt maize. To do this, larval mortality was compared after being exposed to Bt maize in the presence and absence of the larvae's indigenous gut bacteria. Various studies have shown that the susceptibility of Bt maize can be influenced by factors such as insect host, *B. thuringiensis* strain and environmental conditions (Broderick *et al.* 2003; Mostafa *et al.* 2005; Rahman *et al.* 2007; Suzuki *et al.* 2004). In the present study an effort was made to minimize influential factors. These included using two species of lepidopteran larvae. *Busseola fusca* larvae was used as the Bt maize resistant representative and *C. partellus* as the Bt susceptible

representative (van Rensburg, 2007). To lessen the possible influence that different *B. thuringiensis* strains could have, the same cultivar of Bt maize (MON810) was used for all larval groups. All the plants were the same age and the presence of Cry 1Ab proteins were confirmed by using a QuickStix^{TM} strips kit for Cry 1Ab maize leaf and seed (Enviologix, Maine, USA). Environmental factors were kept the same for all the larval groups. For the duration of the feeding study larvae were reared in sterilised falcon tubes, placed in a laminar flow cabinet located in a laboratory at ambient temperatures.

Only culture-dependent methods were used in this study to test the effectiveness of the antibiotics even though it was assumed that the unculturable bacteria present in the mid-gut of the larvae were also exposed to the antibiotics (Broderick *et al.* 2004). The only functional mechanism of Bt investigated in our study was the gut microbiota theory which involves Cry toxins causing extensive cell lysis in the mid-gut and allowing the mid-gut bacteria access to the hemolymph where they cause septicaemia (Raymond *et al.* 2009; Schnepf *et al.* 1998). Although the *in vitro* effectiveness of the antibiotics was not tested in the present study, antibiotics were screened using a rigorous approach. Visotto *et al.* (2009) performed a concentration–response inhibition of viable bacteria from the insect gut which was determined by plating gut bacteria from 5th instar *Anticarsia gemmatalis* larvae reared on a diet containing increased concentrations of either chloramphenicol or tetracycline. Both of these antibiotics had inhibiting effects with strong suppression of bacteria (Visotto *et al.* 2009). Visotto *et al.* (2009) also noted that, even though procedures such as the application of antibiotics are commonly used to achieve the objective of sterilising the gut of insects, the use of antibiotics can cause potentially toxic effects to the host and serve as selective pressure for the gut bacteria. To overcome some of these toxic effects, in the present study only antibiotics that were water-soluble instead of ethanol or HCl soluble were used. The antibiotics selected for incorporation into the diet in equal volumes for the feeding study included ciprofloxacin, ampicillin and doxycycline, each at a concentration of 500 µg/ml each.

Denaturing gradient gel electrophoresis (DGGE) of 16S rDNA fragments amplified by PCR in a study done by Xiang *et al.* (2006) showed that microbial compositions were different between field and laboratory populations. The simplicity of the gut bacteria community of laboratory reared insects may reflect the narrow range of food availability (Xiang *et al.* 2006)**.** In another study, the bacterial community of wild *L. dispar* larval mid-guts were found to be similar to that of laboratory reared larvae (Allen *et al.* 2009). In the present study field collected larvae (5th instar) were used to determine the morphological diversity and the antibacterial susceptibility of the mid-gut microbes. However, laboratory reared larvae (1st to

3rd instar) were used to perform the feeding study. This factor can therefore influence the effect of the antibiotics on the bacteria in the laboratory reared larvae. Xiang *et al.* (2006), however, found little variation among intestinal bacteria of different developmental stages of a laboratory population of *Helicoverpa armigera*. Laboratory reared larvae were used in the present study because it was the only available larvae at the appropriate instar.

Studies show that the potency of Cry 1 decreases with an increase in larval age (George *et al.* 2011; Rausel *et al.* 2000; van Rensburg, 2001). George *et al.* (2011) performed trials to confirm that younger *B. fusca* larvae (2nd instar) are marginally more susceptible to Cry 1Ab (MON810) compared to older larvae. Larvae were used from 1st instar for the feeding study. George *et al.* (2011) observed that the time required for larvae to develop to pupation when reared on Bt maize compared with the time required for larvae maintained on non-Bt maize was significantly longer. There was also a significant reduction in larval mass over the 5 day period that larvae fed on Bt maize (George *et al.* 2011). This effect was also observed in the present study when the size of the larvae reared on the Bt treatments was compared to the size of the larvae reared on non-Bt maize (Figure 4.23). In the present study, neither the stability of Cry 1Ab within the insect mid-gut nor the potential for protease inhibitors to enhance the toxicity of Cry toxin was investigated. Protease inhibitors have been shown to increase the efficacy of Cry toxins, providing further evidence for decreased sensitivity with increasing larval age (George *et al.* 2011). Gilliland *et al.* (2002) also suggested that the variation in potency of Cry toxins for different larval stages.

The parts of the plant that were given to the larvae in the treatments were kept consistent as far as possible. In each falcon tube the larvae were provided with a whorl (leaves) and a thin stem of the same maize plant. All the plants were of the same age and exposed to the same environmental conditions in plant growth tunnels. This was done to minimize the degradation of the protein inside the plant material. Studies have shown that in GM maize a strong decline in Bt toxin occurs with increased plant age, and that different parts of the plant contain different concentrations of the toxin (George *et al.* 2011). Although neither larval weight or pupation time was measured, the larvae remained on the treatments and were observed for 21 days. Another observation that the present study shared with George *et al.* (2011) was the difference in the quantity of Bt maize leaves consumed compared to the non-Bt maize leaves after 7 days. George *et al.* (2011) measured the difference and found that larvae fed non-Bt plants, consumed an average area of 3.3 cm^2/larva/day, whereas those fed Bt maize, consumed an average area of 0.5 cm^2/larva/day. In the present study it was observed that less Bt plant tissue was consumed compared to non-Bt plant tissue.

To illustrate the percentage survival of larvae on the different feeding treatments a line graph over time was used. For each larval group (BF 1, BF 2, CP 1 and CP 2) an individual line graph was constructed starting from day 8 to day 21. This form of illustration was used because it facilitates a visual comparison between the influences of feeding treatments on larvae. After observing the results generated by all the larval survival line graphs, significant differences in the treatments of Bt, NBt, 500 Bt and 500 NBt was investigated by performing a univariate test of the significance. This analysis displayed a p-value < 0.0001 for 3 of the 4 larval groups. The p-value showed that there was significant variation between the treatments. To determine what this variation was, a Post-hoc test in the form of a Tukey's HSD test was conducted. The error bars added to each bar of the bar graph is to show variability in the measures which are plotted in the graph. This is an indication of the potential error amounts relative to each bar.

5.5.1 *Busseola fusca*

Figure 4.19 illustrates the percentage survival of larval group BF 1 (from day 8 to day 21) after being placed on 6 different feeding treatments on day 8. In any experiment, a survival of larvae on a control treatment (in this case non-Bt maize) between 40% and 60% is similar to that observed in other laboratory and semi-field conditions and was therefore considered acceptable (as seen in van Rensburg, 2001; Kruger *et al.* 2011). The survival of BF 1 on non-Bt maize was 44% which means that the larvae survived well enough on the control treatment that the other observations in the experiment can be seen as satisfactory. All the larval groups (BF 1, BF 2, CP 1 and CP 2) survived between these margins (or above them) on the non-Bt treatments. A decline in surviving BF 2 larvae was visible in all the treatments. The focus was to compare the larval survival of the Bt treatment with the larval survival of the 500 Bt treatment (larvae that fed on antibiotic diet with an antibiotic concentration of 500 µg/ml before feeding on Bt) treatment to determine if the absence of mid-gut microbes had an influence on larval survival. Figure 4.19 showed a 19% difference in survival between these two treatments. The larvae that fed on the antibiotic diet (500 µg/ml of ciprofloxacin, ampicillin and doxycycline) prior to feeding on Bt had the highest survival rate. It was crucial, however, to not make assumptions about the contributions of the mid-gut bacteria on the survival of larvae without statistical evidence. A Tukey's HSD test was performed to investigate if significant differences ($p<0.0001$) between larval survival on the different treatments occurred.

This analysis showed that there was a significant difference between the larval survival on the 500 Bt treatment and the Bt treatment (Figure 4.20). The percentage of surviving larvae on the 500 Bt treatment (a; 86%) was significantly higher than those on the Bt treatment (b; 6.25%). The 500 Bt treatment did not differ significantly from the NBt or 500 NBt treatments.
In Figure 4.21, the overall survival of the larvae was less than the survival of larvae illustrated in Figure 4.19. The percentage larval survival depicted by the NBt treatment in Figure 4.21 was much higher than the rest of the treatments. Figure 4.22 shows that there was no significant difference ($p<0.255013$) in the larval survival between the four treatments. However, in Figure 4.22, the mean percentage larval survival for the Bt treatment (a; 59.25%) was lower than the mean larval survival of the 500 Bt treatment (a; 68.75%), even though they did not differ significantly. Resistance development of *B. fusca* has been observed and documented for the past several years, so the survival of this larval group (BF 2) on Bt maize can be attributed to resistance mechanisms (van Rensburg, 2007).

5.5.2 *Chilo partellus*

The larvae depicted in Figure 4.24 survived on the Bt treatment between day 8-14 after which an expected decrease in larval survival occurred. The very steep decline in larval survival on the Bt treatment can be attributed to the fact that *C. partellus* is susceptible to the deleterious effects of Bt maize (Tende *et al.* 2010). Even though the larvae survived on Bt after they fed on the sterile diet (SD Bt), the difference in larval survival when comparing the Bt treatment and the 500 Bt treatment was still visible. When comparing larval survival on the Bt treatment with larval survival of the 500 Bt treatment, survival on the 500 Bt treatment was 10% higher.

The 500 Bt treatment (b; 87.50%) and the NBt treatment (b; 90%) depicted in Figure 4.25 showed no significant difference ($p<0.000014$) but these two treatments differed significantly from the Bt treatment (a; 0%) and 500 NBt treatment (a; 12.50%). As in Figure 4.19, the 500 Bt treatment showed similarity and significance with the NBt treatment with the mean percentage survival of larvae. This means that the larvae treated with Bt maize in the 500 Bt treatment responded a lot like the larvae that that were exposed to NBt maize.
The CP 2 larval group exhibited the larval survival that was expected when testing the efficacy of Bt maize on *C. partellus* (Tende *et al.* 2010). Larval survival was high on the NBt treatments, and all the larvae were killed in the Bt treatment. The susceptibly of *C. partellus* to Bt maize was very clear in larval group CP 2. No larva survived past the 7th day on the Bt treatment. This created a very good opportunity to observe if the absence of mid-gut

microbes contributed to the survival of these larvae on Bt maize (Figure 4.26). Even though the percentage of surviving larvae on the 500 Bt treatment was low, it dominated the complete mortality caused by the Bt treatment. Further evaluation of the influence of different feeding treatments on larval survival (Figure 4.27) showed significant differences ($p<0.000409$). The mean percentage survival of the larvae on the 500 Bt treatment (b; 100%) resembled the results obtained from the NBt treatment (d; 82.50%), when these were compared with the 500 NBt treatment (a; 25%) and the Bt treatment (a; 0%).

5.5.3 Summary of feeding study

All larval groups, with the exception of BF 2, showed significantly higher larval survival on the 500 Bt treatment than on the Bt treatment. Even with the observation of possible resistance, the larvae that were rid of mid-gut microbes survived better on Bt maize when compared to the survival of larvae that fed solely on Bt maize. This result was also observed in a study done by Broderick *et al.* (2006).

To determine whether a possible relationship existed between changes in the gut bacterial community of *L. dispar* and its susceptibility to *B. thuringiensis* toxin, Broderick *et al.* (2006) explored the impact of antibiotics on its survival with regards to *B. thuringiensis*. This was done by rearing larvae on either sterile artificial diet or diet amended with an antibiotic mixture that consisted of penicillin, gentamicin, rifampicin, and streptomycin at a range of concentrations (8–500 µg/ml of diet). Upon molting to the third instar, larvae exposed to the antibiotics were fed with 0, 1, or 10 units of *B. thuringiensis*, and mortality monitored for 7 days (Broderick *et al.* 2006). Broderick *et al.* (2006) found that the mortality of insects fed *B. thuringiensis* was inversely proportional to the antibiotic concentration. A reduction in mortality was accompanied by reduced populations of culturable *Enterococcus* and *Enterobacter* spp. from the mid-guts of larvae. This result suggested that the microbial community in the mid-gut contributed to larval death due to *B. thuringiensis* treatment (Broderick *et al.* 2006). After the reestablishing an *Enterobacter* sp. (a member of the normal mid-gut community) into the gut of *L. dispar*, insecticidal activity of *B. thuringiensis* was restored. These results, coupled with the observation that *B. thuringiensis* did not grow as rapidly as *Enterobacter* sp. and *E. coli* in larval hemolymph indicate that the enteric bacteria, which gained access though the permeabilised epithelium, are responsible for septicemia associated with *B. thuringiensis* toxicity (Broderick *et al.* 2006).

Raymond *et al.* (2009) aimed to test the applicability of the claim that a culturable gut microbiota is required for the pathogenicity of *B. thuringiensis* and its Cry toxins in an alternative host. Raymond *et al.* (2009) had strong opinions about the short comings of the studies performed by Broderick *et al.* (2006) and Broderick *et al.* (2009) in these studies. Raymond *et al.* (2009) highlighted two shortcomings of the study performed by Broderick *et al.* (2006). First, the authors administered high doses of four broad-spectrum antibiotics to hosts in order to eliminate their gut microbiota, but they did not directly control for the effects of antibiotics on *B. thuringiensis* itself. In the present study, this criticism was taken into account and three antibiotics from different classes were tested and used instead of four broad-spectrum antibiotics. An attempt was made to minimise the carry over effects of antibiotics on Bt protein itself by starving the larvae for between 3-5 hours in empty sterile petri dishes before being exposed to Bt maize to reduce food content in the mid-gut and the presence of antibiotic diet (Robinson *et al.* 2010). The antibiotics that were incorporated into the diet were also not viable for the seven days of feeding since it denatures in light exposure and high temperatures.

The second criticising observation Raymond *et al.* 2009 made was that Broderick *et al.* (2006) and Broderick *et al.* (2009) worked exclusively with a strain of Bt (*B. thuringiensis* ssp. *kurstaki* HD-1) that was isolated from a commercial biopesticide (DiPel DF) which grows poorly in some hosts (Raymond *et al.* 2008). In the present study, Bt maize (MON810) plants of the same age was used which implies that the expression level of the toxin was largely similar between plants. This maize cultivar, expressing Cry 1Ab, was specifically developed for insect resistance to lepidopteran pest such as *B. fusca* (George *et al.* 2011). In the study performed by Raymond *et al.* (2009), *Plutella xylostella* was chosen to produce aseptic larvae without directly administering antibiotics. They tested two hypotheses: (i) that strain attenuation in biopesticides leads to a dependence on culturable gut microbiota to pathogenicity and (ii) that the carry-over of ingested antibiotics in host larvae can reduce the pathogenicity of *B. thuringiensis* (Raymond *et al.* 2009). Raymond *et al.* (2009) showed that both Bt toxin and a mixture of spore/toxin are pathogenic in the absence of mid-gut bacteria when carry-over effects of antibiotic are excluded by using aseptically reared hosts. However, for one strain of Bt (HD-1) the presence of culturable gut microbiota led to a small but significant reduction in host mortality. This observation implies that the gut microbiota confers some protection against pathogen attack, as found in other vertebrate and invertebrate hosts (Raymond *et al.* 2009).

Larval mortality after being exposed to *B. thuringiensis* in the presence and absence of the larvae's indigenous gut bacteria was done by Broderick *et al.* (2009) on a range of

lepidopteran species. While the present study made use of Bt maize material to administer the Cry 1Ab protein treatments, Broderick *et al.* (2009) used cell-free formulation of *B. thuringiensis,* DiPel cell-based formulation and a MVPII formulation consisting of Cry 1Ac protoxin encapsulated in NaCl-killed *Pseudomonas fluorescens* cells. Broderick *et al.* (2009) found that the gut bacteria of five Lepidoptera species were reduced below detectable levels after they had been reared on antibiotics. The antibiotics reduced bacterial levels to below levels at which they were previously detectable. Their results suggested that the administration of *B. thuringiensis* without antibiotics was lethal to all six lepidopteran species. The results demonstrate that antibiotics significantly reduced mortality in five of the lepidopteran species. These five species included *Manduca sexta* (Lepidoptera: Sphingidae)*, Vanessa cardui* (Lepidoptera: Nymphalidae)*, Pieris rapae* (Lepidoptera: Pieridae)*, Lymantria dispar* and *Heliothis virescens*. Such results indicate that: (i) although there were differences between species susceptibility, oral administration of antibiotics reduced populations of gut bacteria and (ii) the enteric bacteria have important roles in *B. thuringiensis*-induced killing of Lepidoptera across a range of taxa.

From a pest management perspective, the ability of mid-gut bacteria to influence the survival of lepidopterans such as *M. sexta, V. cardui, P. rapae, L. dispar* , *H. virescens*, as well as *B. fusca* and *C. partellus* may provide opportunities for increasing susceptibility or preventing resistance (Broderick *et al.* 2009). These associations between the efficacy of the *B. thuringiensis* toxin and the gut microbiota of Lepidoptera may provide a useful model with which to further study the mechanisms of Bt maize (Broderick *et al.* 2009).

Chapter 6: Conclusions and recommendations

6.1 General conclusions

In the present study the influence of gut microbes on the efficacy of Bt maize against lepidopteran stem borers was investigated. This investigation started with the collection of *B. fusca* larvae from multiple sampling points in South Africa. The dissection and morphological classification of the mid-gut microbes of the combined larvae yielded 134 different morphological types. Precautions were taken to only excise the mid-gut of the dissected larvae because literature (Anon, 2004; Broderick *et al.* 2004; Anand *et al.* 2009; Engel and Moran, 2013) suggests that this was the section of the gut where the most digestion takes place and bacteria would be found. The results of the present study are in agreement with this statement because of the high numbers of morphological types isolated from the mid-guts of *B. fusca* larvae.

One of the objectives of the present study was to test the antibiotic susceptibility of the mid-gut bacteria of *B. fusca.* This was done by performing the modified Kirby-Bauer method. After the antibiotic susceptibility of the all morphological types was tested, it was concluded that the combination of ciprofloxacin, ampicillin and doxycycline had, potentially, the highest bacteriostatic and bactericidal effect. Even though more Gram positive bacteria than Gram negative bacteria were isolated from the mid-guts, the PCA constructed by using the inhibition zone diameter generated by the antibiotic exposure, shows that the antibiotics did not affect the Gram negative bacteria as much as the Gram positive bacteria. This observation can point to possible antibiotic resistance of the Gram negative isolates. This observation is also supported by literature (Vaara, 1992; Poole, 2001; Idrahim *et al.* 2010; Ash *et al.* 2002).

This combination of antibiotics (ciprofloxacin, ampicillin and doxycycline) was applied to the 134 morphological types in a 96-well plate at different concentrations to visually, in the form of a growth curve, establish the antibiotic concentration with the highest bacteriostatic and bactericidal effect. The results generated showed that the antibiotic concentration of 200 µg/ml had the highest bacteriostatic effect on the isolates, and the antibiotic concentration of 500 µg/ml had the highest bactericidal effect on the isolates. Another objective of the present study was achieved by confirming that the combined antibiotic concentration of 500 µg/ml was effective to rid larvae of mid-gut bacteria. This study also shows that a growth curve is

an effective way to visualise the effect of specific antibiotics and different concentrations on bacteria.

The method used to rid the larvae of gut microbes (another objective of the present study) before exposing them to Bt maize entailed the incorporation of antibiotics into an artificial *B. fusca* diet used by the ARC-GCI. Ciprofloxacin, ampicillin and doxycycline were combined and incorporated in equal volumes into the diet at the concentration of 500 µg/ml each. After the larvae fed on the antibiotic infused diet, they were placed onto Bt maize. The survival of these antibiotic reared larvae on Bt was compared to a parallel treatment which included larvae feeding solely on Bt maize, without any antibiotic treatment. Four experiments (larval groups) were conducted and in three of these (BF 1, CP 1 and CP 2) larva survived significantly better on Bt maize after the removal of their gut bacteria with the use of antibiotics. For that reason it can be concluded that, in the present study, the absence of mid-gut microbes significantly reduced mortality due to Bt maize. This observation can be attributed to the lack of bacteria in the antibiotic reared larvae which cause septicaemia associated with Bt toxicity in the insect host. There were no bacteria present in the guts of the larvae which could gain access through the permeabilised epithelium caused by Bt maize. These results suggested that the *B. thuringiensis* toxin alone was not sufficient to cause larval mortality. The present study correlates with the results obtained from Broderick *et al.* (2006) with regards to antibiotic reared larvae survival on Bt maize.

From these results, it is clear that *C. partellus* is an organism which is much more susceptible to Bt maize when compared to the survival of *B. fusca* on Bt maize. The resistance of *B. fusca* to Bt maize was apparent in larval group BF 2, which showed that there was no significant difference in percentage larval survival between the treatments this larval group was exposed to.

6.2 Recommendations:

- Recommendations for future research include sampling, testing the antibiotic susceptibility and performing the feeding studies on more than two species of lepidopteran larvae. The results generated from a bigger sampling pool will be more indicative of the influence of gut microbes on the efficacy of Bt maize. It will also be very interesting to observe the effect of the absence of gut bacteria on the efficacy of other Bt maize events, for example MON89034 expressing *cry1A.105* and the *cry2Ab2* genes.

- The resistance present in larval group BF 2 that was observed in the present study should be investigated at a genetic level. Heckel *et al.* (2007) reported that resistance to Cry 1A toxins in species of Lepidoptera has a complex genetic basis. At least four distinct, major resistance genes (of which three can possibly be mapped) should be investigated in *B. fusca*.

- The morphological species in the mid-gut of *B. fusca* should be identified up to species level with the help of culture-independent methods. This approach was performed by Broderick *et al.* (2004) which made use of metagenomics to establish the bacterial community of *Lymantria dispar* larva. The identity of the bacteria could also shed a light on their functions in the digestive tract, as well as their documented responses to antibiotics.

- Because it was necessary in the present study to use water soluble antibiotics, it caused limitations to the variety of antibiotics that could be used during the antibiotic susceptibility tests of the mid-gut bacteria of *B. fusca*. The antibiotics needed to be water-soluble to be incorporated into the diet and to rule out any negatives effects the antibiotic solvent could have on the larvae feeding on the diet. Although water soluble antibiotics are limited, alternatives to the antibiotics used in the present study could be more indicative of the specific antibiotic susceptibility of the gut microbes.

- The number of antibiotic resistant isolates we observed in the present study warrants further investigation. By identifying the genes responsible for antibiotic resistance, a more targeted approach could be used when testing the influence of antibiotics on the growth curve of the bacteria, or the usage of antibiotics which can be incorporated into a diet to rid larvae of microbiota.

References:

Agaisse, H. and Lereclus, D. 1995. How does *Bacillus thuringiensis* produce so much insecticidal crystal protein? *Journal of Bacteriology*, 177: 6027 – 6032

Akiba, Y. 1986. Microbial ecology of *Bacillus thuringiensis*.VII. Fate of *Bacillus thuringiensisin larvae of the silkworm, Bombyx mori, and the fall webworm, Hyphantria cunea.* Japanese Journal of *Applied* Entomology and Zoology, 30: 99 –105

Alford, H.G. 1972. Changes in standard for expressing potency of products containing *Bacillus thuringiensis.* Berliner PR Notice 72 (6), October 6, 1972

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: 109 – 117

Allen, H.K., Donato, J., Wang, H.H., Cloud-Hansen, K.A., Davies, J. and Handelsman, J. 2010. Call of the wild: antibiotic resistance genes in natural environments. *Nature Reviews Microbiology*, doi:10.1038/nrmicro2312

Al-Qadiri, H.M., Al-Alami, N.I., Lin, M., Al-Holy, M., Cavinato, A.G. and Rasco, B.A. 2008. Studying of the bacterial growth phases using fourier transform infrared spectroscopy and multivariate analysis. *Journal of Rapid Methods & Automation in Microbiology*, 16: 73 – 89

Amann, R.I., Ludwig, W. and Schleifer, K.H. 1995. Phylogenetic identification and insitudetection of individual microbial cells without cultivation. *Microbiological Reviews,* 59:143 – 69

Anand, A.A.P., Vennison, S.J., Sankar, S.G., Prabhu, D.I.G., Vasan, P.T., Raghuraman, T., Geoffrey, C.J. and Vendan, S.E. 2009. 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: 1 – 20

Andrews, J.M. 2001. Determination of minimum inhibitory concentrations. *Journal of Antimicrobial Chemotherapy,* 48: 5 – 16

Anon, 2012. Cronodon. Date of access: 1 August 2014

Anon, 2012a. Infonet-biovision. http://www.infonet-biovision.org/default/ct/123/crops Date of access: 30 July 2014

Anon, 2012b. Plantwise. http://www.plantwise.org/?dsid=10452&loadmodule=plantwisedatasheet&page=4270&site=234 Date of access: 30 August 2012

Anon. 2004. Journal of Insect Science http://www.faculty.ucr.edu/~insects/pages/teachingresources/stoffolano/3.pdf Date of access: 1 August 2014

Ash, R.J., Mauck, B. and Morgan, M. 2002. Antibiotic Resistance of Gram-Negative Bacteria in Rivers, United States. *Emerging Infectious Diseases*, 8: 713 – 716

Baum, J. A. and Malvar, T. 1995. Regulation of insecticidal crystal protein production in *Bacillus thuringiensis*. *Molecular Microbiology,* 18: 1 – 12

Belda, E., Pedrola, L., Pereto, J., Martinez-Blanch, J.F., Montagud, A., Navarro, E., Urchueguia, J., Ramon, D., Moya, A. and Porcar, M. 2011. Microbial diversity in the mid-guts of field and lab-reared populations of the European corn borer *Ostrinia nubilalis*. *PLoS ONE* 6: 1 – 14

Bernhard, K., Jarrett, P., Meadows, M., Butt, J., Ellis, D.J., Roberts, G.M., Pauli, S., Rodgers, P. and Burges, H.D. 1997. Natural isolates of *Bacillus thuringiensis*: worldwide distribution, characterization, and activity against insect pests. *Journal of Invertebrate Pathology*, 70: 59 – 68

Bietlot, H. P., Schernthaner, J.P., Milne, R.E., Clairmont, F.R., Bhella, R.S. and Kaplan, H. 1993. Evidence that the CryIA crystal protein from *Bacillus thuringiensis* is associated with DNA. *Journal of Biological Chemistry*, 268 : 8240 – 8245

Bourguet, D. 2004. Resistance to *Bacillus thuringiensis* toxins in the European corn borer: what chance for Bt maize? *Physiological Entomology* 29: 251 - 256

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

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: 293 – 300

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: 387 – 391

Broderick, N.A., Raffa, K.F. and Handelsman, J. 2006. Midgut bacteria required for *Bacillus thuringiensis* insecticidal activity. *PNAS*, 103:15196 – 15199

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: 1 – 9

Bush, K. 2010. The coming of age of antibiotics: discovery and therapeutic value. *Annals of the New York Academy of Sciences,* 1213: 1–4

Campagne, P., Kruger, M., Pasquet, R., le Ru, B. and van den Berg, J. 2013. Dominant Inheritance of Field-Evolved Resistance to *Bt* Corn in *Busseola fusca. PLoS ONE* 8: e69675

Carozzi, N. B., Kramer, V.C., Warren, G.W., Evola, S. and Koziel, M.G. 1991. Prediction of insecticidal activity of *Bacillus thuringiensis* strains by polymerase chain reaction product profiles. *Applied and Environmental Microbiology*, 57: 3057 – 3061

Carrière, Y., Crowder, D.W. & Tabashnik, B.E. 2010. Evolutionary ecology of insect adaptation to Bt crops. *Evolutionary Applications,* 3: 561 – 573

Castillo, A., Liébana, J., López, E., Baca, P., Liébana, J.M., Liébana, M.J. and Castillo, F. 2006. Interference of antibiotics in the growth curves of oral streptococci. *International Journal of Antimicrobial Agents,* 27: 263 – 266

Chellaram, C., Venkatesh, S., Prem Anand, T., Kuberan, G., John, A.A. and Priya, G. 2012. Antimicrobial properties of insect gut associated bacteria. *World Journal of Medical Sciences,* 7 : 260 – 263

Davies, J. and Davies, D. 2010. Origins and evolution of antibiotic resistance. *Microbiology and Molecular Biology Reviews,* 74: 417 – 433

de Barjac, H. and Lemille, F., 1970. Presence of flagellar antigenic subfactors in Serotype 3 of *Bacillus thuringiensis*. *Journal of Invertebrate Pathology*, 15: 139 – 140

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: 193 – 199

Demaneche, S., Sanguin, H., Pote, J., Navarro, E., Bernillon, D., Mavingui, P., Wildi, W., Vogel, T.M. and Simonet, P. 2008. Antibiotic-resistant soil bacteria in transgenic plant fields. *Proceedings of the National Academy Sciences*, 105: 3957 – 3962

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

Dow, J. A. T. 1992. pH Gradients in lepidopteran midgut. *Journal of Experimental Biololgy*, 172: 255 – 375

Drummond, L.J., Smith, D.G.E. and Poxton, I.R. 2003. Effects of sub-MIC concentrations of antibiotics on growth of and toxin production by *Clostridium difficile*. *Journal of Medical Microbiology*, 52: 1033 – 1038

Du, C., Martin, P.A. and Nickerson, K.W. 1994. Comparison of disulfide contents and solubility at alkaline pH of insecticidal and non-insecticidal *Bacillus thuringiensis* protein crystals. *Applied and Environmental Microbiology*, 60: 3847 – 3853

Ebenebe, A.A., van den Berg, J. and van der Linde, T.E. 1999. Effect of planting date of maize on damage and yield loss caused by the stalk borer, *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) in Lesotho. *South African Journal of Plant and Soil*, 16: 180 – 185

Engel, P. and Moran, N.A. 2013. The gut microbiota of insects – diversity in structure and Function. *FEMS Microbiology Reviews,* 37: 699 – 735

Erkosar, B., Storelli, G., Defaye, A. and Leulier, F. 2013. Host-intestinal microbiota mutualism: ‘‘Learning on the fly’’. *Cell Host & Microbe*, 13: 8 – 14

Feldhaar, H. 2011. Bacterial symbionts as mediators of ecologically important traits of insect hosts. *Ecological Entomology*, 36: 533 – 543

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

Garcia, M.A. and Altieri, M.A. 2005. Transgenic crops: Implications for biodiversity and sustainable agriculture. *Bulletin of Science, Technology & Society*, 25: 335 – 353

George, D., Ferry, N., Back, E.J. and Gatehouse, A.M.R. 2008. Characterisation of midgut digestive proteases from the maize stem borer *Busseola fusca. Pest Management Science,* 64: 1151 – 1158

George, D.M., Rind, F.C., 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

Gilliland, A., Chambers, C.E., Bone, E.J. and Ellar, D.J. 2002. Role of *Bacillus thuringiensis* Cry1 (delta) endotoxin binding in determining potencyduring lepidopteran larval development. *Applied and Environmental Microbiology,* 68: 1509 – 1515

Gould, F. 1998. Sustainability of transgenic insecticidal cultivars: Integrating pest genetics and ecology. *Annual Reviews of Entomology,* 43: 701 – 26

Govenor, H. L., Schultz, J.C. and Appel, H.M. 1997. Impact of dietary allelochemicals on gypsy moth (*Lymantria dispar*) caterpillars: importance of midgut alkalinity. *Journal of Insect Physiology*, 43: 1169 – 1175

Gwynn, M.N., Portnoy, A., Rittenhouse, S.F. and Payne, D.J. 2010. Challenges of antibacterial discovery revisited. *Annuals of the New York Academy of Sciences,* 1213: 5 – 19

Harley, J.P. 2011. Laboratory exercises in Microbiology. 8th ed. Avenue of the Americas, NY: McGraw-Hill Companies. p. 47 – 289

Heckel, D.G. 2012. Insecticide resistance after *Silent Spring. Science,* 337: 1612 – 1614

Heckel, D.G., Gahan, L.J., Baxter, S.W., Zhao, J.Z., Shelton, A.M., Gould, F. and Tabashnik, B. E. 2007.The diversity of Bt resistance genes in species of Lepidoptera. *Journal of Invertebrate Pathology*, 95: 192 – 197

Heierson, A., Sidén, I., Kivaisi, A. and Boman, H.G. 1986. Bacteriophage resistant mutants of *Bacillus thuringiensis* with decreased virulence in pupae of *Hyalophora cecropia*. *Journal of Bacteriology,* 167: 18 – 24

Höfte, H. and Whiteley, H.R. 1989. Insecticidal Crystal Proteins of *Bacillus thuringiensis*. *Microbiological Reviews*, 53: 242 – 255

Huang, F., Andow, D.A. and Buschman, L. 2010. Success of the high-dose/refuge resistance management strategy after 15 years of Bt crop use in North America. *Entomologia Experimentalis et Applicata,* 140: 1 – 16

Ibrahim, M.K., Galal, A.M. Al-Turk, I.M. and Al-Zhrany, K.D. 2010. Antibiotic resistance in Gram-negative pathogenic bacteria in hospitals' drain in Al-Madina Al-Munnawara. *Journal of Taibah University for Science,* 14: 14 – 22

James, C. 2012. Global Status of Commercialized Biotech/GM Crops: 2012. ISAAA Brief No. 44. ISAAA: Ithaca, NY

Jensen, G.B., Hansen, B.M., Eilenberg, J. and Mahillon, J. 2003. The hidden lifestyles of *Bacillus cereus* and relatives. *Environmental Microbiology*, 5: 631 – 640

Jeong, D.H., Ziemkiewicz, C., Ribarsky, W. and Chang, R. 2009. Understanding principal component analysis using a visual analytics tool. Charlotte visualization center, UNC Charlotte

Jisha, V.N., Smitha, R.B. and Benjamin, S. 2013. An Overview on the Crystal Toxins from *Bacillus thuringiensis. Advances in Microbiology*, 3: 462 – 472

Johnson, K.S. and Felton , G.W. 1996. Physiological and dietary influences on midgut redox conditions in generalist lepidopteran Larvae. *Journal of Insect Physiology,* 42: 191 – 198

Jurat-Fuentes, J.L. and Adang, M.J. 2006. Cry toxin mode of action in susceptible and resistant *Heliothis virescens* larvae. *Journal of Invertebrate Pathology,* 92: 166 – 171

Kfir R. 1991. Duration of diapause in the stem borers, *Busseola fusca* and *Chilo partellus*. *Entomologia Experimentalis et Applicata,* 61: 265 – 70

Kfir, R. 1998. Maize and Grain Sorghum: Southern Africa. (*In* Polaszek, A., *ed.* Cereal Stem Borers in Africa: Economic Importance, Taxonomy, Natural Enemies and Control. UK: CAB International. p. 28 - 37)

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 Reviews of Entomology*, 47: 701 – 731

Kipkoech, A.K., Schulthess, F., Yabann, W.K., Kipsat, M.J. and Mithöfer, D. 2010. Measuring the economic value of redistributing parasitoids for the control of the maize stemborer *Busseola fusca* Fuller (Lepidoptera: Noctuidae) in Kenya. *The African Journal of Agriculture and Resource Economics,* 4: 140 – 158

Kohanski, M. A., Dwyer, D. J., Hayete, B., Lawrence, C. A. and Collins, J. J. 2007. A common mechanism of cellular death induced by bactericidal antibiotics., *Cell* 130: 797 – 810

Kohanski, M.A., Dwyer, D.J. and Collins, J.J. 2010. How antibiotics kill bacteria: from targets to networks. *Nature reviews*, 8: 423 - 435

Koni, P.A. and Ellar, D.J. 1994. Biochemical characterization of *Bacillus thuringiensis* cytolytic δ-endotoxins. *Microbiology*, 140: 1869 – 1880

Krattiger, A.F. 1997. Insect resistance in crops: A case study of *Bacillus thuringiensis* (Bt) and its transfer to developing countries. ISAAA Briefs No. 2. ISAAA: Ithaca, NY. pp. 42

Kronstad, J. W., and H. R. Whiteley. 1984. Inverted repeat sequences flank a *Bacillus thuringiensis* crystal protein gene. *Journal of Bacteriology*, 160: 95 – 102

Kruger, M., Van Rensburg, J.B.J. and Van den Berg, J. 2011a. Transgenic Bt maize: farmers' perceptions, refuge compliance and reports of stem borer resistance in South Africa. *Journal of applied entomology*: 1 – 13

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. 2012. Reproductive biology of Bt-resistant and susceptible field-collected larvae of the maize stem borer, *Busseola fusca* (Lepidoptera: Noctuidae). *African Entomology,* 20: 35 – 43

Kruger, M., Van Rensburg, J.R.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

Kruger, 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 Plant Soil,* 25: 144 – 151

Kumar, P.A. 2003. Insect Pest Resistant Transgenic Crops. (*In* Upadhyay, R.K., *ed.* Advances in Microbial Control of Insect Pests. NY: Kluwer Academic/Plenum Publishers. p. 71 – 82)

Lacey, L.A., Frutos, R., Kaya, H.K. and Vail, P. 2001. Insect pathogens as biological control agents: Do they have a future? *Biological Control*, 21: 230 – 248

Lereclus, D., Ribier, J., Klier, A., Menou, G. and Lecadet, M.M. 1984. A transposon-like structure related to the d-endotoxin gene of *Bacillus thuringiensis*. *EMBO J.*, 3: 2561 – 2567

Lin, J., Lee, S.M., Lee, H.J. and Koo, Y.M. 2000. Modeling of typical microbial cell growth in batch culture. *Biotechnology and Bioprocess Engineering*, 5: 382 – 385

Lin, J., Takagi, M., Qu, Y., Gao, P. and Yoshida, T. 1999. Enhanced monoclonal antibody production by gradual increase of osmotic pressure. *Cytotechnology,* 29: 27 – 33

Lord, J.C. 2005. From Metchnikoff to Monsanto and beyond: The path of microbial control. *Journal of Invertebrate Pathology*, 89: 19 – 29

Martin, P. A. W. and Travers, R.S. 1989. Worldwide abundance and distribution of *Bacillus thuringiensis* isolates. *Applied and Environmental Microbiology*, 55: 2437 – 2442

Marvier, M., Mc Creedy, C., Regetz, J. and Karieva, P. 2007. A meta-analysis of effects of Bt cotton and maize on non- target invertebrates. *Science*, 316: 1475 – 1477

Meadows, M. P., Ellis, D.J., Butt, J., Jarrett, P. and Burges, H.D. 1992. Distribution, frequency, and diversity of *Bacillus thuringiensis* in an animal feed mill. *Applied and Environmental Microbiology*, 58: 1344 – 1350

Milner, R.J. 1994. History of *Bacillus thuringiensis*. *Agriculture Ecosystems and Environment*, 499: 9 – 13

Mostafa, A.M., Fields, P.G. and Holliday, N.J. 2005. Effect of temperature and relative humidity on the cellular defense response of *Ephestia kuehniella* larvae fed *Bacillus thuringiensis*. *Journal of Invertebrate Pathology*, 90: 79 – 84

Murnaghan, I. 2014. Genetically modified crops. http://www.geneticallymodifiedfoods.co.uk/development-history-gm-foods.html Date of access: 30 July 2014

Nakagarwara, S., Goto, T., Nara, M., Ozawa, Y., Hotta, K and Arata, Y. 1998. Spectroscopic characterisation and the pH dependence of bacterial activity of the aqueous chlorine solution. Journal of *Analytical Science and Technology*, 14: 691 – 698

Ndemah, R., Schulthess, F., Korie, S., Borgemeister, C., Poehling, H.M. and Cardwell, K. 2003. Factors affecting infestations of the stalk borer *Busseola fusca* (Lepidoptera: Noctuidae) on maize in the forest zone of Cameroon with special reference to Scelionid egg parasitoids. *Environmental Entomology,* 32: 51 – 60

Obrycki, J.J., Losey, J.E., Taylor, O.R. and Jesse, L.C.H. 2001. Transgenic insecticidal corn: Beyond insecticidal toxicity to ecological complexity. *Journal of BioScience*, 51: 353 – 361

Oerke, E.C. 2006. Centenary Review: Crop losses to pests. *Journal of Agricultural Science*, 144: 31 – 43

Oyebanji, O.B., Nweke, O., Odebunmi, O., Galadima, N.B., Idris, M.S., Nnodi, U.N., Afolabi, A.S. and Ogbadu, G.H. 2009. Simple, effective and economical explant-surface sterilization protocol for cowpea, rice and sorghum seeds. *African Journal of Biotechnology*, 8: 5395 – 5399

Padan, E., Bibi, E., Ito, M. And Krulwich, T.A. 2005. Alkaline pH homeostasis in bacteria: New insights. *Biochimica et Biophysica Acta*, 1717: 67 – 88

Pardo-Lopez, L., Soberon, M. and Bravo, A. 2013. *Bacillus thuringiensis* insecticidal three-domain Cry toxins: mode of action, insect resistance and consequences for crop protection. *FEMS Microbiology Reviews*, 37: 3 – 22

Parker, M.W. and Feil, S.C. 2005. Pore-forming protein toxins: from structure to function. *Progress in Biophysics & Molecular Biology*, 88: 91 – 142

Perlak, F. J., Fuchs, R.L., Dean, D.A., McPherson, S.L. and Fischhoff, D.A. 1991. Modification of the coding sequence enhances plant expression of insect control protein genes. *Proceedings of the National Academy Sciences,* 88: 3324 – 3328

Peyronnet, O., Noulin, J.F., Laprade, R. and Schwartz, J.L. 2004. Patch-clamp study of the apical membrane of the midgut of *Manduca sexta* larvae: direct demonstration of endogenous channels and effect of a *Bacillus thuringiensis* toxin. *Journal of Insect Physiology*, 50: 791 – 803

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

Poole, K. 2001. Multidrug resistance in Gram-negative bacteria. *Current Opinion in Microbiology*, 4: 500 – 508

Prakash, C.S. 2010. Genetically modified crops. http://www.accessscience.com, DOI 10.1036/1097-8542.YB051540 Date of access: 11 August 2012

Rahman, M.M., Roberts, H.L.S. and Schmidt, O. 2007. Tolerance to *Bacillus thuringiensis* endotoxin in immune-suppressed larvae of the flour moth *Ephestia kuehniella*. *Journal of Invertebrate Pathology*, 96: 125 – 132

Rausel, C., Martinez-Ramirez, A.C., Garcia-Nobles, I. and Real, M.D. 2000. A binding site for *Bacillus thuringiensis* Cry1Ab toxin is lost during larval development in two forest pests. *Applied and Environmental Microbiology,* 66: 1553 – 1558

Raymond, B., Davis, D. and Bonsall, M.B. 2007. Competition and reproduction in mixed infections of pathogenic and non-pathogenic Bacillus spp. *Journal of Invertebrate Pathology*, 96: 151 – 155

Raymond, B., Johnston, P.R., Wright, D.J., Ellis, R.J., Crickmore, N. and Bonsall, M.B. 2009. A mid-gut microbiota is not required for the pathogenicity of *Bacillus thuringiensis* to diamondback moth larvae. *Environmental Microbiology*, 11: 2556 – 2563

Raymond, B., Lijek, R.B., Griffiths, R.I. and Bonsall, M.B. 2008. Ecological consequences of ingestion of Bacillus cereus on Bacillus thuringiensis infections and on the gut flora of a lepidopteran host. *Journal of Invertebrate Pathology,* 99: 103 – 111

Reddy, K.V.S. and Zehr, U.B. 2004. Novel strategies for overcoming pests and diseases in India. (In: New directions for a diverse planet:. Proceedings of the 4th International Crop Science Congress, Brisbane, Australia: p 1-8)

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

Romoser, W.S. and Stoffolano, J.G. 1998. Access Science from Mcgraw-Hill. http://www.accessscience.com/search.aspx?topic=BIO:ZOO:ARTH&term=Insect+physiology Date of access: 1 August 2014

Salyers, A.A., Gupta, A. and Wang, Y. 2004. Human intestinal bacteria as reservoirs for antibiotic resistance genes. *Trends* in *Microbiology,* 12: 412 – 416

Schmieder, R and Edwards, R. 2012. Insights into antibiotic resistance through metagenomic approaches. *Future Microbiology*, 7: 73 – 89

Schnepf, H.E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D.R. and Dean, D.H.1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiology and Molecular Biology Review*, 62: 775 – 806

Schwember, A.R. 2008. An update on genetically modified crops. *Ciencia e Investigacion Agraria,* 35: 231 – 250

Shannon, A.L., Attwood, G., Hopcroft, D.H. and Christeller, J.T. 2001. Characterization of lactic acid bacteria in the larval midgut of the keratinophagous lepidopteran, *Hofmannophila pseudospretella*. *Letters in Applied Microbiology*, 32: 36 – 41

Smith, R. A. and Couche, G.A. 1991. The phylloplane as a source of *Bacillus thuringiensis* variants. *Applied and Environmental Microbiology,* 57: 311 – 315

Soberon, M., Pardo-Lopez, L., Lopez, I., Tabashnik, B.E. and Bravo, A. 2007. Engineering modified Bt toxins to counter insect resistance. *Published Online,* 318: 1640 – 1642

StatSoft, Inc. 2013. STATISTICA (data analysis software system), version 12. www.statsoft.com

Stotzky, G. 2004. Persistence and biological activity in soil of the insecticidal proteins from *Bacillus thuringiensis*, especially from transgenic plants. *Plant and Soil,* 266: 77 – 89

Suzuki, M.T., Lereclus, D. and Arantes O.M.N. 2004. Fate of *Bacillus thuringiensis* strains in different insect larvae. *Canadian Journal of Microbiology*, 50: 973 – 975

Tabashnik, B. E. 2008. Delaying insect resistance to transgenic crops. *Proceedings of the National Academy Sciences,* 105: 19029 – 19030

Tabashnik, B. E., A. L. Patin, T. J. Dennehy, Y. Liu, Y. Carrière, M. A. Sims, and L. Antilla. 2000. Frequency of resistance to *Bacillus thuringiensis* in field populations of pink bollworm. *Proceedings of the National Academy Sciences,* 97: 12980 – 12984

Tabashnik, B. E., Gassmann, A.J., Crowder, D.W. and Carrière, Y. 2008. Field-evolved resistance to Bt toxins*. Nature Biotechnology,* 26: 1074 – 1076

Tabashnik, B. E., Y. Carrière, T. J. Dennehy, S. Morin, M. S. Sisterson, R. T. Roush, A.M.Shelton, and J. Z. Zhao. 2003. Insect resistance to transgenic Bt crops: lessons from the laboratory and field. *Journal of Economic Entomology*, 96: 1031 – 1038

Tabashnik, B.E. 1994. Evolution of resistance to *Bacillus thuringiensis. AnnualReview of Entomology,* 39: 47 – 79

Tabashnik, B.E., Brévault, T. and Carrière, Y. 2013. Insect resistance to Bt crops: lessons from the first billion acres. *Nature Biotechnonoly,* 31: 510 – 521

Tabashnik, B.E., van Rensburg, J.B.J. and Carriere, Y. 2009. Field-evolved insect resistance to Bt crops: Definition, theory and data. *Journal of Economic Entomology*, 102: 2011 – 2025

Takatsuka, J. and Kunimi, Y. 2000. Intestinal bacteria affect growth of *Bacillus thuringiensis* in larvae of the oriental tea tortrix, *Homona magnanima* Diakonoff (Lepidoptera: Tortricidae). *Journal of Invertebrate Pathology,* 76: 222 – 226

Takeuchi, O., Hoshino, K., Kawai,T., Sanjo, H., Takada, H., Ogawa, T., Takeda, K. and Akira, S. 1999. Differential roles of TLR2 and TLR4 in recognition of Gram negative and Gram positive bacterial cell wall components. *Immunity*, 11: 443 – 451

Tende, R.M., Mugo, S.N., Nderitu, J.H., Olubayo, F.M., Songa, J.M. and Bergvinson, D.J. 2010. Evaluation of *Chilo partellus* and *Busseola fusca* susceptibility to d-endotoxins in Bt maize. *Crop Protection,* 29: 115 – 120

Ter Braak, C.J.F. and Šmilauer, P. 1998. CANOCO Reference Manual and User's Guide to Canoco for Windows: Software for Canonical Community Ordination (version 4)

Ter Braak, C.J.F. and Šmilauer, P. 2002. CANOCO for Windows version 4.5. Biometris-plant Research International, Wageningen, The Netherlands (1997-2002)

Torres-Cortés, G., Millán, V., Ramírez-Saad, H.C., Nisa-Martínez, R., Toro, N. and Martínez-Abarca, F. 2011. Characterization of novel antibiotic resistance genes identified by functional metagenomics on soil samples. *Environmental Microbiology,* 13: 1101 – 1114

Travers, R. S., Martin, P.A.W. and Reichelderfer, C.F. 1987. Selective process for efficient isolation of soil *Bacillus* sp. *Applied and Environmental Microbiology*, 53: 1263 – 1266

Vaara, M. 1992. Agents that increase the permeability of the outer membrane. *Microbiology Reviews*, 56: 395 – 411

Vachon, V., Laprade, R. and Schwartz, J.L. 2012. Current models of the mode of action of *Bacillus thuringiensis* insecticidal crystal proteins: A critical review. *Journal of Invertebrate Pathology*, 111: 1 – 12

Van Rensburg, J.B.J. 2001. Larval mortality and injury patterns of the African stalk borer, *Bussola fusca* (Fuller), on various plant parts of Bt transgenic maize. *South African Journal of Plant and Soil,* 18: 62 – 68

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: 147 – 151

Van Wyk, A., van den Berg, J. and van Hamburg, H. 2008. Diversity and comparative phenology of Lepidoptera on Bt and non-Bt maize in South Africa. *International Journal of Pest Management*, 54: 77 – 87

Vasanthakumar, A., Handelsman, J., Schloss, P.D., Bauer, L.S. and Raffa, K.F. 2008. Gut microbiota of an invasive subcortical beetle, *Agrilus planipennis* Fairmaire, across various life stages. *Environmental Entomology,* 37: 1344 – 1353

Venette, R. C., W.D. Hutchison, and D. A. Andow. 2000. An in-field screen for early detection and monitoring of insect resistance to *Bacillus thuringiensis* in transgenic crops. *Journal of Economic Entomology*, 94: 1055 – 1064

Visotto, L.E., Oliveira, M.G.A., Guedes, R.N.C. and Ribon, A.O.B. 2009. Contribution of gut bacteria to digestion and development of the velvetbean caterpillar, *Anticarsia gemmatalis*. *Journal of Insect Physiology,* 55(3): 185 – 191

Willey, J.M., Sherwood, L. and Woolverton, C. 2014. Prescott's Microbiology. 9th ed. Penn Plaza, NY: McGraw-Hill Education. p. 32 – 403

Wright, G.D. 2010. Antibiotic resistance in the environment: a link to the clinic? *Current Opinion in Microbiology*, 13: 589 – 594

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

Yeung, K.Y. and Ruzzo, W.L. 2000. An empirical study on Principal Component Analysis for clustering gene expression data. Department of Computer science and Engineering, University of Washington, Seattle, WA

Zhang, H., Tian, W., Zhao, J., Jin, L., Yang, J., Liu, C., Yang, Y., Wu, S., Wu, K., Cui, J., Tabashnik, B.E. and Wu, Y. 2012. Diverse genetic basis of field-evolved resistance to Bt cotton in cotton bollworm from China. *Proceedings of the National Academy Sciences*, 109: 10275 – 10280

Zwietering, M.H., Jongenburger, I., Rombouts, F.M. and van 'T Riet, K. 1990. Modeling of the bacterial growth curve. *Applied and Environmental Microbiology*, 56: 1875 – 1881