

**Diversity of gut-microorganisms in Bt-resistant and Bt-susceptible larvae of the maize stem borer (*Busseola fusca*)
(Lepidoptera: Noctuidae)**

By

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ABSTRACT

Maize is one of Africa's main food sources. It is therefore important to protect this crop against pests such as the maize stem borer *Busseola fusca*. Genetically modified *Bacillus thuringiensis* (Bt) maize has been shown to be very effective against pests like *B. fusca*. However, this pest developed resistance to Bt-maize. The current study was inspired by the lack of knowledge on microorganisms associated with these insects as they play a vital role in the growth and development of herbivorous insects. The aim of the study was to determine and compare the community diversity of microorganisms present in the midgut of resistant and susceptible *B. fusca* larvae. Secondly *Escherichia coli* and *Enterobacter* spp. present in the midgut were quantified and compared with community diversity obtained with DGGE analysis. The midgut of *B. fusca* was removed after which DNA was extracted from the contents. Extracted DNA was subjected to Polymerase Chain Reaction Denaturing Gradient Gel Electrophoresis (PCR-DGGE) analysis after which prominent bands were excised and sequenced. Serial dilutions were also done of the contents of the midgut and plated out on Brilliant Green Bile agar and mFc agar for quantification. DGGE analysis showed no differences in the community structure of the midgut contents of Bt-resistant or susceptible *B. fusca* reared under either laboratory, greenhouse or field conditions. Sequencing results revealed the dominance of *Enterococcus* spp., specifically *Enterococcus mundtii*, *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus hirae* and *Enterococcus durans*. Other organisms isolated included *Staphylococcus* sp., *Agrobacterium tumefaciens*, *Leuconostoc mesenteroides*, *Peptostreptococcaceae* bacterium and several uncultured bacteria. Quantification of *E. coli* and *Enterobacter* spp. suggested that Bt-maize and environmental factors might play a role in the abundance of these organisms in the midgut. Dominance by *E. coli* and *Enterobacter* spp. might suppress the growth of other bacteria in the midgut.

Key word: Bt-maize, *Busseola fusca*, DGGE, *Escherichia coli*, *Enterobacter* spp., resistance

UITTREKSEL

Mielies is een van Afrika se hoof voedselbronne. Dit is daarom belangrik dat hierdie gewas beskerm word teen plae soos die mieliestamrusper, *Busseola fusca*. *Bacillus thuringiensis* (Bt) mielies het getoon dat dit effektiewe weerstand bied teen peste soos *B. fusca*. Onlangse studies toon egter aan dat *B. fusca* weerstand bied teen Bt-mielies. Beperkte kennis oor die mikro-organismes wat geassosieer word met hierdie insekte was die motivering vir die huidige studie, aangesien die mikro-organismes 'n baie belangrike rol speel in die groei en ontwikkeling van insekte. Die doel van die studie was om die diversiteit van mikro-organismes in die middelderm van weerstandbiedende en vatbare *B. fusca* larwes te bepaal en te vergelyk. Tweedens is *Escherichia coli* en *Enterobacter* spp. gekwantifiseer en vergelyk met die diversiteit wat deur middel van DGGE-analises bepaal is. Die middelderm van *B. fusca* is verwyder en DNA is daaruit geëkstraheer. PCR-DGGE-analises is op die DNA gedoen en die mees prominente bande is uitgesny waaruit nukleotiedvolgordes bepaal is. Reeksverdunnings is gedoen met die middelderm inhoud en is uitgeplaas op Brilliant Green Bile agar en mFc agar vir kwantifisering. Volgens die DGGE-analises was daar geen verskil in mikrobiële diversiteit tussen vatbare en weerstandbiedende *B. fusca* larwes wat in verskillende omstandighede groot gemaak is nie. Die dominante spesies was die volgende: *Enterococcus* spp., spesifiek *Enterococcus mundtii*, *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus hirae* en *Enterococcus durans*. Ander spesies sluit in, *Staphylococcus* sp., *Agrobacterium tumefaciens*, *Leuconostoc mesenteroides*, *Peptostreptococcaceae* bakterium en verskeie ongekultiveerde bakterië. Kwantifisering van *E. coli* en *Enterobacter* spp. dui daarop dat die Bt-mielies en omgewingsfaktore moontlik die hoeveelhede van die organismes in die middelderm beïnvloed. Die dominansie van *E. coli* en *Enterobacter* spp. kon ook moontlik die voorkoms van ander organismes in die middelderm onderdruk.

Sleutelwoorde: Bt-mielies, *Busseola fusca*, DGGE, *Escherichia coli*, *Enterobacter* spp., weerstand.

**Ek dra graag hierdie werk op aan my Vader, my ouers,
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DECLARATION

I declare that the dissertation for the degree of Master of Science in Environmental Sciences at the North-West University, Potchefstroom, hereby submitted, has not been submitted by me for a degree at this or another University, that it is my own work in design and execution, and that all material contained herein has been duly acknowledged.

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Daniël Erasmus Brink

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Date

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CHAPTER 1

LITERATURE OVERVIEW

In the following literature review it will be demonstrated that resistance development of *B. fusca*, one of the target pests of genetically modified Bt-maize, could become a major problem in African agriculture. With the possibility of resistance spreading to other areas, it is critical to address the gap in our knowledge of resistance mechanisms of *B. fusca* to Bt maize (Sharma, 2006; Kruger *et al.*, 2011).

There is little information regarding the microorganisms associated with foliage-feeding lepidopterans. These organisms, playing important roles in the growth and development of insects, could play an important role in resistance development (Broderick *et al.*, 2003; Scoble, 1992). The understanding of the types of microbes present in the midgut of a lepidopteran and the role they play in insect development and function, could lead to new strategies for pest and resistance management (Broderick *et al.*, 2003). This literature overview also deals with culture independent and culture dependent methods to determine community diversity and quantity of specific organisms in the midgut of *B. fusca*.

1.1 Background on the importance of agricultural activities with regards to maize

In most developing countries, agriculture is the driving force for broad-based economic growth (Sharma, 2006). In Africa, agricultural productivity is severely limited by a large number of biotic (arthropods, nematodes, diseases, weeds, rodents, birds) and abiotic (drought, low soil fertility, mineral toxicity) (Gressel *et al.*, 2004) constraints. Low agricultural productivity is a major cause of poverty, food insecurity, and malnutrition. The use of high-yielding varieties, irrigation, fertilisers, and pesticides has increased crop productivity five-fold in the past five decades (Sharma, 2006). However, it has been found that this growth in crop productivity has been levelling off in the past two decades. The decrease in land and water resources leaves no option but to increase crop productivity per unit area (Sharma, 2006).

There is a need to examine how science can be used to increase biological productivity without associated ecological costs. Productivity increases are being achieved through the application of modern biotechnological tools in integrated gene management, integrated pest management and efficient post-harvest management. Biotechnology in agriculture can

be a powerful tool to reduce poverty and improve the livelihoods of the rural poor (Sharma, 2006).

Maize is the most important agricultural crop in southern Africa and is used as both human and animal food. In general, maize in Africa is grown by small-scale farmers for local consumption and yields tend to be very low. However, there have been numerous attempts to cultivate more extensive maize monocultures with the aid of irrigation (Polazek & Khan, 1998).

1.2 Stem borer pests of maize in South Africa

Of the various insect pests attacking maize in South Africa, the African maize stalk borer, *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) and the spotted stem borer, *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae), are the most important (Skoroszewski & Van Hamburg, 1987). To a lesser extent, maize is also attacked by the pink stalk borer, *Sesamia calamistis* (Hampson) (Lepidoptera: Noctuidae), which is normally kept under control by parasitoids that prevent serious outbreaks (Kfir, 1998).

1.2.1 Ecology of *Busseola fusca*

Busseola fusca is native to Central Africa but is also distributed southwards where it is associated with maize. It is the dominant stem borer at elevations 900m above sea level in South Africa but also occurs at lower altitudes. *Busseola fusca* is oligophagous and feeds on a variety of related host plants which include crops such as maize, pearl millet and sorghum (Kfir, 1998).

The full-grown diapause larvae of *B. fusca* over-winter in the stalk base of the plant under the soil surface, where they are well protected from natural enemies and adverse climatic conditions (Kfir, 1990). These overwintering larval populations are the source of the first seasonal moth flight during the following spring (Van Rensburg, 1999). The life cycle of *B. fusca* is illustrated in Figure 1.1. Adults emerge from pupae during late afternoon and early evening and are active at night. During the day, they are inactive, resting on plants and plant debris. Mating takes place for several nights after emergence, after which females lay eggs in batches of 30-100 eggs under the vertical edges of leaf sheaths (Kfir, 1998).

Eggs hatch after seven to ten days, depending on the temperature. Young larvae migrate along the outside of the plant to the whorl where it feeds on the rolled up leaves. Some larvae migrate to nearby plants, which often lead to the infestation of up to three other plants shortly after the hatching of an egg batch (Van Rensburg, 1999). As whorl leaves emerge, damage caused by larval feeding can be observed. This is the first indication of an infestation. This type of leaf damage does not cause significant crop damage, but damage done to the growing point of a young plant may cause the whorl of the plant to die. Young plants that are damaged may also be stunted and form excessive tillers without the production of ears (Van Rensburg, 1999).

The majority of stem borer larvae remain in the whorl of the maize plant (although some of the older larvae will bore into the stem) and the most stalk damage is done during the appearance of the tassel when new sources of nutrition and shelter have to be found. The duration of the larval stage is usually six weeks under favourable conditions. Full-grown larvae pupate inside the stem after excavating emergence holes to facilitate exit of moths (Kfir, 1998).

The duration of the pupal stage is about three weeks after which a second seasonal flight of moths appear. These moths lay their eggs on late-planted maize (Van Rensburg, 1999). These second-generation larvae attack maize ears before boring into the stem. Most of the second-generation larvae move into a state of rest during the winter (diapause) to give origin to the moths of the next season. A relative small number of larvae turn into pupae to give origin to a third seasonal flight. These moths usually do not find suitable plants in which to lay their eggs and thus do not produce a progeny (Van Rensburg, 1999).

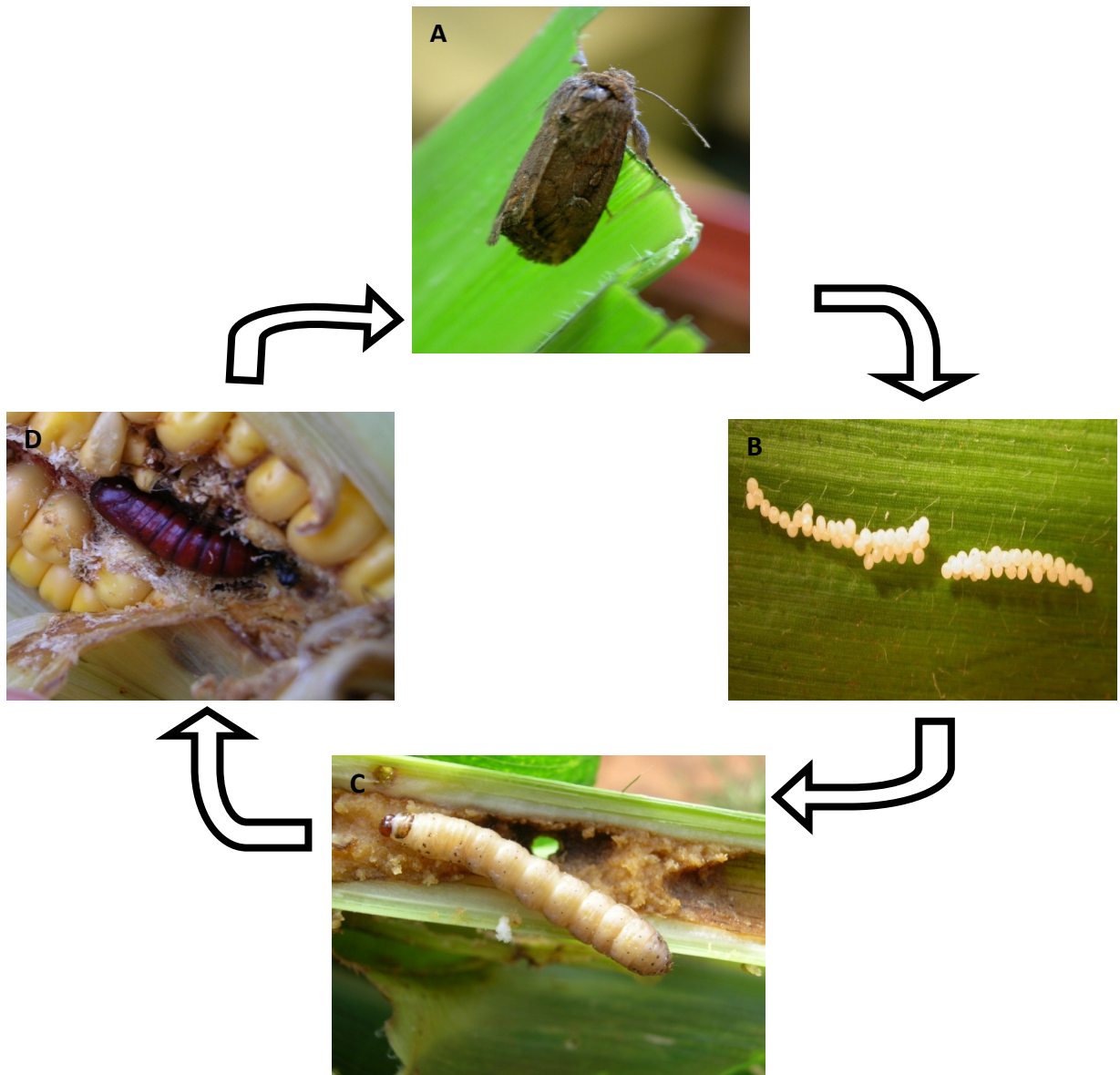


Figure 1.1: A diagrammatic representation of the life cycle of *Busseola fusca*. Different stages are: A (moth), B (eggs), C (larva) and D (pupa).

1.2.2 Control of *Busseola fusca*

There exist various strategies that can be employed to manage maize stem borers. In this section biological control, chemical control and cultural control will be discussed, whereas genetic modification and the use of *Bacillus thuringiensis* (Bt) maize will be discussed in the next section.

1.2.2.1 Biological control

Biological pest management includes the use of sprays of formulations containing microbial organisms such as *B. thuringiensis*. Pest predators, parasites and other beneficial organisms can also be used (Uri, 1998). Natural enemies play an important role in the control of lepidopterous borers in Africa. Approximately 100 genera of parasitoids (Hymenoptera and Diptera) have been recorded attacking cereal stem borers in Africa and surrounding islands (Polazek, 1992).

Inefficiency and high cost of insecticides to control *B. fusca* in South Africa precipitated a biological-control program using exotic parasitoids (Kfir, 1991; 1992). The parasitoid, *Cotesia flavipes* (Cameron) (Hymenoptera: Braconidae), was introduced into South Africa from Pakistan. This parasitoid was reared in the laboratory and released in large numbers in stem borer infested maize fields, but the parasitoid did not establish (Skoroszewski & Van Hamburg, 1987). However, in Kenya the parasitoid used to control *C. partellus* became permanently established during the 1990's and spread to other areas (Omwega *et al.*, 1995). Thereafter, the program was expanded to cover eleven countries in East and southern Africa. Establishment has been recorded in all but one of the eleven countries where it was released (Omwega *et al.*, 2006). Laboratory studies, however, indicated that release of the parasitoid in areas where *B. fusca* is predominant will most likely result in failure to establish since this stem borer is not a suitable host (Ngi-Song *et al.*, 1995; Overholt *et al.*, 2003).

1.2.2.2 Chemical control

Even though chemical control methods are most effective, they are expensive to the smallholder farmer (Tende *et al.*, 2010). The improper use of synthetic pesticides is one of the causes for the current sensitivities regarding environmental pollution, human health hazards and pest resurgence. Natural plant products and biopesticides provide a potentially viable alternative to synthetic insecticides since they are relatively safe to natural enemies, non-target organisms and humans (Sharma, 2006).

1.2.2.3 Cultural control

Crop residues are important for carrying over *B. fusca* larval populations from one growing season to the next. Ploughing during winter in order to bury maize stubble to prevent moths

from emerging is an effective control measure. Stubble that is left on the surface will cause the mortality of the larvae in diapause due to the heat (Van Rensburg, 1999). Little research attention has been given to other aspects of cultural control in southern Africa (Sithole, 1990).

1.3 Genetic modification of plants

Genetic engineering makes it possible to transfer genes from totally unrelated organisms, crossing species barriers that were not possible through conventional genetic enhancement (Sharma, 2006).

Genetically modified (GM) plants have been developed for a variety of reasons that include: a longer shelf life, disease resistance, pest resistance, herbicide tolerance, nutritional improvement, resistance to drought and nitrogen deficiencies. The FlavrSavr tomato, developed to have a longer shelf life, was the first GM crop approved for use in the USA in 1994 (Icoz & Stotzky, 2008). GM crops were first commercialised in 1996 and since then, the planting of GM crops has consistently increased by 10% or more each year worldwide (Sanvido *et al.*, 2007).

The global area of GM crops increased approximately 60-fold during the 11-year period from 1996 to 2006; from 1.7 million hectares to 102 million hectares (James, 2006; 2010). In 2009, biotech crop plantings increased with seven percent to reach 134 million hectares. In 2010, a record 14 million farmers in 25 countries were using agricultural biotechnology. Thirteen million of these were resource-poor farmers in developing countries (James, 2010). South African production of Bt maize has increased from 77 000 ha (2.8 % of total area under maize) in 2000 to 943 000 ha (34.9 %) in 2006 (James, 2006). The total area of biotech crops in South Africa increased from 1.8 million hectares in 2008 to 2.2 million hectares in 2010 (James, 2008; 2010).

1.3.1 *Bacillus thuringiensis* as bio-pesticide

Agricultural land and forests form an important resource to sustain global economical, environmental and social systems. Protection of these resources against pests is a priority; therefore the use of biopesticides is increasing, due to the adverse impacts that chemical insecticides may have. Since the discovery of *B. thuringiensis*, it has received extensive attention in the research community (Brar *et al.*, 2006). Bt insecticides are classified as

biopesticides due to the fact that some bacteria produce insecticidal crystal proteins (Cry proteins) (Höfte & Whitely, 1989).

In a study conducted by Bernhard *et al.* (1997), *B. thuringiensis* was isolated from natural samples that were collected from 80 countries. The majority, 45 % of the 5303 isolates, originated from stored products, with 25 % originating from soil. The materials richest in isolates that are active against insects were mushroom compost and stored products (Bernhard *et al.*, 1997). This indicates that *B. thuringiensis* is a common and wide spread, soil bacterium. It is also abundantly found in grain dust collected from soils and other grain storage facilities (Neppl, 2000).

Sprays of *B. thuringiensis* spores (Figure 1.2) which are relatively specific against certain target species have for decades been used to control pests. Limited effects on most non-target species have been reported (Schnepf *et al.*, 1998). However, the use of the commercial Bt sprays have been limited due to their relatively high cost, poor crop coverage, rapid environmental inactivation, and less than desired levels of pest control, especially when compared with less expensive conventional chemical insecticides (Benedict & Altman, 2001). More recently, toxin-encoding genes from *B. thuringiensis* have been expressed in transgenic crop plants, providing season-long protection from some key pests (Schnepf *et al.*, 1998).



Figure 1.2: *Bacillus thuringiensis* viewed by phase contrast microscopy. The vegetative cells (X) contain endospores (Y) (phase bright) and crystals of an insecticidal protein toxin (delta endotoxins). Most cells have lysed and released the spores and toxin crystals (the structures with a bi-pyramidal shape (Z)) (Deacon, 2008).

Apart from its demonstrated efficacy and relatively narrow host range, one of the key advantages of Bt as a biopesticide, which is applied as a spray formulation, is the ability to mass-produce it in fermented culture. This allows both greater control over quality and the use of either or both the spores and crystal toxin proteins that determine the efficacy of Bt (Evans, 2002). Each strain produces its own unique insecticidal crystal protein, or delta-endotoxin, which is encoded by a single gene on a plasmid in the bacterium. The insecticidal activity of the toxins from each Bt strain differs. The set of Bt delta-endotoxins affect a variety of species from the insect orders Coleoptera, Lepidoptera and Diptera. The Bt toxin is very specific to certain insect groups and it is assumed that these toxins are safe to most beneficial insects and other animals (Neppl, 2006).

1.3.2 Mode of action of Bt toxin

Different factors associated with the mode of action of the Cry protein in insect larvae are related to mortality. These factors include midgut pH, binding of toxin and the role of bacteria.

1.3.2.1 Activation of the toxin produced by *Bacillus thuringiensis*

The specificity of the Bt toxin is associated with the extremely alkaline midgut environment of lepidopteran and dipteran insects when using *B. thuringiensis* as a biological insecticide. The proposed mechanism of the activation of the toxin is illustrated in figure 1.3. Firstly the insecticidal Cry proteins must be ingested and dissolved in the alkaline environment to form protoxins (crystal δ -endotoxins). Alkaline conditions in the midgut, together with midgut proteases, are required to solubilise and cleave the protoxins to produce the biologically active form (Höfte & Whitely, 1989; Broderick *et al.*, 2006; Icoz & Stotzky, 2008). For most Cry toxins, midgut pH must be strongly alkaline (pH>9.5) for the dissolution of the crystal (Evans, 2002). Some coleopteran-specific toxins do, however, function at much lower pH levels. The rate and extend of crystal solubilisation influences toxicity levels in different hosts, and pH may influence the effectiveness and specificity of some toxins (Evans, 2002).

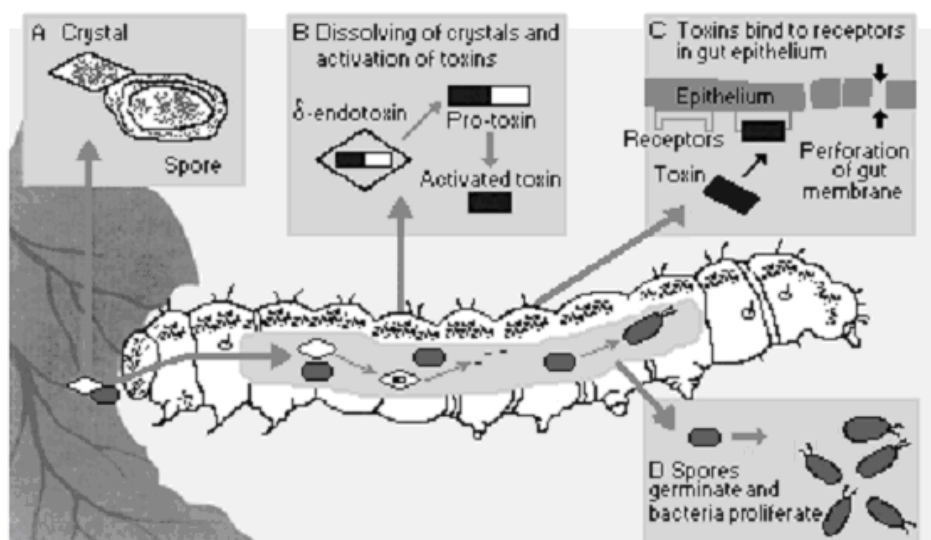


Figure 1.3: The proposed mechanism of toxicity of Bt inside a Lepidopteran larvae (Anon A, 1999).

The truncated genes present in GM plants do however not require a specific midgut pH and solubilisation because the toxin is already in the transcribed active form to the solubilised core (Evans, 2002). Cry genes inserted into most GM plants are in a truncated form, and when expressed in plants, truncated active Cry proteins do not form crystals since they are already soluble and active (Gill *et al.*, 1992; Aronson & Shai, 2001; Stotzky, 2002). Therefore, most of the specificity that accounts for the safety of Cry proteins in commercial bacterial insecticides does not apply to these same proteins when expressed in Bt crops to make them resistant to specific insects (Icoz & Stotzky, 2008).

1.3.2.2 Binding of toxin

Binding to the specific receptors in the brush border membrane of the larval midgut is an essential step in the intoxication process. In Bt-susceptible insects, the toxicity of a particular Cry protein is correlated with the number of receptors as well as its affinity for these sites (Schnepf *et al.*, 1998; Neppl, 2006; Pigott & Ellar, 2007). The binding affinity of these toxin fragments is often directly related to the toxicity, though binding does not assure toxicity (Neppl, 2006). Many chewing insects ingest these insecticidal Cry proteins, but are not sensitive to the toxins, because they do not have the appropriate receptors, even if they have alkaline midguts (Luthy & Ebersold, 1981).

Through a process which is unclear, the toxins appear to insert into the membranes of gut cells of insect larvae (Broderick *et al.*, 2006). The activated protein, whether produced by

the GM plant or activated within the midgut when using the Bt spray, primarily attack mature columnar cells by binding to specific receptors on microvillar membranes. Ion channel pores quickly penetrate the membranes at the sites of attachment, causing leakage of cellular contents, internal messenger disruption, swelling as well as cell lysis due to increased internal osmotic pressure (Pietrantonio & Gill, 1996). The gut subsequently becomes paralysed and the insects stop feeding.

It has been proposed that disruption of the midgut epithelium results in a prolonged termination of feeding and eventually death by starvation. An alternative proposed mechanism of killing is that extensive cell lysis provides Bt spores access to the more favourable environment of the hemocoel of the insect body, where they germinate and reproduce, leading to septicaemia and death (Broderick *et al.*, 2006). According to Broderick *et al.* (2006) these two models are not consistent with experimental observations. For example, *B. thuringiensis*-induced mortality is much faster (2-5 days) than starvation-induced mortality (7-10 days). The septicaemia model is challenged by the observation that the toxin causes mortality when it is separated from the bacterial cells, which has been accomplished with purified toxin and transgenic plants that produce the toxin (Broderick *et al.*, 2006).

1.3.2.3 Role of bacteria in the mode of action of Bt

Bacteria must be able to enter and multiply in the hemolymph to cause septicaemia. It was found that *B. thuringiensis* populations decrease dramatically to below detectable limits and that *B. thuringiensis* spores are typically absent in the hemocoel until well after death of the insect (Broderick *et al.*, 2006). However, *Enterobacter* spp. and *E. coli* showed rapid growth inside the hemolymph. It may be that *B. thuringiensis* enables the enteric bacteria to reach the hemocoel by permeating the gut epithelium (Broderick *et al.*, 2006).

It was observed that the population densities of *B. thuringiensis* were similar in the midguts of larvae that were brought up on sterile artificial diet containing antibiotics and those not containing antibiotics (Broderick *et al.*, 2006). This suggests that the direct elimination of *B. thuringiensis* is not the main reason for reduction in insect mortality. It has also been reported that *B. thuringiensis* does not kill larvae of the gypsy moth, *Lymantria dispar* (Lepidoptera: Lymantriidae), in the absence of indigenous midgut bacteria. This was shown by the abolishment of Bt insecticidal activity through the elimination of the midgut bacteria by oral administration of antibiotics to larvae (Broderick *et al.*, 2006).

This reduction in mortality was accompanied by reduced populations of culturable *Enterococcus* spp. and *Enterobacter* spp. from the midguts of larvae. After *Enterobacter* spp. was reintroduced to the target organism, the insecticidal activity was restored to nearly that of larvae not treated with antibiotics (Broderick *et al.*, 2006). The same procedure was, however, negative for *Enterococcus casseliflavus* and *Staphylococcus xylosus*. This implies that *Enterobacter* spp. is mainly responsible for the septicemia associated with *B. thuringiensis*-induced mortality in gypsy moth larvae (Broderick *et al.*, 2006).

Broderick *et al.* (2006) demonstrated that gypsy moth larvae had been killed independent of the presence of other midgut bacteria, by engineered *E. coli* able to produce the insecticidal toxin of *B. thuringiensis*. Heat-killed engineered *E. coli* fed to gypsy moth larvae, however did not kill the larvae in the absence of other midgut bacteria. They suggested that the microbial community present in the midgut of larvae makes a contribution to the death of larvae subjected to *B. thuringiensis* treatment (Broderick *et al.*, 2006). In another study it was demonstrated that aseptically reared *Homona magnanima* (Diakonoff) (Lepidoptera: Tortricidae) larvae supported 20 times greater growth of *B. thuringiensis* than larvae containing gut microbiota, which suggests that intestinal bacteria influence the growth of the insect pathogen in the larvae (Takatsuka & Kunimi, 2000).

With all the previous deductions, a new hypothesis that states that members of the *Enterobacteriaceae* (*E. coli* and *Enterobacter* spp.) must also be present to induce septicemic death of larvae, could be constructed. The following is a summary from Broderick *et al.* (2006), which indicates that the midgut bacteria residing in insect larvae contribute to mortality associated with the consumption of *B. thuringiensis*:

- insecticidal activity was abolished by eliminating the detectable midgut bacterial community.
- insecticidal activity was restored by reintroducing an *Enterobacter* spp., a member of the normal gut community.
- a live *E. coli* strain that produces the *B. thuringiensis* toxin killed the larvae whether or not they contained other bacteria in their midguts, but if the *E. coli* was heat-killed before feeding, it killed only those larvae that contained the normal gut community (Broderick *et al.*, 2006).

1.3.3 Various genes expressed by Bt maize

Substantial industrial and academic research in Africa, Asia and America has demonstrated the efficacy of a variety of cry proteins from *B. thuringiensis* against various insect groups (Evans, 2002; Gressel *et al.*, 2004). To ensure high levels of control of all Lepidoptera pest species, multiple proteins could be simultaneously introduced into one crop cultivar. By employing a constitutive promoter, all relevant maize tissues could be protected in a way that is not possible when insecticides are applied through spraying. This is especially applicable for stem and ear tissues that are favoured by stem borers and are not accessible through spraying (Gressel *et al.*, 2004).

The different Bt toxins are encoded for by various Cry proteins that have a certain activity spectrum against particular insect orders (Evans, 2002). The specificity of the different Cry genes against different groups of insects is as follows:

- CryI – Lepidoptera active
- CryII – Lepidoptera and Diptera active
- CryIII – Coleoptera active
- CryIV – Diptera active
- CryV – Coleoptera and Lepidoptera active
- Cyt genes – genes with general toxicity (Evans, 2002).

1.4 Resistance development to Bt-maize

Bt-maize, expressing CryIAb insecticidal proteins was introduced for control of two stem borer species, *B. fusca* and *C. partellus* in South Africa after its development for control of other stem borer species in North America (Archer *et al.*, 2001).

Bt-maize has been rapidly adopted in South Africa and has until recently been very effective against the target pest *B. fusca*. However, the first report of field resistance was made in the Christiana area in the North West province in 2006 (Van Rensburg, 2007). Within one year of the first report of resistance another resistant population was recorded in the Vaalharts irrigation scheme, approximately 60 km from the initial site (Kruger *et al.*, 2011). There have also been other reports of survival of *B. fusca* on Bt maize plants (Van Rensburg, 1998, 2001). Knowledge of the mechanisms and inheritance of resistance do not exist. The gaps in our knowledge of mechanisms of resistance, diversity of resistance

sources, and inheritance of resistance have limited our success in developing cultivars with desired levels of resistance (Sharma, 2006).

1.4.1 Evolution of resistance to insecticides

The first report of insect resistance to insecticides was made in 1948. This was within six years of the introduction of the first synthetic insecticide, namely DDT (Hammerton & Stowell, 1963; Burgess, 2004).

The use of insecticides as such does not result in resistance evolution. Resistance develops when naturally occurring genetic mutations allow a small proportion of the population (approximately 1 in 100 000 individuals) to resist and survive the effects of the insecticide. If this advantage is maintained within the target population by continually using the same insecticide, the resistant insects will reproduce and the genetic changes that confer resistance will then be transferred from parents to offspring so that resistant individuals eventually become numerous within the population. This selection process is the same as that which drives other evolutionary changes. The process will take longer if the gene conferring resistance is rare or present at a low frequency (IRAC, 2007).

There exist two types of resistance, namely field-evolved resistance and laboratory-selected resistance. Field-evolved resistance can be defined as a genetically based decrease in susceptibility of a population to a toxin caused by exposure of the population to the toxin in the field (Tabashnik, 1994). Laboratory-selected resistance occurs when exposure to a toxin in the laboratory causes a heritable decrease in susceptibility of a laboratory strain. Because both field-evolved and laboratory-selected resistance entail changes in gene frequency across generations, they exemplify evolution (Tabashnik *et al.*, 2009).

Because microbial preparations of the entomopathogenic bacterium *B. thuringiensis* had been applied as a spray for decades, without substantial resistance developing in field populations of the target pest, it was presumed that evolution of resistance to Bt crops was unlikely (De Barjac, 1987; Krieg & Langenbruch, 1981). Under laboratory conditions, five out of 10 species of moths representing the families Noctuidae, Plutellidae and Pyralidae, that was selected for resistance to Bt, developed more than a 10-fold level of resistance. This suggested that the ability to evolve resistance to Bt was a common phenomenon among the Lepidoptera (Tabashnik, 1994).

1.4.2 Resistance mechanisms to Bt-toxin

Three main resistance mechanisms to Bt have been proposed. These include the binding of the toxin, insect tolerance to toxins and replacement of damaged cells.

1.4.2.1 Binding of toxin

The most common mechanism of resistance is altered binding of the Cry1Ab toxins to target sites in the brush border membrane of the larval midgut (Ferré & Van Rie, 2002). Resistance has also been attributed to genetically acquired decreases in the numbers of toxin binding sites, decreased affinity of binding sites, and lowered titers of enzymes that process the protoxins in the gut (Huang *et al.*, 1999).

A study conducted by González-Cabrera *et al.* (2003) reported a difference between the binding sites for Cry1Ab in resistant and susceptible strains of the pink bollworm *Pectinophora gossypiella* (Lepidoptera: Gelechiidae). In the resistant strain, the common binding site lost affinity for Cry1Ab. This is consistent with the general finding that reduced binding of toxin is a primary mechanism of insect resistance to Cry proteins (González-Cabrera *et al.*, 2003).

1.4.2.2 Insect tolerance to insecticides/toxins

The potential benefits of Bt crops include reduced use of insecticides, conservation of natural enemies, regional suppression of some pest populations and associated increased yield (Herdt, 2006; Huang *et al.*, 2005; Hutchinson *et al.*, 2010). These benefits will, however, be diminished as pests evolve resistance to Bt toxins (Tabashnik *et al.*, 1990; Tabashnik *et al.*, 2008). Resistance should, however, not be confused with tolerance that can occur after sub-lethal exposure to insecticide (IRAC, 2007). Insect tolerance to a given insecticide or other insecticidal product may be expected to develop rapidly when an insect population is characterized by a high rate of development and a quick succession of generations, while being exposed to sub-lethal levels of the toxin (IRAC, 2007).

Sub-lethal doses of Bt toxin does not result in death of target insects since damaged midgut cells are replaced, allowing normal feeding and metamorphosis (Spies & Spence, 1985). Sufficiently high doses of Bt toxin must be administered to kill pest insects (Martinez-Ramirez *et al.*, 1999). Some laboratory reared resistant strains of *Heliothis virescens*

(Fabricius) (Lepidoptera: Noctuidae) have been reported to withstand doses of Bt toxin up to 10 000 times greater than susceptible strains (Heckel *et al.*, 1998).

1.4.2.3 Replacement of damaged cells

Another mechanism of Bt resistance was proposed by Loeb *et al.* (2001). This mechanism suggests the involvement of increased production of stem and differentiating cells inside the insect gut, allowing insects to quickly overcome the loss of mature cells destroyed by Bt toxin. This possibility was anticipated by Martinez-Ramirez *et al.* (1999) who found that resistant and susceptible strains of *H. virescens* sustained the same amount of cellular damage to their midguts in the presence of sublethal doses of pure Bt toxin, and suggested that the resistant strain survived because it could more readily replace its damaged cells.

1.4.3 Insect resistance management

A number of resistance management strategies exist, but only the high-dose/structured refuge strategy, which is used in South Africa will be discussed.

Generally, the high-dose/structured refuge strategy has received the most attention as an optimal insect resistance management strategy (Glaser & Matten, 2003). The success of this strategy is based on several assumptions (Roush, 1994; Gould, 1998; USEPA, 1998, 2001). The basic requirements for the high-dose/refuge strategy include:

- resistance genes must be recessive and resistance is conferred by a single locus with two alleles in three insect genotypes (RR, SS and RS)
- heterozygote survival is less than 5 % of RR
- resistance genes are rare
- non-transgenic refuge plants will sustain a susceptible pest population
- refuge proximity is sufficient to ensure nearly random mating between resistant and susceptible adults
- the crop will express 25 times the toxin concentration required to kill 99 % of the susceptible (S) individuals (Glaser & Matten, 2003)

The success of genetically engineered crops producing insecticidal toxins will be short-lived if pests adapt to them (Butler & Reichhardt, 1999). The primary strategy for delaying insect resistance development to transgenic Bt plants is to provide refuges of host plants that do

not produce Bt toxins (Lui *et al.*, 1999). The development of insect resistance to Bt crops is potentially delayed by providing susceptible insects for mating with resistant insects. However, a study conducted by Lui *et al.* (1999), showed that a resistant strain of *P. gossypiella* larvae on Bt cotton took longer to develop than susceptible larvae on non-Bt cotton. The fact that the two strains have different development rates favours non-random mating that could reduce the expected benefits of the refuge strategy (Lui *et al.*, 1999).

Random mating between susceptible and resistant insects is one of the two critical assumptions for effective functioning of the refuge strategy, while the other is that the inheritance of resistance needs to be recessive. It is thus critical that the two strains of larvae develop at the same rate for the refuge strategy to be effective (Lui *et al.*, 1999). This developmental asynchrony therefore favors assortative mating among resistant moths originally from Bt plants. In the field, the extent of developmental asynchrony and assortative mating would be affected by variation in toxin expression, weather and overlap between generations. Assortative mating would generate a disproportionately high number of homozygous resistant insects, accelerating the evolution of resistance (Lui *et al.*, 1999).

1.5 Microorganisms associated with insects

Microorganisms play important and often essential roles in the growth and development of many insect species (Broderick *et al.*, 2003). Relatively little is known about the microbial associates in insect groups that feed on foliage (Broderick *et al.*, 2003), especially the bacteria associated with Lepidoptera. The Lepidoptera is primarily a phytophagous group and one of the largest insect orders containing over 150 000 species (Scoble, 1992). Studies by Campbell (1990), McKilip *et al.* (1997) and Broderick *et al.* (2003), indicated that lepidopterans harbour mid-gut bacteria. There is, however, a lack of understanding of the types of microbes present in the midgut and of their roles in insect development and function. Knowledge of the gut bacteria of the Lepidoptera and the roles they may play in larval biology could lead to new strategies for pest management. For example, if a higher number of certain bacteria are present in Bt-resistant larvae, and if it affects larval fitness, this would provide novel avenues to manage resistance (Broderick *et al.*, 2003).

There are several possibilities concerning the origin and establishment of the bacterial midgut composition in insects. For one, it is not known whether midgut bacteria are established through ingestion of plant material or whether different host plant chemistries select for specific bacterial genotypes (Broderick *et al.*, 2003). It is assumed that many

insect species derive their microbiota from the surrounding environment such as the phylloplane of food plants, but the degree of persistence of strains of the ingested species is unknown. It is also not known if strains of these species occupy particular niches in the gut and colonize gut epithelia, or whether they are present in all members of the same insect species (Dillon & Dillon, 2004).

1.5.1 Factors affecting diversity of microbiota

Microbial communities are dynamic and often experience change in composition and structure. These changes can result from variations in nutrient availability, physical aspects of the environment and proximity to other organisms (Butler *et al.*, 2003; Carrero-Colon *et al.*, 2006; Kiorboe *et al.*, 2003; Vinas *et al.*, 2005). Many microbial communities, for example those inhabiting lakes, soil, insects, humans, and other animals, experience temporal changes associated with factors such as season, nutrient availability, and host development (Höfle *et al.*, 1999; Mackie *et al.*, 1999; Smalla *et al.*, 2001; Van der Wielen *et al.*, 2002; Vasanthakumar *et al.*, 2006; Villa-Costa *et al.*, 2007).

The diversity of the microbiota in insects relates in part to the variety of specialized structures present in the gut and the effect of pH, reduction-oxidation conditions, digestive enzymes present, and type of food ingested. In view of the diversity of insects and the absence of detailed information about many genera, it is impossible to generalize about the occurrence of gut microbiota in digestive tracts of insects (Dillon & Dillon, 2004).

There are no specialized structures in the gut of lepidopteran larvae that are usually associated with microorganisms, and in many of these folivores there is a rapid food throughput. It was therefore previously assumed that gut microorganisms contributed little towards nutrition and digestion (Appel, 1994).

However, the biological effects and role of gut-microorganisms have been underestimated. Molecular approaches, instead of culture techniques, for the detection and characterization of microbes have resulted in a dramatic change in the understanding of microbial diversity (Amann *et al.*, 1995). Through molecular techniques, it is now known that insects with a rapid food throughput harbor indigenous microbiota (Dillon & Dillon, 2004). For example, the locust *Schistocerca gregaria* (Forsskål) (Orthoptera: Acrididae) maintains a throughput time of 1.5 h while constantly feeding, but this decreases rapidly during periods without food (Dillon & Charnley, 1988). Despite the absence of specialized structures, there is a

substantial population of bacteria residing in the hindgut cuticle of the desert locust, *Schistocerca gregaria* (Hunt & Charnley, 1981). Bacteria have also been found incorporated into the peritrophic matrix of migratory grasshoppers (Mead *et al.*, 1988). Bacteria have also been found to occupy the gut lumen of *Manduca sexta* (Linnaeus) (Lepidoptera: Sphingidae) (Toth-Prestia & Hirshfield, 1988). In the case of the fruit fly *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae) the main site of microbial colonization is the midgut lumen inside the peritrophic matrix (Murphy *et al.*, 1994). In insect species where the main indigenous bacterial community is confined to the gut lumen, the doubling time of bacteria needs to exceed the food transit time in order to maintain a stable population unless there is some counter current movement of bacteria (Dillon & Dillon, 2004).

Experimental models of microbial communities in insects are either batch systems (closed system) or flow-through systems such as chemostats (fresh medium is continuously added, while culture liquid is continually removed to keep the culture volume constant). For these models nutrient concentrations are growth-limiting (Atlas & Bartha, 1998). These two models give different microbial growth kinetics and there are elements of these models found in different types of insect gut and insects in different feeding states. A constantly feeding insect with a high food throughput is therefore likely to possess a microbial community profile different from that of an insect that has not fed for several days (Dillon & Dillon, 2004). Bacterial division can occur as often as every 20 minutes and viable bacterial mutations are generated during every cycle, allowing the indigenous microbiota to adapt rapidly to changes in the gut environment. This adaptation and its consequences for the insect host are almost completely unknown (Dillon & Dillon, 2004).

1.5.2 Microbiological methods used to study insect gut microbiota

Previous studies using traditional microbiological methods characterize the gut microbiota by phenotypic characterization of isolates (Dillon & Dillon, 2004). These studies established an essential basis for the current interest in gut microbiota, but they often presented a biased description of the insect gut microbiota. This was largely due to the fact that the media used for isolation were often the same as those used in medical studies. Hence some microorganisms that were recorded as numerically dominant were possibly biologically unimportant. Culture media should account for environmental factors such as the pH and available nutrients encountered inside the insect gut. The presence of unculturable bacteria was also previously largely ignored. It is now recognized that

approximately 99 % of the microbes in the environment cannot be cultivated (Amann *et al.*, 1995).

Although nucleic acid-based approaches have provided new information, they also have their own limitations and inherent bias, for example, there are differences in the ease of extraction of nucleic acids from different bacterial cell types. Sequenced-based studies are often flawed in terms of descriptions of sampling strategies, lack of information on sample size, and generally limited use of statistical tests of hypotheses (Ward *et al.*, 1990). The availability of culture independent tools provides an opportunity to detect and classify microorganisms that cannot be cultured using existing methods (Ward *et al.*, 1990). Profiling the insect gut microbiota is now feasible using methods based on the 16S rRNA genes such as denaturing gradient gel electrophoresis (DGGE) (Schabereiter-Gurtner *et al.*, 2003).

1.5.3 Brief overview of methods used in the current study

Two main approaches were followed to conduct the current study. Community diversity of the gut-microorganisms was determined using molecular methods including PCR, DGGE and 16S rDNA sequencing. The second approach was to determine the relative quantity of *Enterobacter* spp. and *Escherichia coli*, using cultural methods.

1.5.3.1 Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE is considered a molecular fingerprinting technique that was initially designed to study microorganisms that are difficult to culture. It is therefore used to study these organisms as they occur in their natural habitat, such as soil and water, without having to culture them prior to any experimental procedures (Muyzer & Smalla, 1998). DGGE provides an indication of the diversity and succession of the community of microbes and usually exploits the comparison of rDNA of different microbes (Hori *et al.*, 2006).

The principles on which DGGE rely are illustrated in Figure 1.4. PCR-amplified 16S rRNA gene fragments are electrophoresed in an increasing solution gradient of polyacrylamide gel (Fischer & Lerman, 1979). The gradient is provided by denaturants such as urea and formide (Ercolini, 2004). One of the PCR primers usually contains a GC-rich sequence which is referred to as the GC-clamp. The function of the GC-clamp is to maintain the integrity of the one end of the heteroduplex, thus maintaining a partially double stranded molecule (Myers *et*

al., 1985; Sheffield *et al.*, 1989). Certain regions show discrete melting preferences and are thought to be melting domains. These are stretches of base pairs possessing the same melting temperature. When the region with the lowest melting temperature achieves its melting temperature at a certain position in the DGGE gel, the migration of the DNA molecule will be retarded due to the partially melted conformation. When the base sequences differ, so will the melting temperature and the migration distance of the different fragments of DNA as indicated by Figure 1.4. Organisms A, B and C migrated to different positions on the DGGE gel, indicating different base sequences. DNA fragments of similar size but different sequences can be separated using this technique (Muyzer *et al.*, 1993).

The visualization of DNA bands under UV light is made possible by ethidium bromide staining of the gel (Muyzer & Smalla, 1998). SYBR Green I (Muyzer *et al.*, 1997) and silver staining (Felske *et al.*, 1996) can also be used. SYBR Green I allows for the visualization of low concentration DNA fragments because of less background staining (Muyzer *et al.*, 1997). Increased sensitivity is achieved by silver staining but single stranded DNA is also stained (Heuer & Smalla, 1997).

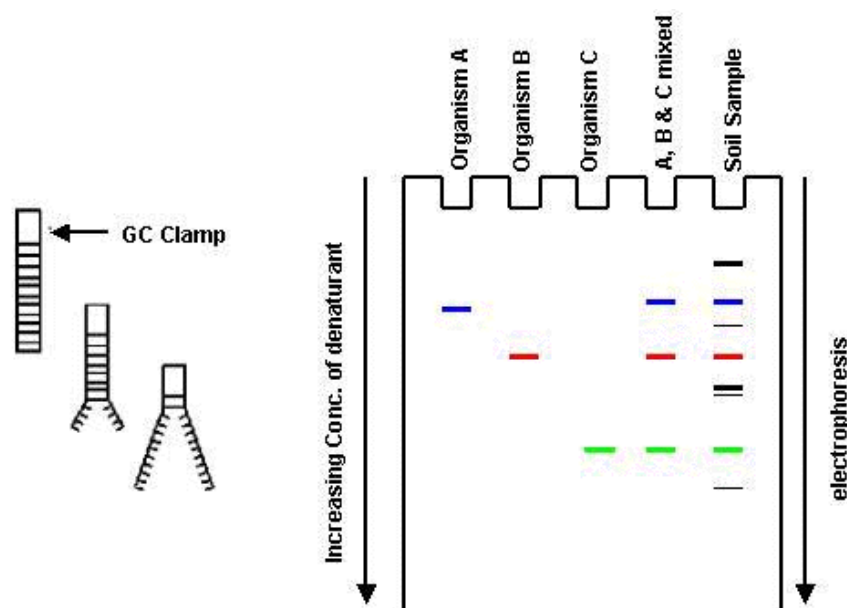


Figure 1.4: A simplified diagram illustrating the principles of DGGE. (Source: www.environmental-expert.com).

PCR-DGGE is a method which provides information on community structure and diversity without the need to culture the microorganisms. Thus, more accurate analysis can be done in terms of the presence and diversity of bacteria within the gut of the maize stem borer *B. fusca*, which was the insect species used in this study.

1.5.3.2 Cultural methods

The current study was based on the isolation and purification of coliform bacterial colonies. Coliform bacteria are commonly used as bacterial indicator of the sanitary quality of foods and water (Ashbolt *et al.*, 2001). These bacteria are rod-shaped, gram-negative, non-spore forming, oxidase-negative organisms that ferment lactose (with β -galactosidase) to acid and gas within 24-48 hours when incubated at 35-37°C. Coliforms are bacteria that are not specific indicators of faecal pollution and they include the following genera: *Enterobacter*, *Klebsiella*, *Citrobacter*, *Hafnia*, *Escherichia*, *Serratia*, and *Yersinia* (Ashbolt *et al.*, 2001).

Escherichia coli is a rod-shaped organism and can be distinguished from most other coliforms by its ability to ferment lactose at 44°C, and by its growth and colour reaction on certain types of culture media (Ewing, 1986). *Enterobacter* are the most important organisms considered in this study. It is a genus of common Gram-negative, facultative-anaerobic, rod-shaped bacteria of the family Enterobacteriaceae (Ashbolt *et al.*, 2001). Two important species from this genus considered in the current study are *E. aerogenes* and *E. cloacae*.

1.6 Research aim and objectives

Microorganisms play important roles in the growth and development of insects, and possibly also in resistance development. However, no information exists on gut-microbes of *B. fusca*. The aim of the study was to determine the community diversity of gut-microorganisms in Bt-resistant and Bt-susceptible larvae of the maize stem borer (*Busseola fusca*) (Lepidoptera: Noctuidae).

The specific objectives of the study were:

- i) to determine the gut microbe community diversity in Bt-resistant and Bt-susceptible *B. fusca* larvae collected from different geographical areas.
- ii) to identify the microbes occurring inside the midgut of *B. fusca*.
- iii) to determine the quantity of members of the Enterobacteriaceae family (specifically *E. coli* and *Enterobacter* spp.) in Bt-resistant and Bt-susceptible *B. fusca* larvae

collected from different geographical areas and comparing the prevalence of these bacteria with community diversities obtained with DGGE analysis.

CHAPTER 2

MATERIAL AND METHODS

2.1 Sample collection

Stem borer larvae were collected from maize fields in different geographical areas. The areas were Venda (Limpopo province), Ventersdorp and Potchefstroom (North-West province), Delmas (Mpumalanga province) and the Vaalharts area (Northern Cape). These areas were selected based upon current knowledge of Bt-resistant strains of *B. fusca*, with the Vaalharts area being the site with a confirmed Bt-resistant population (Kruger *et al.*, 2011).

Larvae were collected at the different localities by dissecting Bt maize plants that exhibited symptoms of stem borer damage. Larvae were brought to the NWU microbiology laboratory in cooler-bags. A number of larvae of each field-collected population were immediately dissected while other were divided into two groups and reared on Bt and non-Bt maize respectively.

Eggs were obtained from moths originating from the larvae which were reared on Bt and non-Bt maize respectively. Larvae obtained from these eggs were put on Bt and non-Bt maize tissue (cut maize stems in test tubes) in the laboratory and in one instance they were also put on Bt and non-Bt plants in the field. Eggs were specifically used to ensure that larvae were exposed to the same conditions throughout the assays.

Table 2.1: A summary of samples collected from different geographical areas and reared on different food.

Collection site	Experiment	Food source	Resistance status of larvae	What was done?
Delmas	Field and Lab	Non-Bt maize	Susceptible to Bt	Plated out, DGGE and sequenced
Ventersdorp	Field and Lab	Non-Bt maize	Susceptible to Bt	Plated out, DGGE and sequenced
Ventersdorp	Lab	Bt-maize	Susceptible to Bt	Plated out, DGGE and sequenced
Vaalharts	Greenhouse (Tunnel)	Bt-maize	Resistant to Bt	Plated out, DGGE and sequenced
Vaalharts	Field and Lab	Bt-maize	Resistant to Bt	Plated out, DGGE and sequenced
Vaalharts	Field and Lab	Non-Bt maize	Resistant to Bt	Plated out, DGGE and sequenced

Larvae were reared on maize stems collected from both the field as well as from the greenhouse tunnels of the NWU. Larvae were reared until the fourth and fifth instar after which dissection took place.

Bt-susceptible larvae were collected from Delmas and Ventersdorp, but during the rearing process in the latter, it was revealed that these larvae were resistant to Bt and that they survived on Bt-maize. This was observed after which 98% of larvae collected from Ventersdorp, reared on Bt-maize, survived.

Fourth/fifth instar larvae were placed in 95% ethanol. Alimentary tracts were removed using aseptic dissection techniques. The dissection procedure is illustrated in figure 2.1. Midguts of three larvae were isolated and placed in a 1.5 ml sterile Eppendorf tube (Separations, US) containing 500 µl sterile distilled water. This was done in triplicate, thus nine midguts in total were used. The tubes were sonicated for 60 seconds and centrifuged for six seconds, to disintegrate the midgut for release of midgut contents.

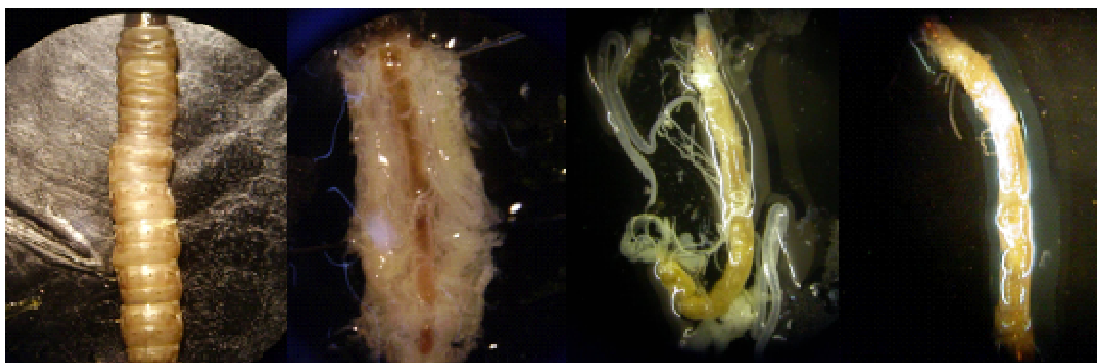


Figure 2.1: Dissection of *B. fusca*, using a stereomicroscope, to obtain the midgut (far right).

2.2 DNA isolation

2.2.1 DNA isolation of gut microorganisms

DNA was extracted directly from the gut contents of *B. fusca* larvae using a DNA isolation kit (Nexttec, Germany). The manufacturer protocol was followed. Bacterial cells present in the gut contents were lysed by the addition of two lysis buffers, RNase A and lysozyme. Purification was achieved by centrifuging the lysate through a Nexttec clean column. DNA concentrations and quality ($A_{260nm}:A_{280nm}$ ratios) were determined by a NanoDrop™ 1000 Spectrophotometer (Thermo Fischer Scientific, US).

2.2.2 DNA isolation of marker microorganisms (ATCC pure cultures)

Overnight cultures of *E.coli*, *E. cloacae* and *E. aerogenes*, grown in nutrient broth (Merck, SA) were used for DNA extractions. The same protocol was followed as described in Section 2.2.1.

2.3 Agarose gel electrophoresis of extracted DNA from midgut microorganisms and marker microorganisms

DNA quality was determined by using a 1 % (w/v) agarose gel containing EtBr (0.01%) (Bio-Rad, UK). Electrophoresis conditions were set at 80 V for 45 min. Extracted DNA (5 µl) was mixed with 5 µl 6 x Orange Loading dye (Fermentas Life Science, US). A 1 x TAE buffer (20 mM Acetic acid (Merck, US), 40 mM Tris (Sigma Aldrich, US) and 1 mM EDTA (Merck, US), pH 8.0) was used. A 100 bp molecular marker (O'GeneRuler, Fermentas Life Science, US) was used to confirm the fragment sizes by loading it into each gel. A Gene Bio Imaging

System (Syngene, Synoptics, UK) and GeneSnap (version 6.00.22) software was used to capture the gel images (Bezuidenhout *et al.*, 2006).

2.4 DNA amplification

DNA, obtained from marker microorganisms and midgut microorganisms, was amplified using a nested-PCR approach in conjunction with touchdown cycling conditions. An ICycler thermal cycler (Bio-Rad, UK) was used to amplify the DNA. Each 25 µl reaction mixture consisted of 12.5 µl double strength PCR master mix (0.05 U/µl *Taq* DNA Polymerase in reaction buffer, 0.4 mM of each dNTP, 4 mM MgCl₂) (Fermentas Life Science, US), 9.3 µl PCR-grade water (Fermentas Life Science, US), 100 ng sample DNA, 0.2 µl additional *Taq* DNA Polymerase (9 U), 0.5 µl additional MgCl₂ (0.5 mM) and 0.5 µl 27F and 1492R primer mix (Table 2.1; Inqaba Biotech, SA). Cycling conditions were set at 95°C for 10 min, a total of 30 cycles consisting of 94°C for 1 min denaturation, 65°C for 1 min annealing and 72°C for 2 min extension. Annealing temperature was lowered by 2°C every 4 cycles up until it reached 55°C. Seven cycles were carried out at 55°C.

Second round amplifications were carried out under the same conditions and reaction mixture with the exception of the primer set and first round amplification products (2 µl) being used. Primer sets 341F-GC and 907R (Table 2.1; Inqaba Biotech, SA) were used for second round amplification.

2.5 Agarose gel electrophoresis of PCR products

Agarose gel electrophoresis as described in Section 2.3 was used to confirm DNA amplification. PCR products were run on a 1.5 % (w/v) agarose gel (PeQlab, Germany). Agarose gel electrophoresis was only conducted after second round amplification.

Table 2.2: Primer sets used for PCR reactions for DGGE and amplification of excised bands for sequencing analysis

Gene	Primer Sequence	Size (bp)
16S rRNA (1 st Round PCR)	27F: 5'-AGAGTTTGATCMTGGCTCAG-3' 1492R: 5'-TACGGYTACCTTGTTACGACTT-3'	1465*
16S rRNA GC (2 nd Round PCR)	341F-GC: 5'-CGCCCGCCGCGCGCGGCGGGCGGGGC GGGGGCACGGGCCTACGGGAGGCAGCAG-3' 907R: 5'-CCGTCAATTCCTTTGAGTTT-3'	566**
16S rRNA (Sequencing PCR)	338F: 5' – ACTCCTACGGGAGGCAGCAG-3' 518R: 5' – ATTACCGCGGCTGCTGG-3'	180

* Lane (1991)

** Muyzer *et al.* (1993)

2.6 Denaturing gradient gel electrophoresis (DGGE)

DGGE was performed to determine the community diversity of bacteria present in the gut contents of *B. fusca* with special attention to the presence of *E. coli* and *Enterobacter* spp. (marker microorganisms).

DGGE analysis was done using a D-Code Universal Mutation Detection System (Bio-Rad, UK). A 1-mm-thick 8.0% (w/v) polyacrylamide gel with a denaturing gradient ranging from 30 to 50% (100% denaturant contained 7 M urea and 40% formamide). Electrophoresis was conducted at 60 °C for 16 h at a constant voltage of 100 V. Staining was done for 20 min in 1 x TAE containing ethidium bromide (0.01%) (Bio-Rad, UK) for visualization of bands. Gene Genius Bio Imaging System (Syngene, Synoptics, UK) and GeneSnap software (version 6.00.22) were used for visualization.

2.7 Excision of prominent bands from DGGE gel

Prominent bands and bands with the same migration potential as the marker organisms (the ATCC pure cultures of *E. coli*, *Enterobacter cloacae* and *Enterobacter aerogenes*) were excised using a sterile scalpel. Excised bands were placed in sterile Eppendorf tubes after

which 50 µl of PCR-grade water (Fermentas Life Science, US) was added. The gel containing the band was stabbed with sterile tips for DNA to be released into the water.

2.8 PCR of DNA from excised bands

Each 25 µl reaction mixture consisted of 12.5 µl double strength PCR master mix (0.05 U/µl *Taq* DNA Polymerase in reaction buffer, 0.4 mM of each dNTP, 4 mM MgCl₂) (Fermentas Life Science, US), 9.3 µl PCR-grade water (Fermentas Life Science, US), 2 µl of the water and DNA mixture, 0.2 µl additional *Taq* DNA Polymerase, 0.5 µl additional MgCl₂ and 0.5 µl 338F and 518R primer mix (Table 2.1; Inqaba Biotech, SA). Cycling conditions were set at 95°C for 5 min denaturing; 30 sec at 95°C for melting, 30 sec at 56°C for annealing, 1 min at 72°C for extension, these conditions were repeated for 30 cycles; final extension was done at 72°C for 5 min.

2.9 Agarose gel electrophoresis of PCR products

Agarose gel electrophoresis as described in Section 2.3 was used to confirm DNA amplification. PCR products were run on a 1.5 % (w/v) agarose gel (PeQlab, Germany). DNA concentrations and quality ($A_{260\text{nm}}:A_{280\text{nm}}$ ratios) were determined using a NanoDrop™ 1000 Spectrophotometer (Thermo Fischer Scientific, US).

2.10 Sequencing

Amplicons were sequenced by Inqaba Biotech, SA using the 518R primer. Chromatograms were viewed with Geospiza Finch TV (version 1.4) software. Amplified sequences were identified using BLAST searches (<http://www.ncbi.nlm.nih.gov/BLAST>).

2.11 Enumeration on selective agar

Suspensions containing the midguts as described in Section 2.1 and Figure 2.2 were serially diluted to 10⁻⁵. The dilutions were then spread-plated onto mFc agar (Difco Laboratories, US) and Brilliant Green Bile Agar (Difco Laboratories, US) using aseptic techniques. mFc agar plates were incubated at 44.5°C for 24 hours. Brilliant Green Bile agar plates were incubated at 37°C for 24 hours. Colonies (between 10 and 300 per plate) were counted based upon colour. *Enterobacter* spp. represented the purple/pink colonies

on Brilliant Green Bile agar. Feecal *coliforms* (*E. coli*) represented the blue colonies on mFc agar.

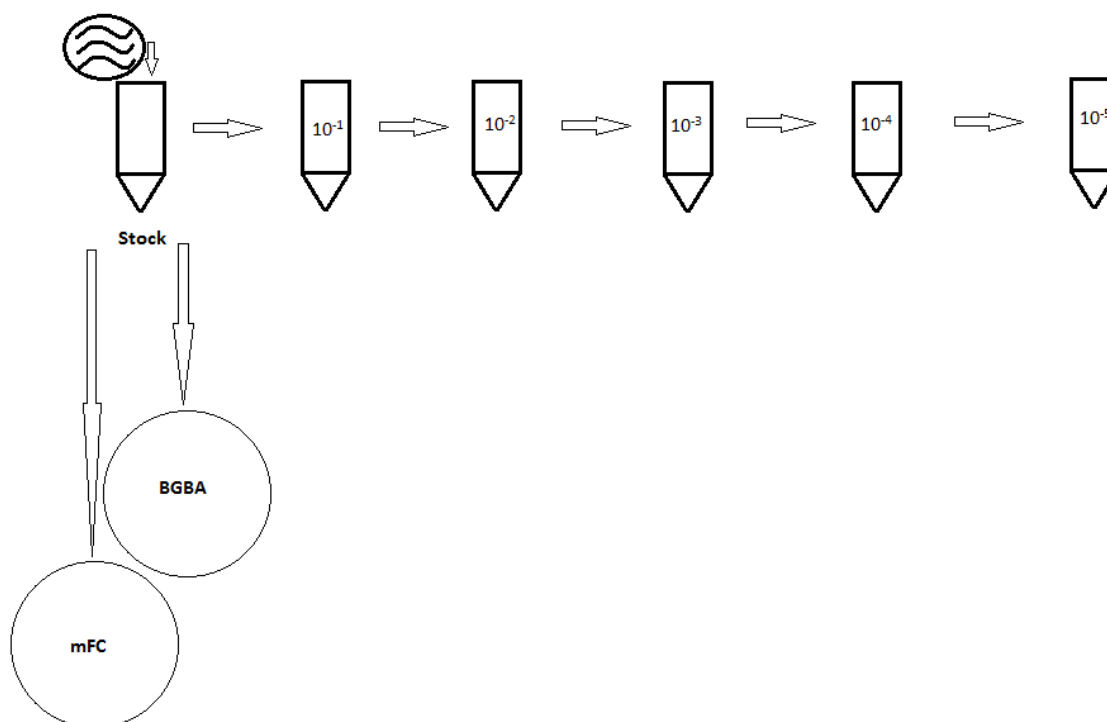


Figure 2.2: Diagrammatic illustration of the serial dilution. The stock solution contained mid-guts from three larvae. The stock and each of the dilutions were plated out three times on both the mFc and Brilliant Green Bile agar.

2.12 Isolation and purification

Single colonies were selected from the mFc and Brilliant Green Bile agar and streaked onto nutrient agar (Merck, US) plates to obtain pure colonies. This was repeated three times to assure the purity of the colonies. Plates containing the colonies from mFc plates were incubated at 44.5°C for 24 hours. Plates containing colonies isolated from Brilliant Green Bile agar were incubated at 37°C for 24 hours.

2.13 Confirmation identification of selected isolates

Gram staining and triple sugar iron tests were performed on the pure colonies as a confirmation for the presence of *E. coli* (Feecal *coliforms*) and/or *Enterobacter* spp. (Enterobacteriaceae).

2.14 Data analysis

Colonies of faecal *coliforms* and *Enterobacter* spp. were counted and expressed as colony forming units (cfu's) per ml. The numbers of colony forming units (cfu's) were then compared to the DGGE analysis to determine if there were similarities.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Introduction

This study compared the community diversity of microorganisms present in the midgut of different *B. fusca* populations which are either resistant or susceptible to Bt maize. DNA was extracted from the contents of the midgut after which it was amplified using PCR. These PCR products were subjected to DGGE analysis for determination of the community diversity. Prominent bands, normally representing the most abundant organisms present in the gut were excised and subjected to 16S rDNA sequencing. Furthermore, the contents of the midgut were serially diluted and plated out on Brilliant Green Bile Agar and mFc agar to enumerate *E.coli* and *Enterobacter* spp. present in the midgut.

3.2 DNA extractions

Genomic DNA was extracted from three overnight ATCC pure cultures (*E.coli*, *E. cloacae* and *E. aerogenes*) to serve as marker organisms on each DGGE gel. DNA was also isolated from 24 Eppendorf tubes containing the gut contents of three larvae per tube. This was executed using the genomic DNA isolation kit from Nexttec (Section 2.2.1). The quantity and quality of the DNA extracted from the ATTC cultures are indicated in Figure 3.1. The extractions were done in duplicate. The quantity of the extracted DNA was sufficient. There was no fragmentation or RNA observed.

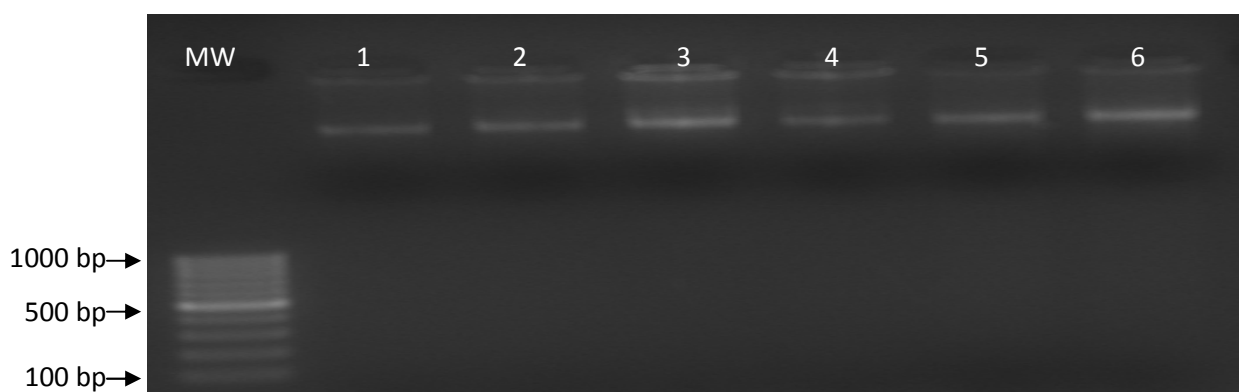


Figure 3.1: An ethidium bromide stained agarose gel (1 % w/v) indicating DNA isolated from ATCC pure cultures used as marker organisms. MW represents the 100bp molecular size marker (O'GeneRuler™ 100bp DNA ladder, Fermentas Life Science, US). Lanes 1 & 2 – *Enterobacter cloacae*; lanes 3 & 4 – *Escherichia coli*; lanes 5 & 6 – *Enterobacter aerogenes*.

The results, as indicated in Figure 3.1, were confirmed by determination of $A_{260\text{nm}}:A_{280\text{nm}}$ ratios and DNA concentrations making use of a NanoDrop™ 1000 Spectrophotometer. The 260:280 ratios varied between 1.35 and 1.55. Low ratios (below 1.8) usually indicate protein contaminations. DNA concentrations ranged between 150 and 400 ng/μl. These DNA concentrations were sufficient for subsequent analysis and were concordant with the results obtained from gel electrophoresis indicated in Figure 3.1.

Extracted total microbial DNA from the midgut contents of larvae could, however, not be visualized on the 1% agarose gels as the DNA concentrations obtained after extraction were too low. Concentrations varied between 20 and 30 ng/μl. However, the 260:280 values were between 1.40 and 1.70. A reason for the low concentration of DNA obtained could be due to no prior cultivation of the organisms as the DNA was extracted directly from the midgut contents in order not to eliminate the non-culturable organisms. This was, however, not a problem for subsequent analysis because DNA was first amplified using a nested PCR approach before subjecting the amplified DNA to DGGE analysis.

Zhang and Jackson (2008) extracted DNA from the alimentary tract of *Costelytra zealandica* (Coleoptera: Scarabaeidae) to indicate autochthonous bacterial flora, using the Fast Prep DNA isolation kit (Fast Prep™, Bio101) (Zhang & Jackson, 2008). In the current study, DNA was isolated using a DNA isolation kit (Nexttec, Germany). Broderick *et al.* (2003), on the other hand, extracted DNA from Gypsy moth larval midguts for a census of the bacterial community using a modified version of the extraction method described by Ausubel *et al.* (1994). The method used by Broderick *et al.* (2003) involves mechanical sheering of bacterial cells present in the midgut contents after which DNA was extracted using phenol and chloroform. Rani *et al.* (2009) used a similar approach to that of Broderick *et al.* (2003) to extract DNA from the midgut of the mosquito *Anopheles stephensi* (Rani *et al.* 2009). Both these methods, DNA extraction by means of a kit and DNA extractions using phenol and chloroform provided good quality DNA (Rani *et al.* 2009; Broderick *et al.* 2003). Phenol:chloroform extractions are however, time consuming and a kit was therefore the preferred technique for the present study.

3.3 DNA amplification for DDGE

DNA was amplified using a nested PCR approach under conditions described in Section 2.4. The 16S primer sets (Table 2.1) were used to amplify genomic DNA of the marker organisms and bacteria isolated from the midgut of *B. fusca* larvae. Genomic DNA of the

marker organisms and isolated bacteria were subjected to two PCR reactions, first with the 1465 bp primer and then with the 566 bp primer. DNA amplification with the 1465 bp primer set could, however, not be visualized on the agarose gels and therefore only the results obtained with the 566 bp primer set (2nd round amplification) are displayed in Figures 3.2, 3.3 and 3.4. Although the band in lane 6 is unclear (Fig. 3.2), DNA product yield was satisfactory.

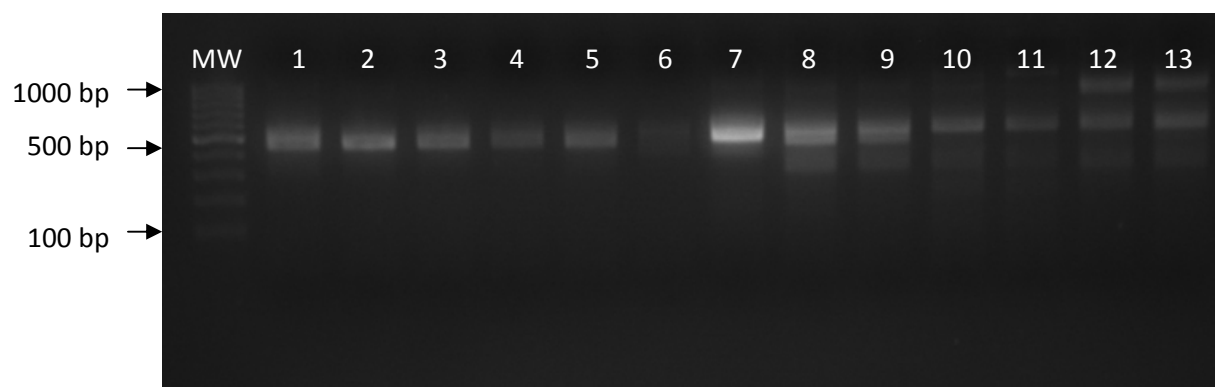


Figure 3.2. A 1.5% (w/v) agarose gel stained with ethidium bromide illustrating the amplified products of the samples and marker organisms of DGGE gel 1 (Figure 3.5). MW represents the 100bp molecular size marker (O'GeneRuler™ 100bp DNA ladder, Fermentas Life Science, US). Lanes 11-13 represent marker organisms and lanes 1-10 represent samples of microbial DNA present in the mid-gut of *Busseola fusca*.

DNA product yield was satisfactory for samples in lanes 1 to 13 (Fig. 3.2). Lanes 1 to 7 displayed no smears and multiple bands were not observed. Samples in lanes 8 and 9 displayed smears. Samples in lanes 10 to 13 displayed multiple bands even after multiple optimization and clean-up attempts. Primer dimers were not present.

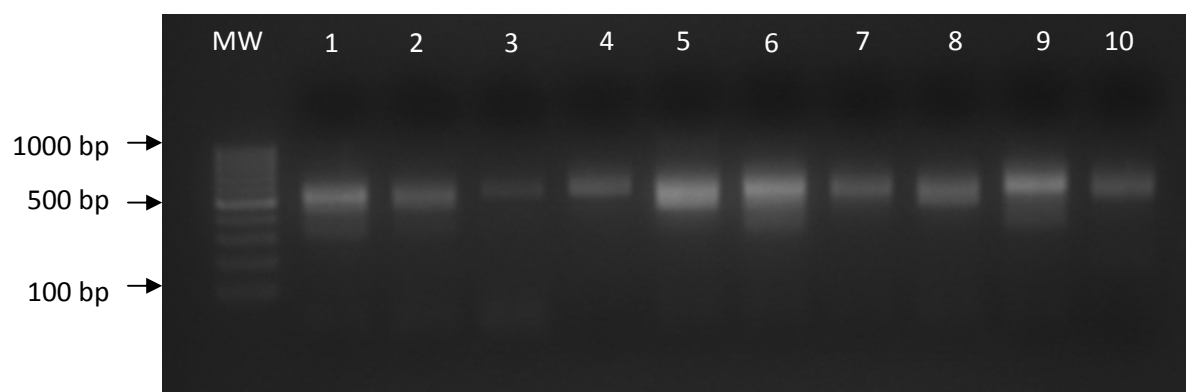


Figure 3.3. A 1.5 % (w/v) agarose gel stained with ethidium bromide illustrating the amplified products of the samples of DGGE gel 2 (Figure 3.6). MW represents the 100bp molecular size marker

(O'GeneRuler™ 100bp DNA ladder, Fermentas Life Science, US). Lanes 1-10 represent samples of microbial DNA present in the mid-gut of *Busseola fusca*.

DNA product yield was satisfactory for the samples in lanes 1 to 10 (Fig. 3.3). Smears were observed for samples in lanes 1, 6 and 9. Despite an increased annealing temperature and decreased quantity of primer used, there were still some primer dimers present, but these had no effect on further analysis, as it ran off the DGGE gels.

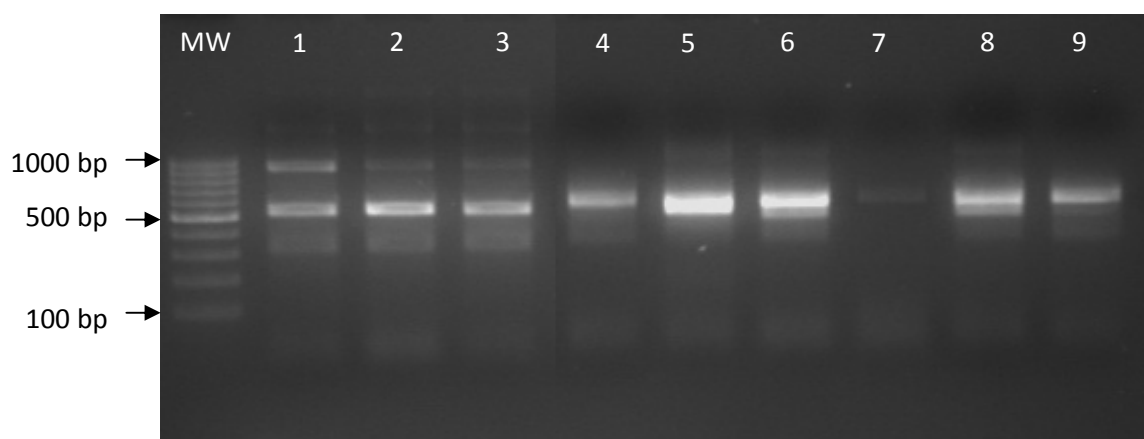


Figure 3.4. A 1.5 % (w/v) agarose gel stained with ethidium bromide illustrating the amplified products of marker organisms and samples of DGGE gel 3 (Figure 3.7). MW represents the 100bp molecular size marker (O'GeneRuler™ 100bp DNA ladder, Fermentas Life Science, US). Lanes 1-3 represents marker organisms and lanes 4-9 represents samples of microbial DNA present in the mid-gut of *Busseola fusca*.

After several optimization attempts, multiple bands were still observed for samples 1, 2, 3, 6, 8 and 9 as indicated in Figure 3.4. The sample in lane 7 displayed a low product yield, but was still sufficient for DGGE analysis. Primer dimers could be observed, but would have run off a DGGE gel and would thus have no effect on further analysis.

A 566bp 16S fragment was amplified. The primer contained a GC-clamp ensuring the DNA molecule remains partially intact when it reached a point in the DGGE gel where most of the fragment is single stranded (Sheffield *et al.*, 1989). The annealing temperature varied between at 65°C and 55°C as a touchdown approach was followed which supplied the best PCR product for DGGE analysis.

Zhang and Jackson (2008) were able to obtain good quality DNA with one round of amplification at an annealing temperature of 56°C. They used a bacterial-specific primer described by Nubel *et al.* (1996). Broderick *et al.* (2003) followed a hot-start approach where the *Taq* polymerase was only added after the first minute of initial denaturation.

Annealing temperature was set at 55°C with primers 27F and 1492R (Lane, 1991) being used. This was the same primer used in the current study for first round amplification after which another primer with a GC-clamp had to be used in order to do DGGE analysis. The current study made use of a nested PCR approach to obtain sufficient DNA of good quality in order to use this DNA for DGGE analysis. Good quality DNA will produce better DGGE results. Primers used are mostly dependant on the application of the product for further analysis.

3.4 Denaturing gradient gel electrophoresis of larvae samples and pure cultures

DGGE was employed to determine the community diversity in Bt-resistant and susceptible larvae as well as to determine if marker organisms were present in the whole gut samples. A 30-50 % denaturing gradient polyacrylamide gel was employed for the analysis. The procedure is described in Section 2.6. Figures 3.5, 3.6 and 3.7 depict the results obtained for the different samples isolated from the midgut of larvae.

The molecular fingerprinting technique, DGGE, had previously been used to give a more accurate indication of the community diversity and to identify and monitor microbes common to different individuals and/or environments (Reeson *et al.*, 2003; Hayashi *et al.*, 2007; Zhang & Jackson, 2008, O'Callaghan *et al.*, 2003). Researchers have shifted from culture dependant to culture-independent techniques to study the organisms that are difficult to culture (Farnleitner *et al.*, 2004; Hoefel *et al.*, 2005a; Hoefel *et al.*, 2005b; Tokajian *et al.*, 2005; Burtscher *et al.*, 2009). It is also used to study the organisms as they occur in their natural habitat, for example in an alimentary tract, as was the case in this study (Muyzer & Smalla, 1998). The rDNA of different microbes was compared as seen in studies by Hori *et al.* (2006) and Schabereiter-Gurtner *et al.* (2003).

A DGGE banding pattern indicating community diversity within the midgut of *B. fusca* is shown in Figure 3.5. The midgut content of *B. fusca* was analysed after a feeding experiment. The larvae were from different maize production areas (Vaalharts, Ventersdorp and Delmas). Representatives were fed on Bt- and non-Bt maize. All those from Ventersdorp and Delmas were presumed susceptible and those from Vaalharts resistant.

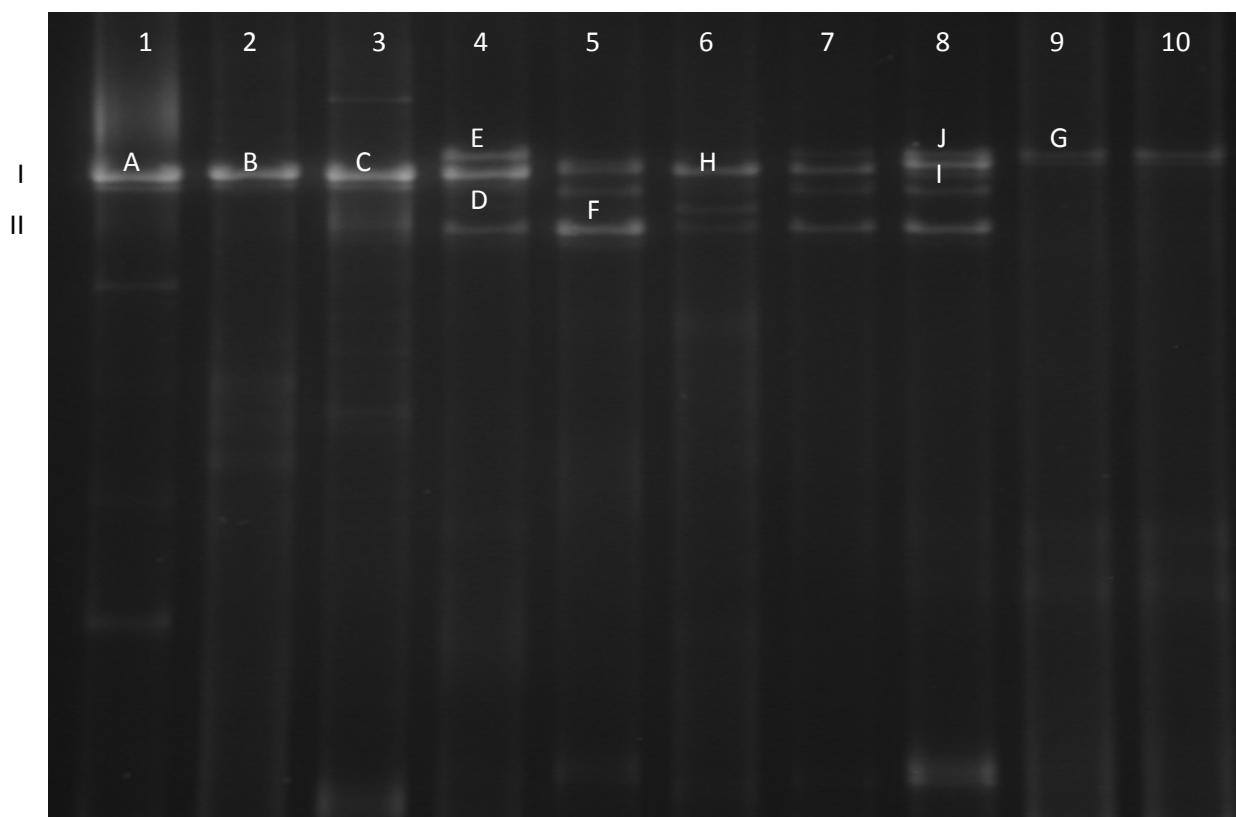


Figure 3.5. DGGE analysis of the 566bp 16S rDNA fragments of midgut bacteria of *Busseola fusca* and ATCC pure cultures as a ladder. A 30-50% denaturing gradient was used on an 8% polyacrylamide gel. Electrophoresis was carried out at 100V for 16 hours. A to J represents the bands excised (Table 3.1).

Sample description:

Lane 1: *Enterobacter cloacea*, Lane 2: *Escherichia coli*, Lane 3: *Enterobacter aerogenes*

Lane 4: Resistant larvae (Vaalharts) reared on non-Bt maize plants in the field

Lane 5&6: Susceptible larvae (Ventersdorp) reared on non-Bt maize plants in the field

Lane 7&8: Resistant larvae (Vaalharts) reared on Bt maize in a test tube

Lane 9&10: Susceptible larvae (a) (Delmas) reared on non-Bt maize in a test tube

The DNA of three ATCC pure cultures (lanes 1 to 3) were used as marker organisms based on their specific importance in the current study (Fig. 3.5). The DNA of these three isolates migrated to more or less the same positions on the gel. Profiles of bands in lanes 4 - 8 were similar. Few variations were observed. One band at migration position I (A,B,C,H,G) was present in all lanes (1-10). A band at migration position II (D and F) was present in lanes 3 to 8. Band E, J and G was present in lanes 4, 7, 8 and 9. Band I was present in lanes 5, 7 and 8. The major bands were excised and sequenced.

Even though pure cultures were used in lanes 1-3, multiple bands could be observed. This could be due to the fact that the 566 bp primer used in the amplification of DNA for DGGE, amplified more than one region of the genome, resulting in multiple bands. Those multiple bands resulted in multiple bands on the DGGE gel. Several attempts were made to optimize

the PCRs in order to eliminate the multiple bands. These attempts were unsuccessful. The prominent bands for each of the cultures were, however, sequenced to ensure the identity of the organism used as marker organisms.

There was no clear difference in community diversity observed (Figure 3.5) between larvae being resistant or susceptible irrespective of whether it was feeding on Bt or non-Bt maize. Community diversity for larvae obtained from Delmas (Lanes 9 and 10) was, however, lower than for larvae from Ventersdorp (Lanes 5 and 6) and Vaalharts (Lanes 4, 7 and 8) (Figure 3.5). Due to migration potentials being similar for *E.coli* and *Enterobacter* it was difficult to determine the presence of these in the midgut fluid. Thus, there is no clear evidence obtained from this analysis and none of the parameters seem to have an impact on diversity. There was also no difference in community diversity for larvae from the field and larvae from test tubes.

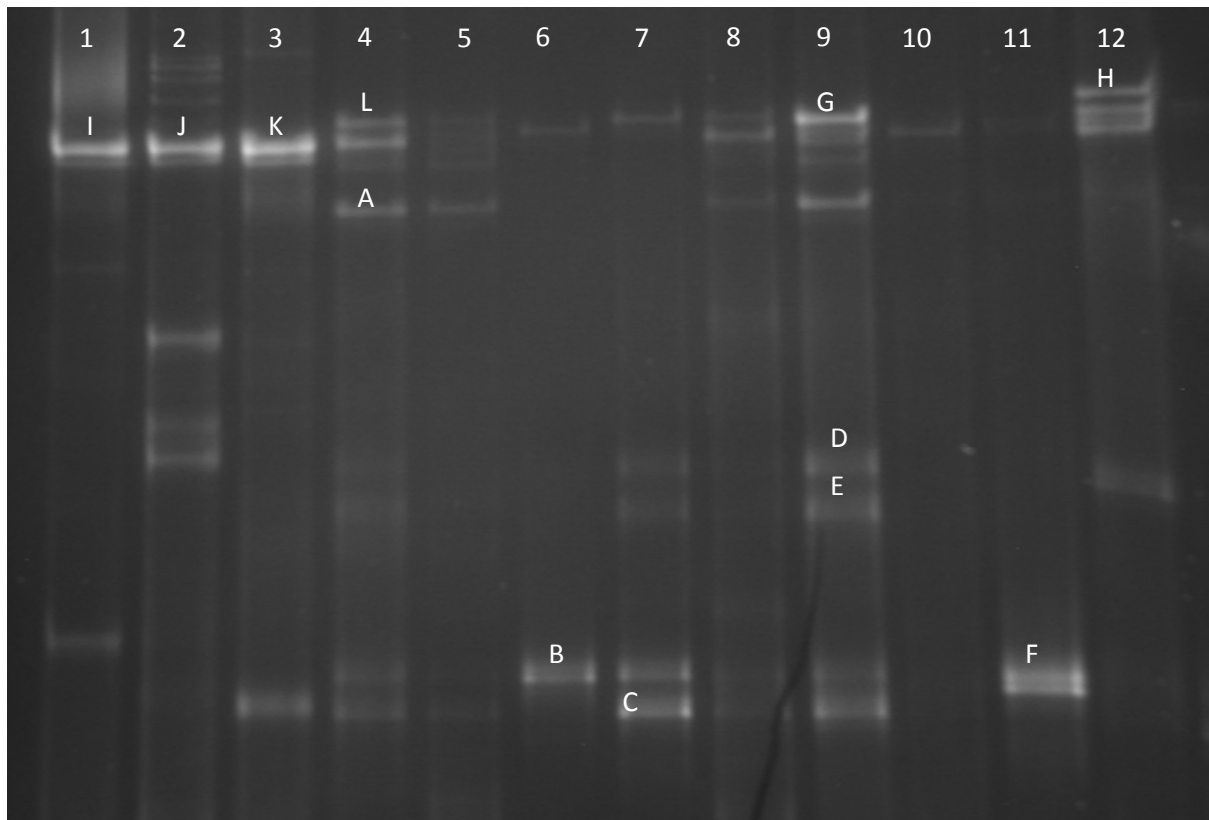


Figure 3.6: DGGE analysis of 566bp 16S rDNA fragments of midgut bacteria of *Busseola fusca* and ATCC pure cultures as a ladder. A 30-50 % denaturing gradient was used on an 8 % polyacrylamide gel. Electrophoresis was carried out at 100V for 16 hours. A to L represents the prominent bands that were excised (Table 3.1). All larvae were reared in test tubes on either Bt or non-Bt maize.

Sample description:

Lane 1: *Enterobacter cloacea*, Lane 2: *Escherichia coli*, Lane 3: *Enterobacter aerogenes*

Lane 4&5: Resistant larvae (Vaalharts) from Bt maize

Lane 6&7: Susceptible larvae (Ventersdorp) from non-Bt maize

Lane 8&9: Susceptible larvae (Ventersdorp) from Bt maize

Lane 10&11: Resistant larvae (a) (Vaalharts) from non-Bt maize

Lane 12: Susceptible larvae (a) (Delmas) from non-Bt maize

The same three ATCC pure cultures (lanes 1 to 3) were used as marker DNA. Bands (A-L) were also excised and sequenced (Fig. 3.6).

As previously mentioned, multiple bands were observed for the marker organisms. More or less the same pattern was observed for the second gel (Fig. 3.6) compared to the first gel (Fig. 3.5), with the exception of a greater number of bands displayed by samples ran on this gel (Fig. 3.6).

Figure 3.6 displays community diversity for larvae obtained from test tubes from different regions including Vaalharts, Ventersdorp and Delmas. More bands were observed for

resistant larvae from Vaalharts (Lanes 4 and 5) feeding on Bt maize than resistant larvae from Vaalharts (Lanes 10 and 11) feeding on non-Bt maize. Susceptible larvae from Ventersdorp (Lanes 6, 7, 8 and 9) feeding on Bt-maize and non-Bt maize displayed a few differences in DNA migration with susceptible larvae feeding on non-Bt maize having a greater number of bands. No clear conclusion could be drawn from these results as to what factors determines community diversity in resistant and susceptible larvae. It could be a combination of the source of food being consumed together with environmental conditions that larvae were exposed to.

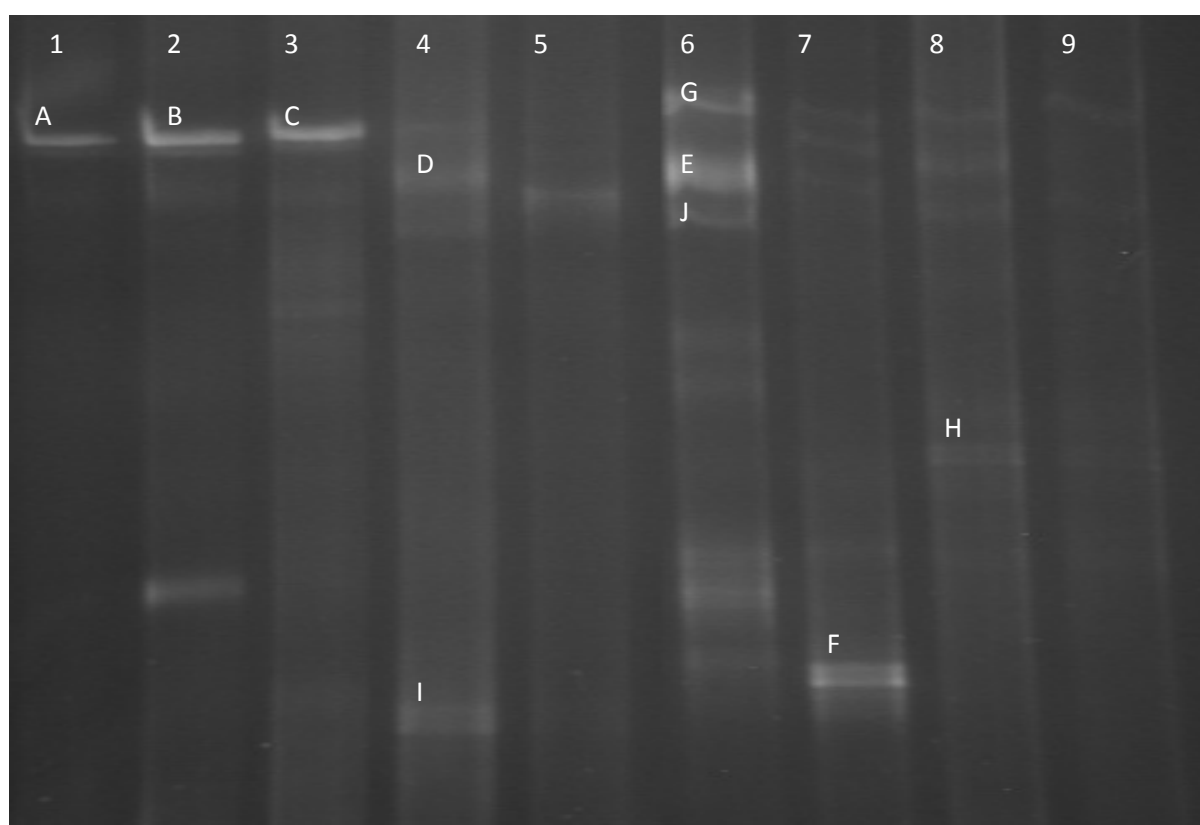


Figure 3.7: DGGE analysis of 566bp 16S rDNA fragments of midgut bacteria of *Busseola fusca* and ATCC pure cultures as a ladder. A 30-50 % denaturing gradient was used on an 8% polyacrylamide gel. Electrophoresis was carried out at 100V for 16 hours. A to I represents the prominent bands excised (Table 3.1).

Sample description:

Lane 1: *Enterobacter cloacea* Lane 2: *Escherichia coli* Lane 3: *Enterobacter aerogenes*

Lane 4&5: Susceptible larvae (Delmas) reared on non-Bt maize plants in the field

Lane 6&7: Resistant larvae (Vaalharts) reared on Bt maize plants in the greenhouse

Lane 8&9: Resistant larvae (Vaalharts) reared on Bt maize plants in the field

The same three ATCC pure cultures (lanes 1 to 3) were used as marker DNA and were again excised and sequenced (Fig. 3.7).

Some multiple bands were again present for the marker DNA. DNA from the midgut of *B. fusca* had the same migration potential as the DNA of marker organisms. Organisms with different migration potentials were also present. Prominent bands were again excised and sequenced. There was no clear difference in number of bands observed for resistant larvae from Vaalharts reared in the plant growth tunnel (Lanes 6 and 7) or from the field (Lanes 8 to 9) (Figure 3.7). Thus the difference in number of bands observed can therefore not solely be ascribed to environmental conditions. Susceptible larvae from Delmas (Lanes 4 and 5) again displayed a few bands.

Traditional microbiological methods were used in the past to characterize the gut microbiota (Dillon & Dillon, 2004). These methods could not detect the presence of unculturable bacteria, thus these bacteria were not presented in the results obtained (Amann *et al.*, 1995). A culture independent method provides the means to detect microorganisms that cannot be cultured by existing methods (Ward *et al.*, 1990). Thus the current study made use of culture independent methods in order to obtain results representing both culturable and non-culturable bacteria.

In this study, PCR-DGGE was employed to determine the community diversity of microorganisms present and if species used as marker organisms (*E. coli*, *E. cloacae*, *E. aerogenes*) were also present in the midgut samples of *B. fusca*. DNA fragments of ATCC pure cultures migrated to more or less the same position and could therefore not be used to construct a ladder. Co-migration could have been due to species being closely related, 16S rDNA sequence do not always provide enough sequence difference to allow separation of amplified DNA of all species on a DGGE gel (Ercolini, 2004). It was consequently decided to use them as marker organisms. Marker organisms were selected after considering results found in a previous study by Broderick *et al.* (2006). The same migration potentials of the marker organisms and the samples of *B. fusca* made it difficult to compare the extracted DNA for potential identification of bacteria. Thus, no unambiguous identification of species could be made from Figures 3.5-3.7, which is a disadvantage of this particular application of DGGE (Ercolini, 2004). This is due to the co-migration of different species (Muyzer *et al.*, 1993; Ercolini *et al.*, 2001; Sekiguchi *et al.*, 2001; Meroth *et al.*, 2003). This is, however, technically, a relatively uncomplicated and cost effective technique in establishing the presence of certain bacteria as well as determining the community diversity of bacteria present (Ercolini, 2004). Farnleitner *et al.* (2004) demonstrated that prominent bands can be excised and sequenced to obtain identities of the various isolates. Due to DGGE analysis not providing sufficient results to distinguish between species, prominent

bands were excised to identify species using sequencing as an alternative method. The present study used a similar approach to that of Zhang and Jackson (2008) to identify the organisms present in the gut of the grass grub larvae, *Costelytra zealandica* (White) (Coleoptera: Scarabaeidae). Genomic DNA was extracted and amplified after which it was subjected to DGGE analysis and prominent bands were excised for identification with 16S rDNA sequencing. *Clostridium* spp. was amongst the most dominant occurring in the hindgut of grass grub larvae.

Rani *et al.* (2009) also used culture dependant and culture independent techniques for the identification of bacteria present in the larvae and adult midgut of the *Anopheles stephensi* (Diptera: Culicidae) mosquitoes. These mosquitoes serve as a vector for malaria. They used a rather different approach to that of the current study. DNA was extracted, amplified and cloned. The DNA was sequenced and phylogenic analysis was done. Bacteria that dominated within the gut of the male mosquitoes caught in the field were *Paenibacillacea* whereas *Serratia marcescens* bacteria dominated in the gut of the field-collected females and larvae. *Serratia marcescens* and *Cryseobacterium meningosepticum* dominated within the gut of laboratory reared mosquitoes (Rani *et al.*, 2009).

3.5 DNA amplification of excised DGGE bands for sequencing

DNA was excised under conditions described in Section 2.7 and amplified using PCR under conditions described in Section 2.8. The 16S primer sets, 338F and 518R, (Table 2.1) were used and the excised DNA bands of the ladder and sample isolates amplified without any problems. Amplification products were of the expected 180bp size (Fig. 3.8-3.10). A primer producing a larger fragment did not give desired results, thus the set amplifying a 180bp fragment was used.

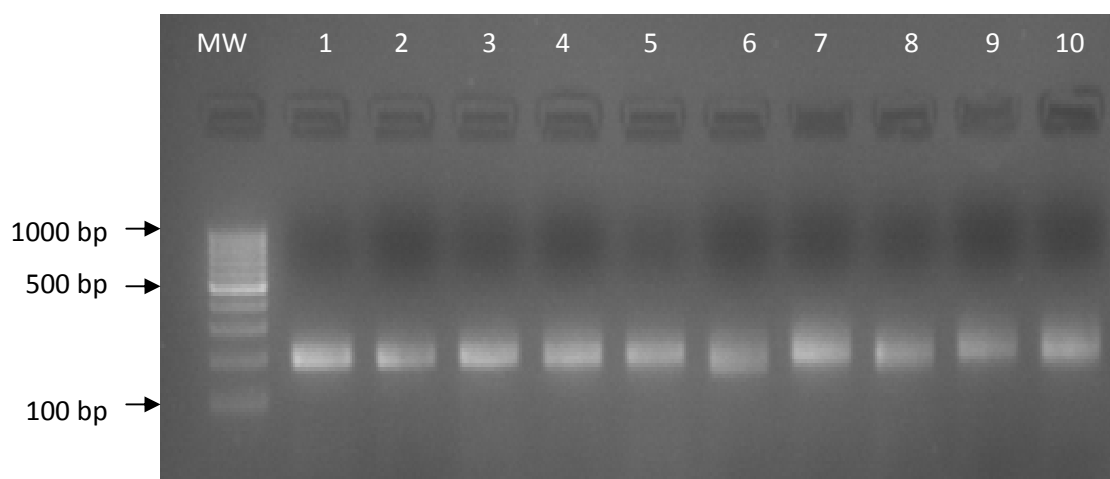


Figure 3.8: A 1.5 % (w/v) agarose gel stained with ethidium bromide illustrating the amplified products of the excised bands from the first gel (Fig. 3.5) (lanes 1-10). MW represents the 100bp molecular size marker (O'GeneRuler™ 100bp DNA ladder, Fermentas Life Science, US).

Product yield was very similar for all the isolates. No optimization of the process was needed, because very little traces of smears were observed. No non-specific products or primer dimers were observed for both gels.

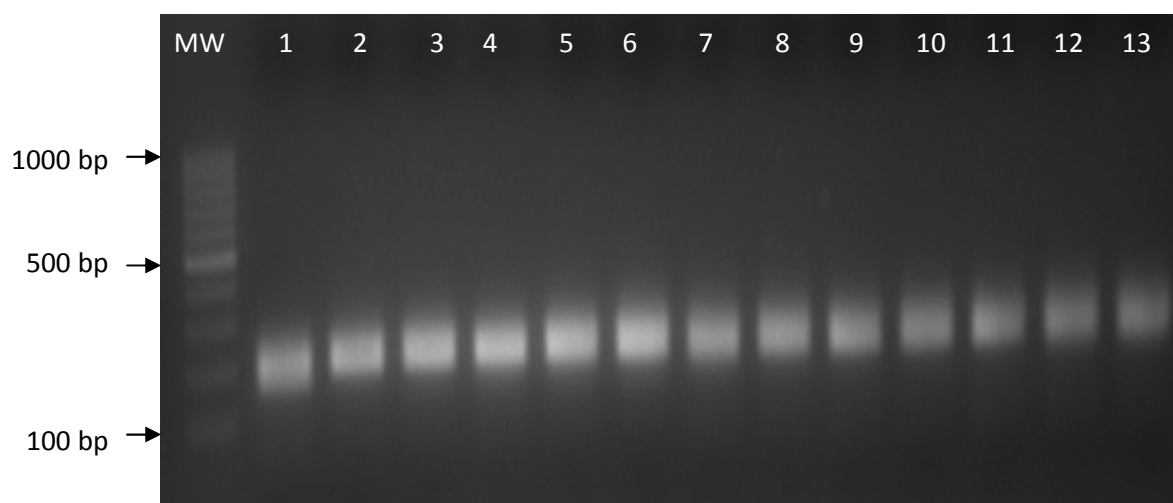


Figure 3.9: A 1.5 % (w/v) agarose gel stained with ethidium bromide illustrating the amplified products of the excised bands from the second gel (Fig. 3.6) (lanes 1-13). MW represents the 100bp molecular size marker (O'GeneRuler™ 100bp DNA ladder, Fermentas Life Science, US).

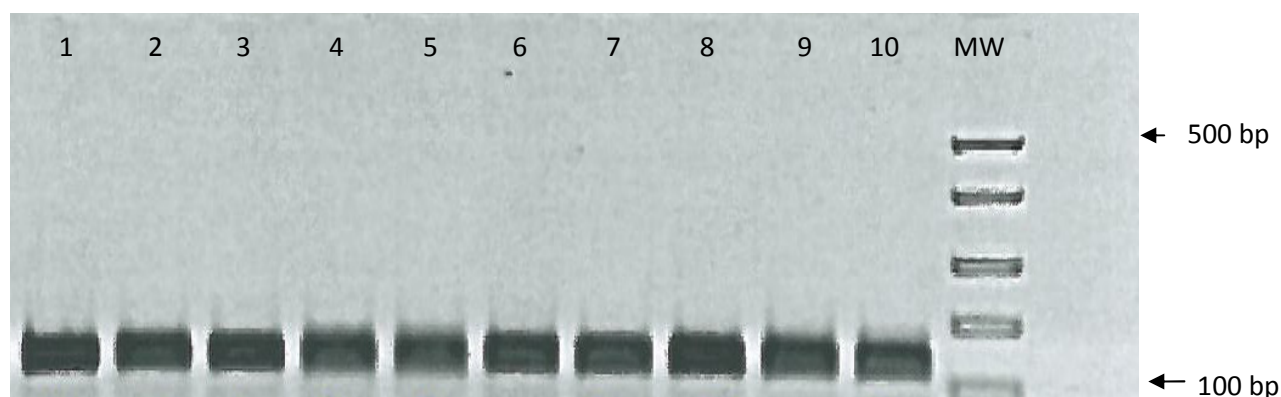


Figure 3.10: A 1.5 % (w/v) agarose gel stained with ethidium bromide illustrating the amplified products of the excised bands from the third gel (Fig. 3.7) (lanes 1-10). MW represents the 100bp molecular size marker (O'GeneRuler™ 100bp DNA ladder, Fermentas Life Science, US).

Product yield was again satisfactory with no primer dimers and non-specific products present. The appearance of this gel (Fig. 3.10) differs from those in Fig. 3.8 and Fig. 3.9 due to the fact that this gel was run at another facility (Inqaba Biotech, SA).

3.6 Sequencing of excised bands

Sequencing was done by Inqaba Biotech, SA, using the 518R primer. DNA as illustrated in Figures 3.8 to 3.10, were used for the sequencing reactions. GenBank was used to identify the amplified sequences by BlastN searches (<http://www.ncbi.nlm.nih.gov/BLAST>). Results based on the 16S rRNA gene sequence are illustrated in Table 3.1 for each of the different bands excised from the DDGE gels. BlastN searches revealed a variety of bacteria, with *Enterococcus* species dominating in the midgut contents of *B. fusca*. Background noise was present for 5 of the 32 identified organisms, but high sequence similarities were still obtained in these samples.

Enterobacter spp. and *Enterococcus* spp. have been recognized as inhabitants of the gut of several insect species (Demaio *et al.*, 1996; Straif *et al.*, 1998; Gonzalez-ceron *et al.*, 2003; Campbell *et al.*, 2004; Rani *et al.*, 2009) thus suggesting that these bacteria may play various important roles (Geiger *et al.*, 2009).

Of the 23 prominent bands excised in this study, only one was identified belonging to *Enterobacter* spp. (Table 3.1). This organism was found only in larvae collected at Delmas and in only one of the five sets of the susceptible larvae feeding on non-Bt maize. *Enterobacter* has previously been identified in numerous insects, including gypsy moths

(Lepidoptera: Lymantriidae) (Broderick *et al.*, 2003), grasshoppers (Orthoptera: Acrididae) (Hunt & Charnley, 1981; Mead *et al.*, 1988), mosquitoes (Diptera: Culicidae) (Straif *et al.*, 1998), thrips (Thysanoptera: Thripidae) (Wells *et al.*, 2002) as well as the cabbage white butterfly (Lepidoptera: Pieridae) (Robinson *et al.*, 2009).

The fact that only one *Enterobacter* spp. was found in all of the lanes (Figures 3.8-3.10) supports the study by Broderick *et al.* (2009) in which they reported that *Enterobacter* spp. is not required for *B. thuringiensis*-induced killing of Lepidoptera. A reduction of gut bacteria have been reported to increase *P. gossypiella* susceptibility to *B. thuringiensis*. Antibiotic treatment reduced *B. thuringiensis*-induced mortality of *H. virescens* larvae, even though no bacteria tested for restored killing (Broderick *et al.*, 2009).

Heliothis virescens presents a model in which bacteria may play a role, but in a more complex manner than in the other host species. This is due to the fact that *Enterobacter* spp. was unable to restore *B. thuringiensis* mediated killing of the larvae (Broderick *et al.*, 2009). Indicating that while antibiotics may alter larval susceptibility to *B. thuringiensis*, the mechanism by which gut bacteria mediate *B. thuringiensis*-induced killing requires further elucidation, as do the different responses to gut bacteria in various insect species (Broderick *et al.*, 2009).

Broderick *et al.* (2006) found that this reduction in mortality of larvae was accompanied by reduced populations of culturable *Enterococcus* and *Enterobacter* spp. from the midguts of gypsy moth larvae. After *Enterobacter* spp. was reintroduced to the target organism, the insecticidal activity was restored to nearly that of larvae not treated with antibiotics. The same procedure was negative for *Enterococcus casseliflavus* and *Staphylococcus xylosus*. This implicates that *Enterobacter* spp. is mainly responsible for the septicemia associated with *B. thuringiensis*-induced mortality in gypsy moth larvae (Broderick *et al.*, 2006).

In the current study, *Enterococcus* spp. were amongst the most dominant (10 of the 32 excised bands) (Table 3.1) from the isolated species from the midgut contents. This result depicted another interesting finding, which was not anticipated. *Enterococcus* spp. was found in five of the lanes, of which four was from the sets of resistant larvae. In a study by Broderick *et al.* (2009), they found that the Gram-positive bacterium, *Enterococcus casseliflavus*, might protect its host (*P. gossypiella*) against *B. thuringiensis*. Such a protective role has also been proposed for gut bacteria of the tortricid *Homona magnanima* (Diakonoff) (Lepidoptera: Tortricidae) (Takatsuka & Kunimi, 2000). It was previously

reported by Broderick *et al.* (2009), that *Enterococcus faecalis* was not detectable in the guts of *L. dispar* larvae reared on antibiotics and on reintroduction, this bacterium did not restore larval susceptibility as *Enterobacter* sp. did (Broderick *et al.*, 2009).

In the current study, 2 of the 23 excised DNA bands (Table 3.1) were identified as *Enterococcus faecalis* and 7 of the 23 (Table 3.1) were identified as *Enterococcus* species. *E. faecalis* is commonly found at higher pH values (8 to 9) and acidifies its environment through its metabolism (Manero & Blanch, 1999). This might serve as an advantage to *B. fusca*, as *B. thuringiensis* toxins are activated by alkaline conditions (Wilson & Benoit, 1993). In other words, *E. faecalis* might help protect *B. fusca* from the toxin by decreasing the midgut pH. This is, however, only applicable when a product containing the Cry toxin is used to control *B. fusca* as the toxin is activated in alkaline conditions. Previous studies have shown that larvae that are more susceptible to *B. thuringiensis* toxin have smaller populations of *E. faecalis* in their midguts (Broderick *et al.*, 2003). Bacteria may also contribute to their own survival by managing midgut pH to enhance their own growth or exclude competing microorganisms (Broderick *et al.*, 2004). In addition, few studies have examined bacteria in highly alkaline environments, particularly in non-aquatic ecosystems (Fry *et al.*, 1997; Gsell *et al.*, 1997). Further studies are needed to identify adaptations that may permit these bacteria to function in this extreme environment, and whether they have a role in maintaining it (Broderick *et al.*, 2004).

Broderick *et al.* (2006) suggested that the microbial community present in the midgut of larvae contributes to the death of larvae subjected to *B. thuringiensis* treatment. In another study it was demonstrated that aseptically reared *Homona magnanima* (Lepidoptera: Tortricidae) larvae supported 20 times greater growth of *B. thuringiensis* than larvae with a gut microbiota. Thus suggests that intestinal bacteria influence the growth of the insect pathogen in the larvae (Takatsuka & Kunimi, 2000).

In the current study, 7 of the 23 excised bands (Table 3.1) containing the bacterial DNA could not be identified or were unculturable bacteria. Broderick *et al.* (2009) using both culturing and direct 16S rRNA, was also unable to identify some of the gut bacteria in *H. virescens*, which like *B. fusca* is a lepidopteran belonging to the family Noctuidae.

In a study conducted by Geiger *et al.* (2009), 39 bacterial isolates were identified from a tsetse fly (*Glossina palpalis palpalis*) (Diptera: Glossinidae). Of the 39 isolates, 32 were identified as belonging to *Enterobacter* spp. and only two as *Enterococcus* spp.

Furthermore, of 13 analysed flies, only one contained *Enterococcus* spp. Bacteria were only isolated from 54 % of the flies used in their study, which could be an indication that the majority of the flies in the population acquired the bacteria, but their absence may also suggest that bacteria may not be permanent midgut residents (Geiger *et al.*, 2009).

The enteric bacteria that occupy lepidopteran larvae may affect susceptibility to *B. thuringiensis*, and the extent of this impact may vary across lepidopteran species. These factors are not mutually exclusive and in some cases may interact. For example, host diet can alter the composition of enteric bacteria (Broderick *et al.*, 2003; Walenciak *et al.*, 2002). Broderick *et al.* (2009) demonstrated that enteric bacteria have important roles in *B. thuringiensis*-induced killing of Lepidoptera across a range of taxa and relative susceptibility to *B. thuringiensis*. This impact of enteric bacteria therefore differs between species (Broderick *et al.*, 2009). Broderick *et al.* (2003) also demonstrated through culturing and sequence analysis of 16S rRNA genes amplified directly from larval midguts, that diet had a significant impact on midgut microbial diversity.

Table 3.1: GenBank identification of the prominent bands excised from the DGGE gels. (* indicates reference bacteria – ATCC cultures)

Samples	Accession number #	Genbank ID	Background noise		% Similarity	Sequence ID
			Yes	No		
Gel 1	Figure 3.5					
1-A*	HQ434623.1	Enterobacter cloacae		X	100%	111/111
1-B*	AB599716.1	Escherichia coli		X	100%	119/119
1-C*	HM480361.1	Enterobacter aerogenes		X	100%	118/118
1-D	no identity					
1-E	HQ225023.1	Uncultured bacterium		X	90%	79/87
1-F	EU789974.1	Uncultured bacterium		X	94%	66/70
1-G	HM053761.1	Uncultured Enterobacter sp.	X		83%	76/91
1-H	no identity					
1-I	no identity					
1-J	no identity					
Gel 2	Figure 3.6					
2-A	EU445264.1	Agrobacterium tumefaciens		X	90%	65/72
2-B	GU458344.1	Leuconostoc mesenteroides		X	91%	99/108
2-C	AB596997.1	Enterococcus faecium		X	100%	109/109
2-D	HQ415819.1	Enterococcus sp.		X	97%	114/117
2-E	FR717468.1	Enterococcus hirae		X	96%	114/118
2-F	HQ327128.1	Staphylococcus sp.		X	98%	109/111
2-G	FJ821314.1	Enterococcus faecalis		X	92%	104/113
2-H	GU429172.1	Peptostreptococcaceae bacterium	X		82%	75/91
2-I*	HQ434623.1	Enterobacter cloacae		X	99%	112/113
2-J*	AB599716.1	Escherichia coli		X	99%	99/100
2-K*	HM480361.1	Enterobacter aerogenes		X	100%	118/118
2-L	AY134904.1	Eubacterium sp.	X		82%	75/91
Gel 3	Figure 3.7					
3-A*	HQ434623.1	Enterobacter cloacae		X	100%	101/101
3-B*	AB599716.1	Escherichia coli		X	99%	109/110
3-C	HM480361.1	Enterobacter aerogenes		X	100%	112/112
3-D	HM163497.1	Enterococcus mundtii	X		88%	100/113
3-E	AB596997.1	Enterococcus faecium		X	100%	118/118
3-F	HQ262547.1	Staphylococcus sp.		X	95%	106/111
3-G	FR717468.1	Enterococcus hirae		X	100%	115/115
3-H	AB470333.1	Enterococcus sp.	X		89%	67/75
3-I	HQ293064.1	Enterococcus faecalis		X	97%	109/112
3-J	HM218738.1	Enterococcus durans		X	100%	119/119

3.7 Quantitative analysis of selected bacteria

Faecal *coliforms* (*E. coli*) and *Enterobacter* spp. were quantified using mFc agar and Brilliant Green Bile agar respectively. Faecal *coliforms* are represented by blue colonies on the mFc agar whereas *Enterobacter* spp. are represented by pink colonies on the Brilliant Green Bile agar. These colonies were counted and reported as colony forming units per millilitre. Figures 3.11, 3.12 and 3.13 represent the mean number of colony forming units of faecal coliforms and *Enterobacter* spp. isolated from the midgut of *B. fusca* larvae. Each graph indicates the samples presented on DGGE gels from Figures 3.5, 3.6 and 3.7.

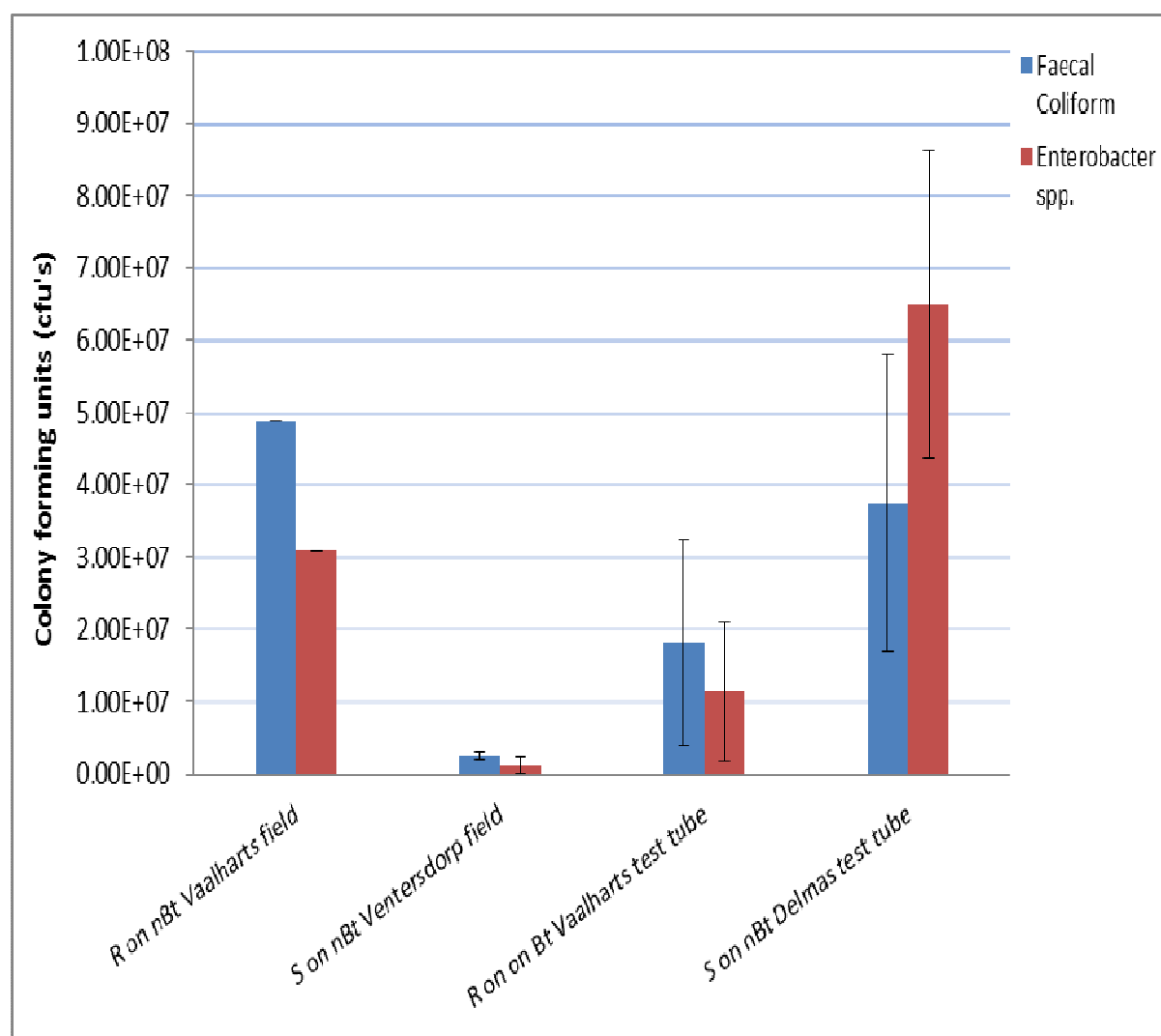


Figure 3.11: Mean number of colony forming units of faecal coliforms and *Enterobacter* spp. isolated from the midgut of *Busseola fusca* larvae feeding on Bt and non-Bt maize. These results represent the samples ran on DGGE gel (Figure 3.5). (R-resistant and S-susceptible; nBt = non-Bt maize; Bt = Bt maize).

The first three samples in Figure 3.11 displayed a higher number of faecal *coliforms* than *Enterobacter* spp. whereas the sample from Delmas displayed a higher *Enterobacter* spp. number. No trend could be observed as to what the determining factor would be for numbers being higher or lower for certain bacteria. There was, however, a difference in the number of total *coliforms* and *Enterobacter* spp. present in the midgut fluid of resistant and susceptible *B. fusca* larvae despite the fact that both these were fed on non-Bt maize from the field from different locations. However, susceptible *B. fusca* larvae from Delmas feeding on non-Bt maize displayed high numbers of both species of bacteria. These larvae were reared in test tubes under a controlled environment. Thus, environmental conditions might play a role in the presence of these bacteria. Even under controlled conditions in test tubes, with no environmental factors playing a role, the resistant larvae from Vaalharts feeding on Bt-maize displayed lower numbers of these bacteria than resistant larvae feeding on non-Bt maize. Thus, the type of food might also play a role in determining the abundance of these bacteria in the midgut fluid of the larvae.

When comparing the results displayed in Figure 3.11 with the community diversities displayed in Figure 3.5 the only clear difference is that the community diversity of the Delmas sample was much lower than the rest. This sample had the highest number of total *coliforms* and *Enterobacter* spp. which might indicate a dominance of these bacteria suppressing the occurrence of other bacteria in the midgut of the larvae.

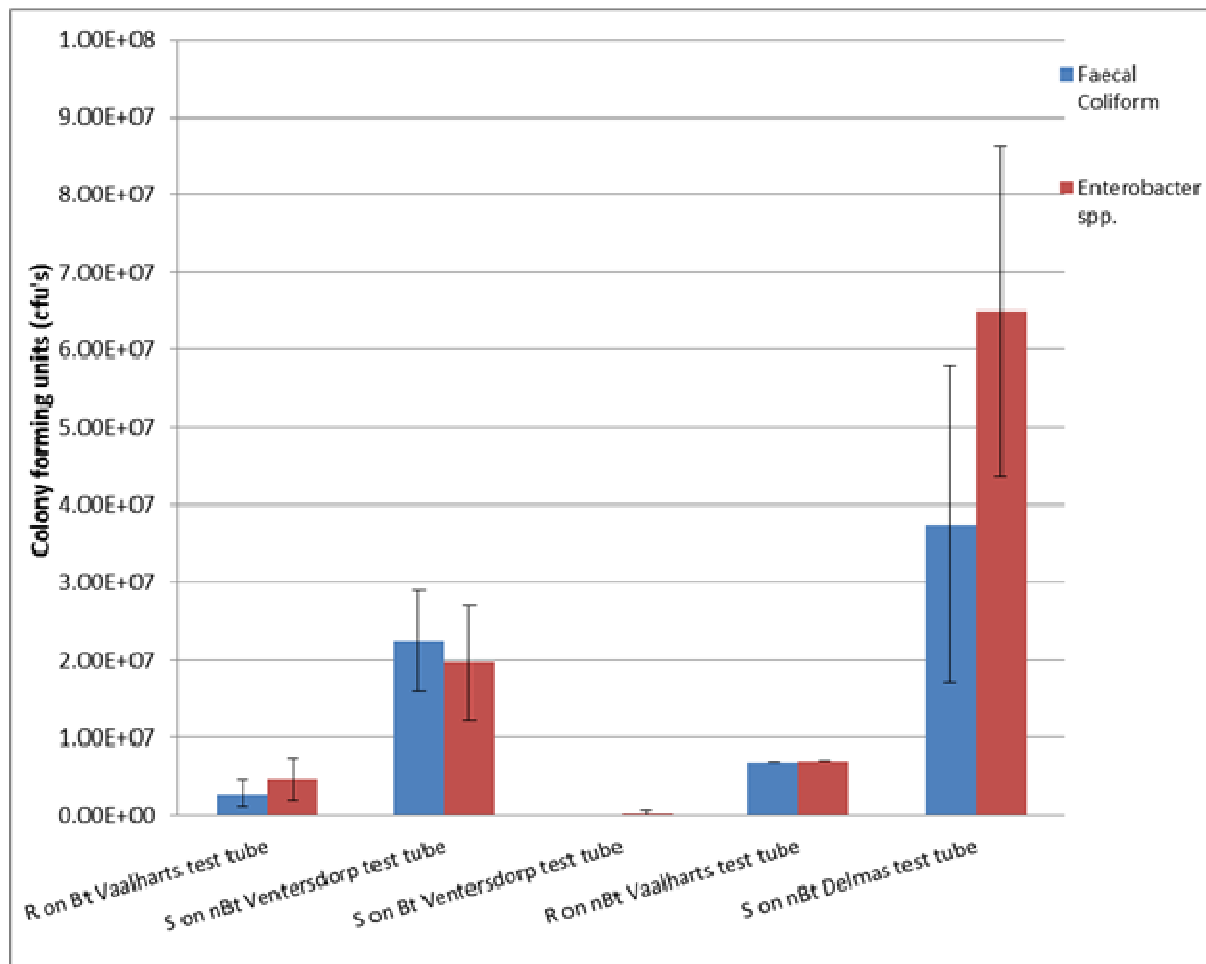


Figure 3.12: Mean number of colony forming units of faecal *coliforms* and *Enterobacter* spp. bacteria isolated from the midgut of *Busseola fusca* larvae feeding on Bt- and non-Bt maize. These results represent the samples ran on DGGE gel Figure 3.6. (R-resistant and S-susceptible; nBt = non-Bt maize).

The results displayed in Figure 3.12 were obtained from larvae reared in test tubes, thus environmental factors were eliminated. In both resistant and susceptible larvae, higher numbers of the bacteria were present when larvae were feeding on non-Bt maize. The Delmas sample also displayed high numbers of the bacteria when feeding on non-Bt maize. This might be an indication that the type of food plays a role in the abundance of these bacteria in the midgut of larvae. The Delmas sample (non-Bt) had more bacterial numbers than the samples from Vaalharts (Bt) and Ventersdorp (non-Bt).

When comparing the results displayed in Figure 3.12 with the results displayed in Figure 3.6, it is evident that the higher the number of these bacteria the lower the community diversity as displayed in Figure 3.6. It is therefore possible that dominance by these bacteria suppresses the occurrence of other bacteria in the midgut of larvae.

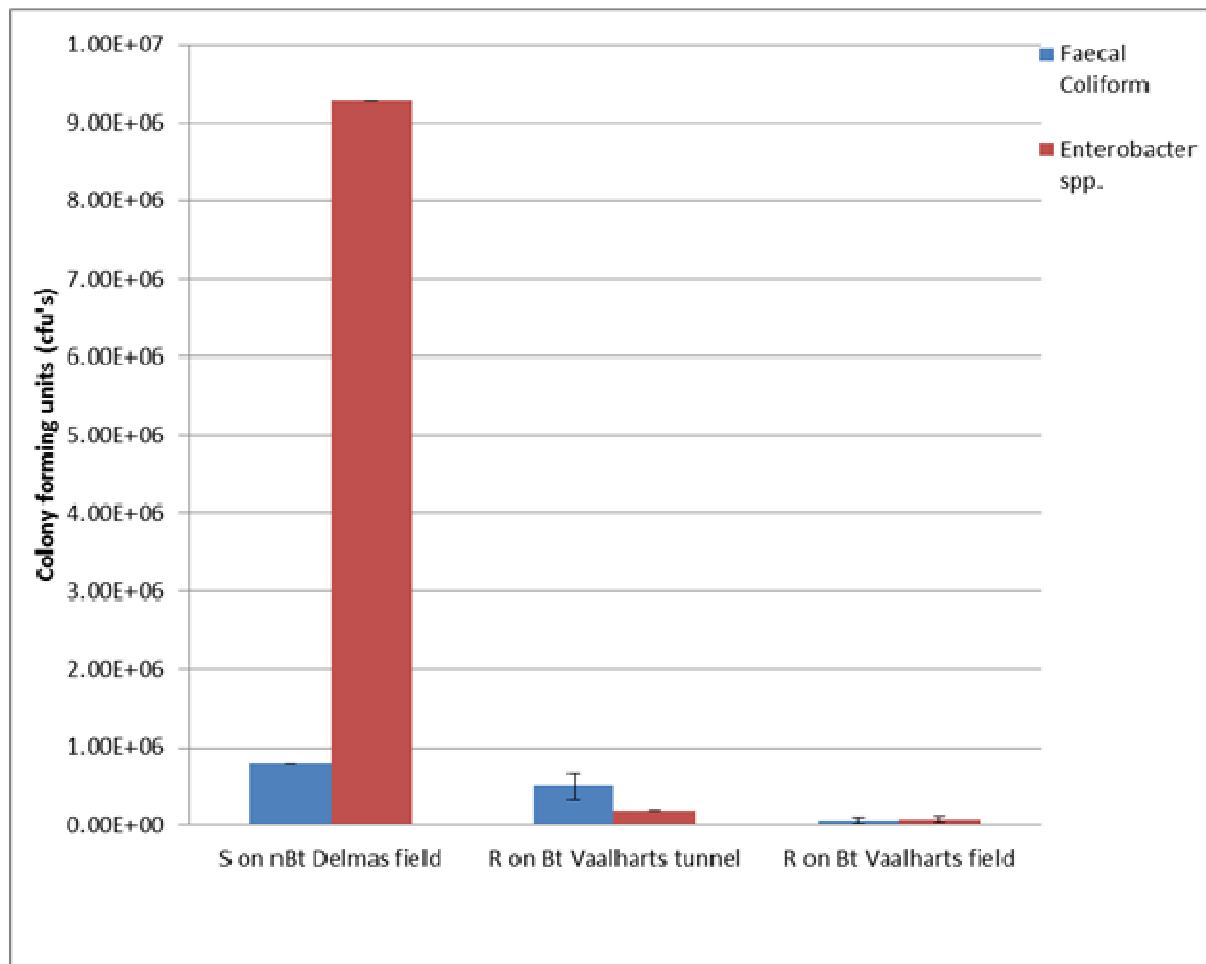


Figure 3.13: Mean number of colony forming units of faecal coliform and *Enterobacter* spp. bacteria isolated from the mid-gut of *Busseola fusca* larvae feeding on Bt- and non-Bt maize. These results represent the samples ran on DGGE gel Figure 3.7. (R-resistant and S-susceptible; nBt = non-Bt maize).

In another sample the Delmas sample displayed the same trend as previously observed, *i.e.* very high numbers of *Enterobacter* spp. Again low community diversity was observed for this sample (Figure 3.7). When resistant larvae from Vaalharts were fed with Bt-maize but reared under different climatic conditions, in a plant growth tunnel and in the field lower numbers of bacteria were present in larvae reared in the field. This again might be an indication of environmental conditions playing a role as the growth tunnel provided a more controlled environment compared to the field where larvae were exposed to harsh environmental conditions. These two samples however, displayed similar community diversities (Figure 3.7).

In general, colony counts varied greatly among individual sets of larvae (Figs. 3.11 - 3.13). Similar variations were observed in a study by Van Frankenhuyzen *et al.* (2010) on gypsy moths and spruce budworm. Even though the counts varied greatly, a general pattern was apparent. The effect that environmental conditions or type of food plays in the abundance of

these bacteria and its effects on community diversity should be studied further in more controlled environments.

3.9 Gram Staining and TSI as confirmative identification of selected bacteria

Certain colonies were selected from the agar plates that displayed characteristics of faecal *coliforms* and *Enterobacter* spp. These isolates were then subjected to gram staining and TSI analysis. All the isolates were Gram negative. *Enterobacter* spp. displayed no significant reaction, while the *E. coli* species produced a yellow reaction with gas formation when subjected to TSI analysis.

In a study conducted by Geiger *et al.* (2009), they found that the prevalence of Gram negative bacteria in the midgut of the tsetse fly (*G. palpalis*) was 94.8% in comparison to the 5.1% for Gram positive bacteria. In these two studies it was found that there are more Gram negative than Gram positive bacteria present in an insect midgut.

CHAPTER 4

CONCLUSION AND RECOMMENDATIONS

4.1 Conclusion

The aim of the study was to compare the community diversity of gut-microorganisms in Bt-resistant with Bt-susceptible larvae of *Busseola fusca* and to quantify the total *coliforms* and *Enterobacter* spp. present in the midgut of the larvae. Three objectives were formulated to achieve the aim of the study.

(i) Community diversity of gut-microorganisms using DGGE

DGGE banding patterns did not reveal any differences in the community diversity of microorganisms in the midgut contents of *B. fusca* reared under the different conditions. There were, however, several dominant bands that were excised and sequenced (ii below). Most of the bands observed were in the lower denaturing area of the gel. Although no difference in community diversity between *B. fusca* reared under different conditions were observed by means of DGGE, this technique was successful in demonstrating microbial community structure. However, the observed patterns were highly variable.

(ii) Identification of prominent microorganisms through 16S rDNA sequencing

Sequencing of the DGGE bands indicated that the most dominant species in the midgut of *B. fusca* were *Enterococcus* spp. The specific species identified were *E. mundtii*, *E. faecalis*, *E. faecium*, *E. hirae* and *E. durans*. Other organisms identified included *Staphylococcus* sp., *Agrobacterium tumefaciens*, *Leuconostoc mesenteroides*, *Peptostreptococcaceae* bacterium and several sequencing were similar to previously uncultured and unidentified bacteria. These results demonstrate that DGGE combined with the 16S rRNA sequencing is a powerful technique and resulted in the identification of several bacterial species.

(iii) Quantification of faecal *coliforms* and *Enterobacter* spp.

Based on results from a previous study (Broderick *et al.*, 2006) one of the objectives were to quantify faecal *coliforms* and *Enterobacter* spp. levels in midgut of *B. fusca* larvae fed on Bt and non-Bt maize. This study did not conclusively demonstrate consistent differences in the levels of the target species in the midguts of the Bt and non-Bt maize fed *B. fusca* larvae. Environmental conditions and/or the type of food (Bt and non-Bt maize) might be the factors determining the abundance of faecal *coliforms* and *Enterobacter* spp. in the midgut of *B. fusca* larvae. This in turn could have affected the community diversity. From the

results it was evident that there were differences in levels of faecal *coliforms* and *Enterobacter* spp. associated with rearing conditions. However, what is clear from the DGGE results is that the dominant species in the midgut of *B. fusca* may not be members of the Enterobacteriaceae family, but rather from *Enterococci* spp.

Finally, the aim and objectives of the study were achieved in terms of quantifying coliforms and *Enterobacter* spp. and also determining the community diversity within the midgut of *B. fusca*.

4.2 Recommendations

- Studies on how the bacteria present in the midgut of *B. fusca* are transferred (from the female or through the environment) should receive further attention (Geiger *et al.*, 2009). Thus, do the bacteria naturally occur in the midgut or is it dependent on other factors?
- It is not known whether midgut bacteria are established through ingestion of plant material and whether different host plant chemistries select for specific bacterial genotypes. This is an aspect that may be investigated.
- The current study indicated the presence of *Enterococcus* spp. in the midgut of *B. fusca*. Investigating the role *Enterococcus* spp. play in the resistance or susceptibility of *B. fusca* to Bt-maize may lead to a better understanding of the role of this species in *B. fusca* midgut.
- In the current study it was also observed that the food rot after a day in the test tubes. It is recommended that when using test tubes as controlled environment food should be replaced on a daily basis as the decomposition of the food may alter the microbial community composition in the midgut of *B. fusca*.
- DGGE may be a useful tool in establishing community diversity as the current study indicated. This application, however, needs further optimization using different and longer primer sets, particularly for sequencing.

- It is advisable that in future studies the variables are kept to a minimum. For instance food and environmental conditions should be kept constant if the influences of microbes are investigated in terms of the effect it has on susceptibility or resistance to Bt-maize.

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