The effect of *Ricinus communis* on larval behaviour and midgut microbe communities of *Spodoptera frugiperda* (Lepidoptera: Noctuidae)

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Abstract

The Fall Armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) is an invasive pest species that spread throughout sub-Sahara Africa after its introduction into west Africa during 2016. It is a destructive pest of many cultivated plant species. The greatest damage by the larvae is however done to its main hosts, maize and sorghum. This study aimed to determine the midgut microbiota community complex of fourth instar FAW larvae that fed on maize (*Zea mays*) and castor oil plants (*Ricinus Communis)*. To identify the midgut microbial community the isolated bacteria were sequenced through the 16S rRNA gene. Molecular phylogenetic analyses revealed that the bacteria are affiliated to Proteobacteria, Actinobacteria and Firmicutes for both maize and castor oil reared larvae. The microbial midgut community structure and composition differed between larvae that fed on the two respective host plants. Cannibalism was also evaluated when larvae were kept at different densities on maize and castor oil plants to determine if host plants affect their cannibalism behaviour. Y-tube bioassays were conducted to determine if the larvae emit possible chemical compounds that either could attract or repel conspecific larvae and which could in turn enhance or suppress cannibalistic behaviour. The study showed that the castor oil plant affects cannibalism behaviour and midgut microbial community structure. Cannibalism occurs less when the larvae feed on castor oil plants, but the larvae are still cannibalistic when stressed in terms of higher numbers and food source. This study generated information regarding the gut microbe complex of FAW larvae as well as its cannibalism behaviour.

Keywords: Castor oil plant, Fall armyworm, maize, pest management, *Ricinus communis.*

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Chapter 1: Literature review

1.1.General introduction

The premise on which this study is based was that observations showed Fall armyworm (*Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) larvae to be less cannibalistic when they are reared on castor oil plants than on maize plants. This observation promted questions such as: could the castor oil plants have more nutrients available than maize plants and could this be the reason for this type of change in behaviour in the Fall armyworm larvae? Furthermore, was this change in behaviour linked to the fact that the gut microbiota of the larvae that were reared on castor oil plants differ from the larvae that were reared on maize plants?

The Fall armyworm is an invasive and destructive pest on crops in America, Africa and Asia. Gut microbiota or bacteria symbionts are known to help and assist the larval host with its evolution, nutrition, physiology, defence and protection, immunity and reproduction. These gut microbiota can change in different geographical regions or when there is a change in the larvae's host plants. This happens so that the larvae benefits the most from their host plant and possibly change their behavioural characterisics. Manipulation and exploitation of insect microbiota could be an avenue towards developing sustainable strategies for their management of insect pests.

1.2 Maize as food source in Africa

Maize is one of the first plant species that were cultivated by farmers in South America about 7 000 to 10 000 years ago (Ranum *et al.*, 2014, Piperno and Flannery, 2001). The world's population keeps on growing at an exponential rate and it is estimated that it will be around 9.2 billion by 2050 (United Nations, 2008). The largest producer of maize in the world is the USA which produces 37% of the annual harvest (Figure 1.1). Approximately 1 038 million tons of maize was produced during the 2016-2017 cropping period (Figure 1.1).

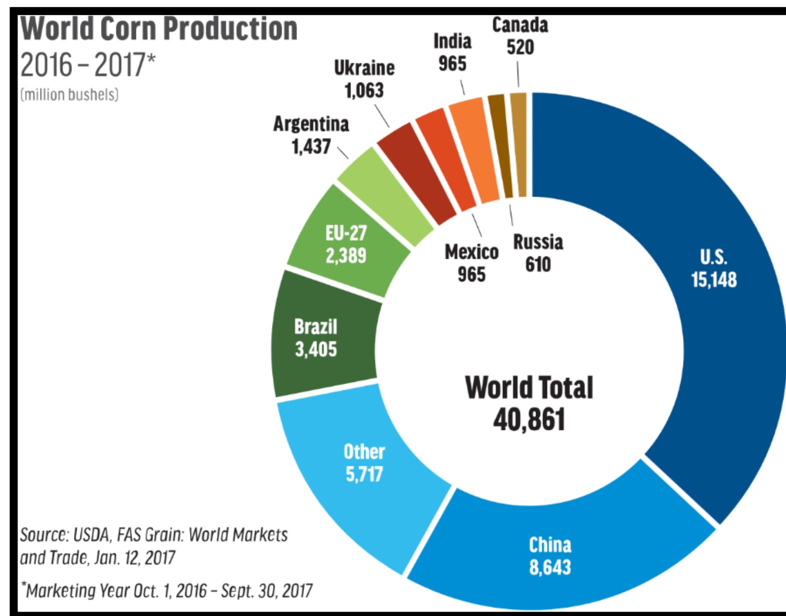

Figure 1.1: Proportional production of maize by the leading maize growing countries in the world (*AgUpdate, 2018*). One bushel/hectare of maize equals 63 kg/hectare (*Johanns, 2013*).

Food insecurity continues to cause problems for millions of Africa's poor people and is likely to worsen with climate change and exponential population growth (Midega *et al.,* 2015). Africa produces 6.5% of the world production of maize, where Nigeria is the largest produser in Africa (8 million tons) followed by South Africa (IITA, 2019).It is therefore important that food production increases at a rate that satisfies the growing world population. One strategy to ensure sustainable yields and sufficient production is to apply biotechnology such as genetic modification of crops to introduce traits such as herbicide and drought tolerance and pest resistance (Zhang *et al.*, 2016).

Maize is vulnerable to insect pests, especially lepidopteran species (Kfir, 1997). The most important lepidopteran species that attack maize in Africa are stem borers such as *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) and *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae) (Kfir, 1997) and *Sesamia calamistis* (Lepidoptera: Noctuidae) (Mengistu *et al.,* 2009). The African bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) and the invasive Fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) also attack maize and cause significant damage to maize ears and leaves, causing an estimated annual loss of US$2 billion, excluding the socio-economic and environmental costs associated with its control (Goergen *et al.*, 2016; Tay *et al.*, 2013).

The FAW recently invaded West Africa and spread throughout the continent within one year (Goergen *et al.*, 2016). Recent investigations revealed that the pest is present in nearly all of sub-Saharan Africa, where it causes damage to maize, sorghum and other crops. Currently, over 30 countries have identified the pest within their borders including the island countries of Cape Verde, Madagascar, São Tomé and Príncipe, and the Seychelles (Prasanna *et al.*, 2018). The FAW was

first reported in South Africa during January 2017 where it was reported to damage maize in the Limpopo Province (Jacobs *et al.*, 2018).

Various strategies have been employed to limit insect pest damage to maize. These include chemical control (Van den Berg and Nur, 1998), biological control, and host plant resistance (Tefera *et al.*, 2011). Cultural control strategies, which make the environment unfavourable for a pest to colonise and survive in maize fields, are also commonly used by African famers (Van den Berg *et al.*, 1998). Genetically modified maize has been used with success in South Africa for control of lepidopteran stem borers in maize since 1998 (Kruger *et al.*, 2012). *Bt* (genetically modified maize with an inserted gene sequence from the bacteria: *Bacillus thuringiensis*) maize has been registered for control of FAW in South Africa from November 2018 onwards.

1.3. Botanical pesticicdes and chemical compounds

Host plant resistance (HPR) can help to protect a plant from insect damage and it is an effective, environmental friendly and economical method of pest control (Sharma and Ortiz, 2002). What makes host plant resistance so attractive to farmers is that fewer chemical insecticide applications are needed and it is cost effective.

Botanical pesticides (natural compounds found in plants) are also commonly used against lepidopteran pests all over the world (Isman, 1997). There are five main types of botanical pesticides, namely essential oils, alkaloids, flavonoids and isoflavonoids, glucosides and fatty acid methyl esters (Hikal *et al.*, 2017).

The use of essential oils as insecticides has increased considerably over the last decade. These oils are extracted from aromatic plants and their increased use thereof is ascribed to their popularity with organic growers and environmentally conscious consumers (Hikal *et al.*, 2017). Essential oils may have repellent, insecticidal, antifeedant, growth limiting, oviposition inhibitory as well as ovicidal effects on a variety of insect species (Don-Perdo, 1996; Elzen and Hardee, 2003; Koshier and Sedy, 2001; Lu, 1995; Pereira *et al.,* 2006; Regnault-Roger *et al.*, 2012; Shelton *et al.*, 2002).

Alkaloids are the most important group of natural substances that contain insecticidal compounds (Rattan, 2010). Wachira *et al.* (2014) reported that pyridine alkaloids extracted from *Ricinus communis* L. (Euphorbiaceae) against the malaria vector *Anopheles gambiae* (Diptera: Culicidae) were toxic to larvae. Furocoumarin and quinolone alkaloids extracted from *Ruta chalepensis* L. (Rutaceae) leaves also showed larvicidal and antifeedant activities against the larvae of *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) (Emam *et al.*, 2009).

Both flavonoids and isoflavonoids are known to protect plants against insect pests by influencing their behaviour, growth and development (Simmonds, 2003). Flavonoids are one of the groups of

chemicals reported to regulate oviposition and feeding of insects on several crops such as vegetables (Mierziak *et al.*, 2014). Naringenin, hesperetin-7-O-rutinoside and quercetin-3-O-rutinoside, along with other active compounds, stimulated oviposition in the swallowtail butterfly (*Papilio* sp.) on young leaves of citrus plants (Nishida and Fukami, 1989). Nishida (1994) also found similar results for luteolin 7-O-(6''-malonyl glucoside) on the black swallowtail, *Papilio polyxenes* (Stoll) (Lepidoptera: Papilionidae) and for isorhamnetin glucoside on *Luehdorfia japonica* oviposition on the leaves of *Asarum* plants species. Flavonoids can also prevent insects from laying eggs for example quercetin-3-O-rutinoside acts as a stimulant to monarch butterfly, *Danaus plexippus* (Linnaeus, 1758) (Lepidoptera: Nymphalidae), but acts as a deterrent to *Pieris rapae* (Linnaeus, 1758) (Lepidoptera: Pieridae), the small cabbage white butterfly (Tabashnik, 1987).

The following flavonoids: 5-hydroxyisoderricin, 7-methoxy-8- (3-methylbutadienyl)-flavanone and 5-methoxyisoronchocarpin, and the isoflavonoids: judaicin, judaicin-7-O-glucoside, 2-methoxyjudaicin and maackiain, were also reported as direct feeding deterrents to lepidopteran larvae (Mierziak *et al.*, 2014). Flavone induces polysubstrate monooxygenases (PSMO), general esterases (GE), and glutathioneS-transferases (GST) in *S. frugiperda*, yet this species is affected deleteriously by low dietary concentrations of this allelochemical (Wheeler *et al.*, 1993).

Cyanogenic glucosides (CGNs) present in plant species are considered to have an important role in plant defence against herbivores (Zagrobelny *et al.*, 2004). The latter authors reported that these compounds are present in more than 2,500 different plant species, including ferns, gymnosperms and angiosperms. When plant tissue is disrupted or damaged by herbivores, CNGs are brought into contact with β-glucosidases and α-hydroxynitrile lyases that hydrolyze the CNGs and then cause the release of toxic hydrogen cyanide (HCN) (Figure 1.2). This binary system provides plants with an immediate defence mechanism against intruding herbivores and pathogens that cause tissue damage in the plants (Zagrobelny *et al.*, 2004). Many *Cynodon* grasses are cyanogenic (Mahmoodzadeh, 2010), whereas maize plants release low levels of hydrogen cyanide (Jones, 1998) upon tissue disruption. This raises the possibility that differential resistance to cyanogenic glycosides could be a factor in strain-specific host preference (Hay-Roe *et al.*, 2011). Hay-Roe *et al.* (2011) found that maize and stargrass leaves have very different levels of cyanogenic compounds, suggesting that cyanide toxicity may explain elevated Fall armyworm mortality when maize and stargrass are utilized as host plants. Differences in the ability to metabolize or eliminate cyanide may be the physiological basis for the plant host biases exhibited by Fall armyworm host strains (Hay-Roe *et al.*, 2011).

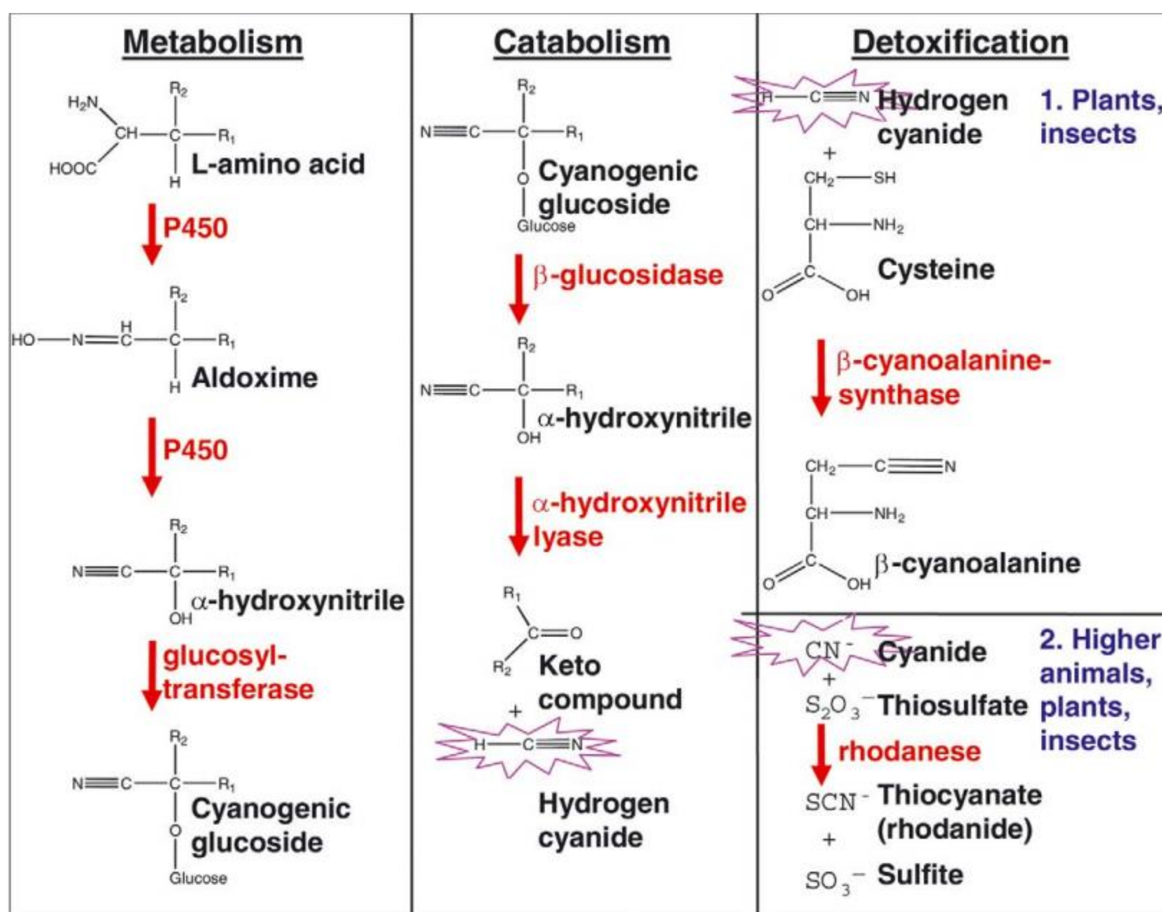

Figure 1.2: Biosynthesis, catabolism and detoxification of CNGs in plants, insects and higher animals. Enzymes involved are shown in red. HCN is highlighted in purple (Zagrobelny *et al.*, 2004).

Another example of secondary plant metabolism products ate the benzoxazinoids. These are important defence chemicals that are widespread in grasses (Poaceae), including crops such as maize, wheat and rye, but they are not present in rice, oat, sorghum and cultivated barley (Niemeyer, 2009). Fatty acid methyl esters were also isolated from *Solanum lycocarpum* (Solanaceae) and have larvicidal activity against *S. littoralis* (Yousef *et al.*, 2013).

1.4. Castor oil plant: its origin and uses

Castor oil plant (*Ricinus communis*) (Euphorbiaceae) is a monotypic species and is a very useful tropical foliage plant. It is grown from seed and can easily reach a height of 3 m in a single season, depending on the variety of the plant (Cronk and Fuller, 1995). The leaves of castor oil plants are mostly green but may have a purple colour (Figure 1.3). Flower spikes are bright red in colour and appear at the end of the season. These spikes contain highly toxic seeds for animals and humans.

The castor oil plant is a tropical perennial shrub that originated in Africa (Chan *et al.* 2010). It is also cultivated in tropical and subtropical regions around the world. It is believed that the Egyptians first

used castor oil about 4 000 years ago after which it spread to other parts of the world such as Greece and Rome where it was used as a laxative 2 500 years ago (Scarpa and Guerci, 1982). Castor oil plants can be cultivated with ease, even in unfavourable environments which makes it an appealing crop to cultivate in tropical developing countries.

Figure 1.3: Castor oil plant: *Ricinus communis* (Gardenia.net).

Ricinus communis seeds contain up to 60% unique oil, 90% of it being ricinoleic acid (12-C hydroxyoleic acid) (Maheshwari and Kovalchuk, 2016). Some special characteristics of this acid, such as its high molecular weight, low melting point (5 °C), very low solidification point (−12 °C to −18 °C), and the highest and most stable viscosity, render it extremely useful for industrial purposes. Vegetable oils that are rich in ricinoleic acid have properties that are desirable in the production of nylon, lubricants, soaps and resins (Dyer *et al*., 2008).

The nearly uniform ricinoleic acid content of castor beans have unique chemical properties that this fatty acid confers to the oil (Chan *et al*., 2010). Castor bean oil is also used as lubricant, in the cosmetic and medical industry as well as in speciality chemical applications (Chan *et al*., 2010; Okechukwu *et al*., 2015; Razzazi *et al*., 2015). Castor beans have also been proposed as potential sources of biodiesel because of the high oil content of the seeds (Lima Da Silva *et al*., 2006). The main suppliers of castor oil worldwide are India (the world's leader with 60% of the total production), Brazil and China (Maheshwari and Kovalchuk, 2016). According to Chan *et al*. (2010) the area under castor oil crop cultivation in the USA is limited due to concerns that the ricin toxin can be used as a

bioterrorism tool. Therefore, USA remains one of the largest importers of castor oil and its derivatives from developing countries that are threatened by political and economic instability (Chan *et al.,* 2010).

1.5. The use of plant extracts for pest control

Insects are major causes of crop and grain losses worldwide (Ferry *et al*., 2004). There are many synthetic chemical pesticides that are widely used to control pests, but ethical standards and problems associated with the extensive and over use of pesticides, for example development of pesticide resistance, negative impact on natural enemies and the environment as well as health impacts stand in the way of using these chemicals (Ramos-Lopez *et al.,* 2010). The abovementioned facts, together with the consumer's demand for residue free food and strict environmental regulations governing pesticide use created new opportunities for agrochemical companies to exploit the potential of plants with their natural toxic products in pest management (Isman, 2000).

Because of the adverse effects of especially chemical pest control methods on the environment, organic crop production contributed to reduce the use of chemical insecticides (Uchino *et al*., 2015; Alves *et al*., 2014). Alves *et al.* (2014) indicated that a promising alternative method to control pests such as the FAW is the use of plant secondary metabolites. These chemical compounds are naturally produced by plants and can induce deleterious effects in insects such as weight loss, reduction in fertility and reproduction, increasing development time of immature stages, feeding deterrence, structural changes in body tissues, changes in nutritional parameters and the inhibition of digestive enzymes in the gut (Malau and James, 2008).

The use of proteinase inhibitors (PI) is an example of these alternative strategies to control pests because it is a class of substances involved in plants defence mechanisms (Carvalho *et al.,* 2015). The levels of these proteinase inhibitors in plants are usually low, but as soon as plants get attacked by insects and suffer mechanical damage or gets exposed to plant hormones, these levels start to increase (Sharma, 2015). According to Jongsma and Bolter (1997) proteinase inhibitors also affects the amino acids in insects and cause deficiencies that influence their growth and development. These deficiencies may lead to their death by inhibiting gut proteinases or cause a large over production of digestive enzymes (Jongsma and Bolter, 1997; (Sharma, 2015).

1.6. The use of castor oil plant in pest control

Extracts of *R. communis* have been used to control insect pests in several crops (Ramos-Lopez *et al*., 2010). According to Upasani *et al*. (2003) and Aouinty *et al*. (2006) aqueous castor bean leaf extract possesses insecticidal activity against several Coleoptera and Diptera species. For example, *Callosobruchus chinensis* (Coleoptera: Bruchidae), *Cosmopolites sordidus* (Coleoptera: Curculionidae), *Culex pipiens*, *Aedes caspius*, *Culiseta longiareolata* and *Anopheles maculipennis*

(Diptera: Culicidae) were found susceptible to castor bean leaf extract. *Ricinus communis* is therefore a promising plant species for use in integrated pest management against several pest species (Carvalho *et al.*, 2015).

Bioactivity of aqueous extracts from green fruits of *R. communis*, when added to artificial diet, reduced the FAW larval development time and also caused a significant reduction in the weight of FAW larvae (Santiago *et al.*, 2008). These effects on FAW were mainly ascribed to the high fatty acid content (Ramos Lopez *et al.*, 2010) and the ricin found in *R. communis* plants.

Castor oil plant extracts have successfully been used to control several pest species using different formulations. Aqueous castor bean leaf extract has been shown to possess insecticidal activity against a few Coleoptera and Diptera species whereas a methanolic leaf extract had insecticidal activity against *C. chinensis* (Upasani *et al.*, 2003). In addition, both aqueous and acetone leaf extracts had different activity against *Acromyrmex lundi* (Hymenoptera: Formicidae) (Caffarini *et al.,* 2008). Castor oil insecticidal activity was also reported against *Zabrotes subfasciatus* (Coleoptera: Bruchidae) (Mushobozy *et al.*, 2009). It was also established that the aerial parts of plants had insectistatic activity against FAW (Kumar and Mihm, 2002; Molina *et al.*, 2003). Fall armyworm has been used as a model species for evaluation of the insecticidal and insectistatic activities of many plant species (Céspedes *et al.,* 2005).

1.7. Ricin, the most toxic component in nature

Because of the high concentration of ricin (extremely toxic protein) that constitutively occurs in the seeds, it is extremely difficult to cultivate castor beans on a widespread basis (Knight, 1979). Ricin is one of the most toxic natural toxins and leads to death when the seeds are eaten or when fine particles of the seeds are inhaled (Chan *et al.*, 2010). Ricin has in the past been used as a chemical weapon (Knight, 1979), and specifically as an immunotoxin for therapeutic purposes in the treatment of different cancers (Schnell *et al.*, 2002; Fidias *et al.*, 2002).

Ricin biochemical activity has been characterized as a type 2 ribosome-inactivating protein (RIP2) and consists of two subunits that are linked by a disulphide bond (Figure 1.4). The two subunits are as follows:

- Ricin toxin A (RTA) chain that harbours the ribosome-inactivating activity (32 kDa)
    - N-glycosidase that depurinates adenine in residue of the 28S ribosomal RNA (Endo *et al.,* 1987).

- Ricin toxin B (RTB) chain with a galactose-binding lectin domain (34 kDa)
    - Allows ricin to enter eukaryotic cells by binding to the cell surface galactosides and endocytosis (Lord *et al.,* 1991).

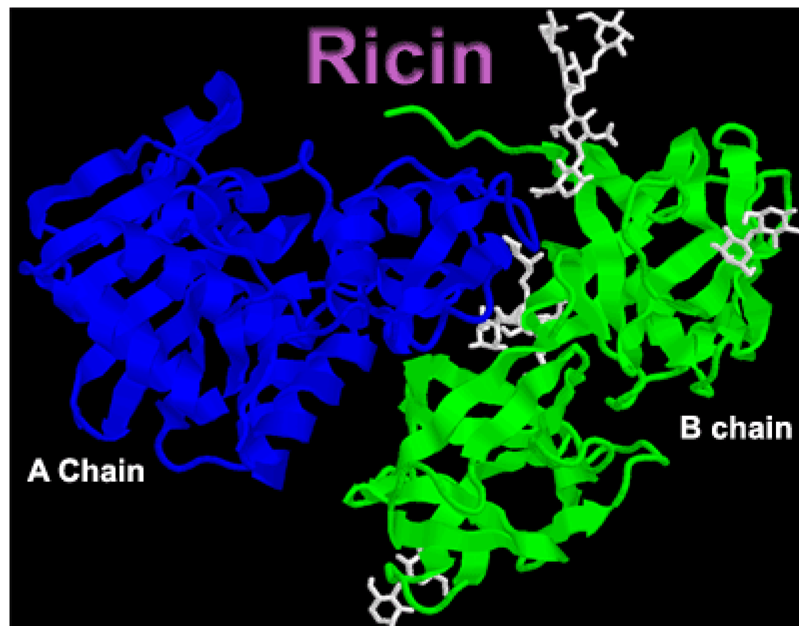

Figure 1.4: Structural domains of the ricin protein (Pastura, 2014).

According to Lord (1985), ricin is synthesized as a precursor that encodes both the above-named subunits in the endoplasmic reticulum of the endosperm cells after which it is translocated into protein bodies. Maize responds to Fall armyworm damage by producing a RIP2 protein (Chuang *et al.,* 2013).

1.8. The distribution of Fall armyworm

FAW has a tropical-subtropical origin in the Western Hemisphere (Luginbill, 1928). In Georgia in 1797 the first outbreak of FAW was recorded on grasses and grains (Smith and Abbot, 1797; Johnson, 1987). FAW is an important pest in South America, all of central America and the Caribbean Islands (McGuire and Crandall, 1967). After its invasion in Africa in 2016, the pest's distribution was recorded in over 43 African countries (Cowan and Johnson, 2018; Prasanna *et al.*, 2018). It was reported that in early May-June of 2018, FAW has also invaded India (Sharanabasappa *et al.*, 2018). Snow and Copeland (1969) illustrated the seasonal distribution of FAW in the United States (Figure 1.5). This species has no diapause mechanism to survive cold winters, but some individuals can overwinter in south Florida and Texas where host plants are continually available with moderate temperatures (Luginbill, 1928).

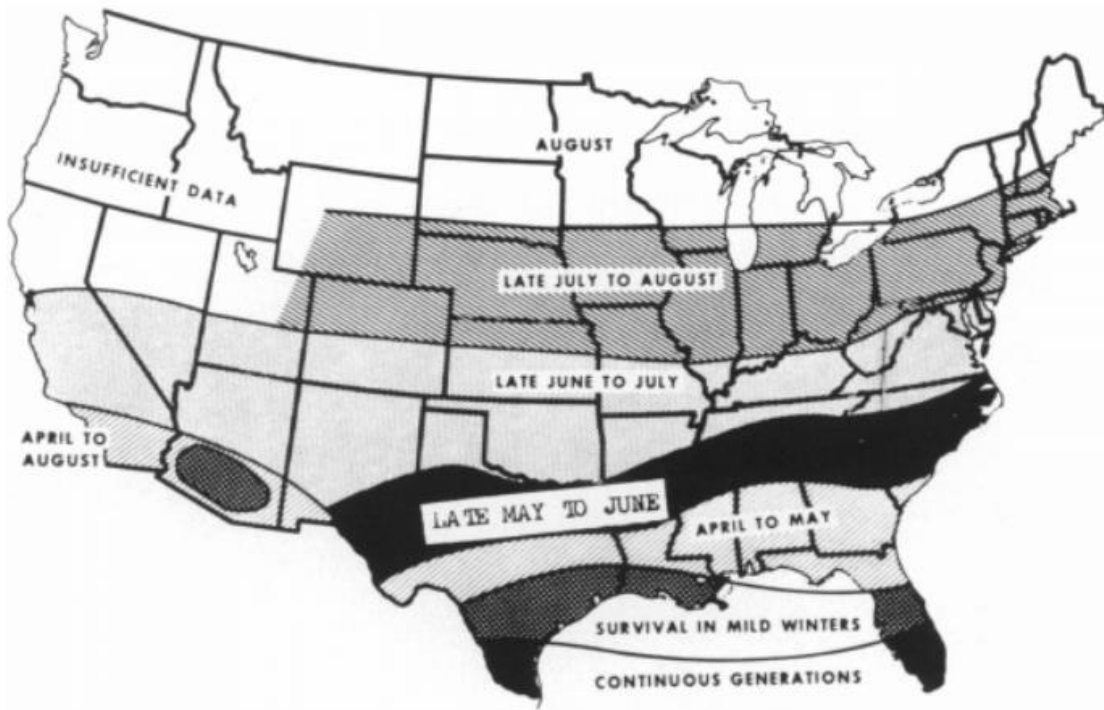

Figure 1.5: Seasonal distribution of Fall armyworm in the United States (Snow and Copeland, 1969).

According to a media release of South African Department of Agriculture, Forestry and Fisheries (DAFF) in May 2017, the presence of FAW in South Africa was confirmed on 3rd February 2017 with positive morphological and molecular identification of the larvae and adult moths. They also stated that the pest was mainly detected in the following provinces in South Africa: Limpopo, Gauteng, North West, Mpumalanga, KwaZulu-Natal, Free State and the Eastern Cape. In the Northern Cape it was only detected in the Hartswater area. FAW moths were also found in pheromone traps in the Western Cape Province in June 2018.

1.9. Life cycle of Fall armyworm

An illustration of the life cycle of the FAW is presented in figure 1.6.

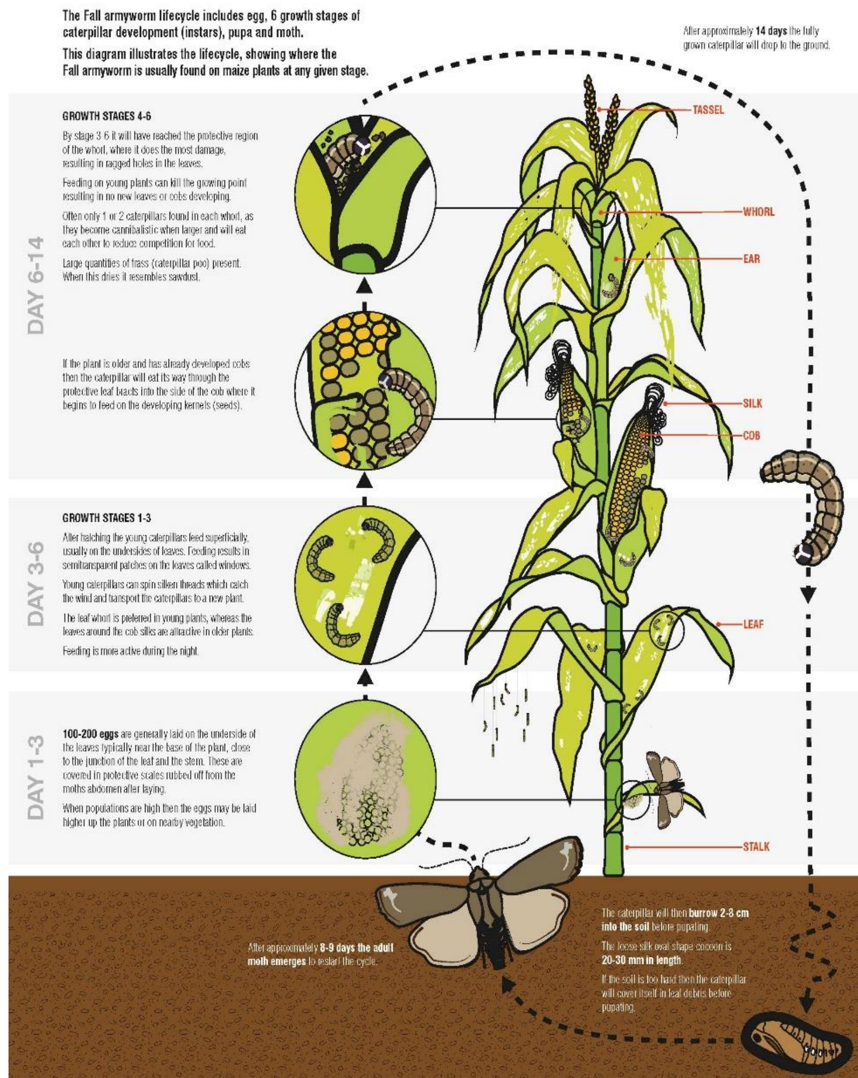

Figure 1.6: The life cycle of Fall armyworm, *Spodoptera frugiperda* (Curry, 2017).

1.9.1. Eggs

The eggs are spherical (0.75 mm diameter) in shape and green at the time of oviposition. Eggs become light brown prior to eclosion (Sparks, 1979) and mature in about 2-3 days at temperatures between 20-30°C. They are laid at night in masses of approximately 150-200 eggs per batch and a female can lay up to 1000 eggs during her life time. The female usually lays her eggs in batches, two to four layers deep, on the surface of leaves. The egg mass is usually covered with a protective, felt-like layer of grey-pink scales (setae) from the female abdomen (Figure 1.7A-B). Hatching usually takes place after 3-5 days (Sparks, 1979).

Figure 1.7 A: Egg mass of Fall armyworm, *Spodoptera frugiperda*. B: First instar larvae hatching.

1.9.2. Larvae

The larvae develop through six larval stages (Sparks, 1979). The duration of the development period of the different instars is controlled by a combination of larval diet and weather conditions. Development under favourable conditions is completed within 14-21 days. Larger larvae are nocturnal unless they enter the armyworm phase when they swarm and disperse, seeking other food sources (Capinera, 2017). Larvae are a light green to dark brown colour with longitudinal stripes. Sixth instar larvae are about 3-4 cm long (Figure 1.8). Larvae have four pairs of prolegs of which one pair of prolegs is on the last abdominal segment. Upon hatching they are green with black lines and spots, and as they grow they either remain green or become buff-brown and have black dorsal and spiracular lines (Capinera, 2017). If crowded (by a high population density and food shortage) the final instar can turn to almost black in its armyworm phase. Large larvae are characterized by an inverted white to yellow Y-shape on the head, black dorsal pinaculae with long primary setae (two each side of each segment within the pale dorsal zone) and four black spots arranged in a square on the last abdominal segment (Capinera, 2017).

Figure 1.8: Larvae of Fall armyworm, *Spodoptera frugiperda*.

1.9.3. Pupa

The pupae are shorter than mature larvae (1.3-1.5 cm in males and 1.6-1.7 cm in females), and are shiny brown (Figure 1.9) (Capinera, 2017). Pupation takes place inside a loose cocoon in an earthen cell, or rarely between leaves on the host plant. The pupal stage lasts nine to 13 days.

Figure 1.9: Pupa of Fall armyworm, *Spodoptera frugiperda*, (Agro Slide Bank).

1.9.4. Moths

The body length of a male moth is approximately 1.6 cm and its wingspan 3.7 cm (Capinera, 2017). The forewing is mottled (light brown, grey, straw) with a discal cell with a straw colour on three quarters of the area and dark brown on one quarter of the area (Figure 1.10). The female body length is 1.7 cm and the wingspan are approximately 3.8 cm. The forewing is mottled (dark brown, grey) with hind wings that have a straw colour with a dark brown margin (Figure 1.10) (Capinera, 2017).

Figure 1.10: Moths of the Fall armyworm, *Spodoptera frugiperda*, female left and male right.

The adults emerge at night and they typically fly for many kilometres during their natural pre-oviposition period, sometimes migrating for long distances before they settle to oviposit. On average, adults live for 12-14 days (Capinera, 2017).

1.10. Host plants of Fall armyworm

FAW is highly polyphagous and have a wide range of different plant species that have been recorded as its hosts (Luginbill, 1928). The most preferred hosts are in the Poaceae family, for example: maize, sweet corn, sorghum, Bermudagrass and crabgrass (Capinera, 2017). Montezano *et al.* (2018) stated that there are currently 353 plant species, belonging to 76 families, that are recorded as hosts of FAW. Pashley (1988) and Capinera (2017) reported that there are two strains of FAW. Female moths are presumed to be largely responsible for selecting hosts (Rojas *et al*., 2018). There is some evidence that FAW strains exist, based primarily on their host plant preference (Pashley, 1988; Dumas *et al*., 2015; Nagoshi *et al*., 2007). The first known as maize strain feeds primarily on maize, but it also on sorghum and cotton, while the other one called rice strain feeds primarily on rice, Bermuda grass and Johnson grass. They may be biotypes in which genetic differences are due to a selectively-mediated polymorphism within a single randomly-mating species. They may be sibling species that are either capable of hybridizing to a limited degree or completely reproductively isolated (Diehl and Bush 1984; Prowell *et al*., 2004; Meagher *et al.,* 2004).

The larvae of FAW can cause serious damage to its cultivated host plants. Young larvae consume the leaf tissue from one side of the leaf, resulting in a “window” - type of damage on the leaf (Curry, 2017). During the second and third instar phases, larvae begin to eat and make holes through the leaf tissue (Figure 1.11). The larvae usually feed inside the whorl, causing perforations in the leaves. The larval densities are usually reduced to one or two per plant due to cannibalism (Capinera, 2017).

Figure 1.11: Maize leaf damage caused by Fall armyworm, *Spodoptera frugiperda* larvae.

1.11. Management approaches of Fall armyworm

FAW can be managed by means of insecticides, cultural control strategies, host plant resistance and biological control (Capinera, 2017). Since FAW is a serious pest of maize, it is important to control this pest during both the plant's vegetative and reproductive stages. Because FAW has a short life cycle, it is necessary to employ effective management strategies to reduce the risk of pest outbreaks and economical damage (Curry, 2017).

The control of FAW populations is mostly done by application of chemical insecticides (Figure 1.12). Pesticide application has however been overused throughout the years, leading to insect resistance (Yu *et al*., 2003), environmental contaminations (Starner and Goh, 2012) and adverse effects on natural enemies, human and animal health (Loewenherz *et al*., 1997).

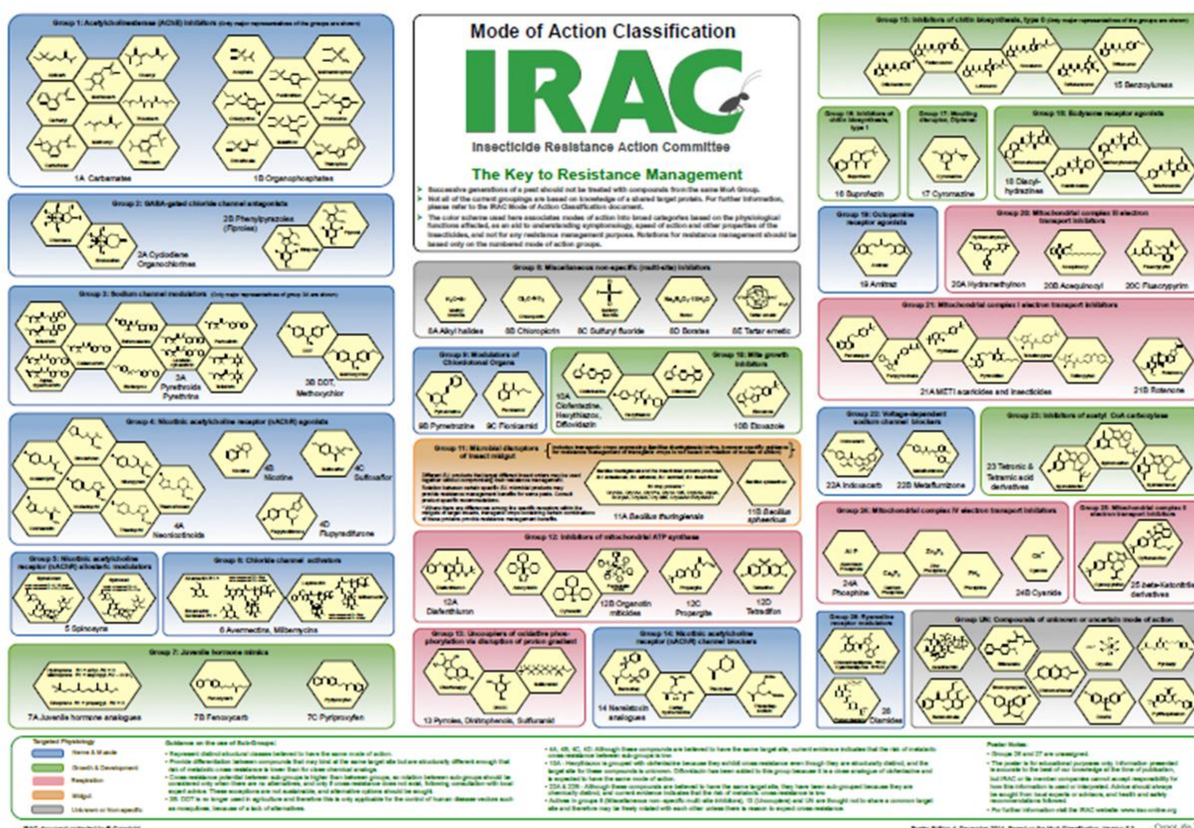

Figure 1.12: IRAC Mode of Action Classification (IRAC, 2019).

The different modes of action according to IRAC (2019) is as follow: Neuromuscular toxins, Insect growth regulators (IGR's), respiratory poisons/Metabolic poisons, gut disruptors and non-specific multi-site inhibitors.

1.11.1. Insecticides

According to Foster (1989), FAW larvae feed deep inside the whorl of young maize plants and a high volume of insecticide spray is needed for adequate penetration into the whorl. Insecticides can also be applied through irrigation systems, especially during the silking phase of the maize. Granular insecticides may also be applied into the whorls of young plants because these particles fall deep into the whorl and reaches the region where larvae prefer to feed (Foster, 1989).

1.11.2. Cultural control strategies

The most important and commonly used cultural practice is adaptation of planting date or the use of early maturing maize varieties. As a result, the maize plants escape pest infestation during the most vulnerable stages of the crop (Mitchell, 1978). Reduced tillage has little to some effect on FAW infestation levels and the presence of large amounts of crop residue results in delayed emergence of moths from the ground, resulting in reduced needs for chemical application onto crops (All, 1988; Roberts and All, 1993).

1.11.3. Transgenic resistance

According to Capinera (2017) there is partial resistance in some sweet corn varieties, but it is not sufficient to provide effective protection against FAW larval feeding damage. Studies in Brazil showed that certain genetically modified *Bt* maize events are no longer effective against FAW (Santos-Amaya *et al.*, 2017). They reported that FAW showed significant levels of resistance to the Cry1F protein expressed in *Bt* maize. The evolution of this resistance against Cry1F protein is ascribed to selection pressure by exposure to *Bt* maize and *Bt*-based sprays. Santos-Amaya *et al.* (2017) reported that Cry1F maize failed to express high enough levels of the *Bt* toxin to kill FAW. The pest individuals that are resistant, are able to survive and reproduce on Cry1F maize, thus increasing the number of resistant individuals in a population.

1.11.4. Biological control

There are many natural enemies of FAW, but only a few of these are sufficiently effective against the pest. Climatic conditions such as a cool, wet spring seasons, followed by warm and humid weather allow FAW to develop quickly and escape parasitization by natural enemies (Capinera, 2017).

Several Hymenoptera and Diptera species have been reported to parasitize FAW larvae, for example *Cotesia marginiventris* (Cresson) and *Chelonus texanus* (Cresson) (Hymenoptera: Braconidae), and *Archytas marmoratus* (Townsend) (Diptera: Tachinidae) (Luginbill, 1928). Other parasitiods of FAW in the families Ichneumonidae (*Ophion flavidus*, *Phryneta spinator* and *Campoletis flavicincta*), Braconidae (*Cerobasis insularis* and *Meteorus laphygmae*), Eulophidae (*Euplectrus plathypenae*)

(Hymenoptera) and Tachinidae (Diptera) have also been recorded in the Mexican states (Molina-Ochoa *et al.,* 2004).

The general predators of FAW larvae are the same predators that attack larvae of other arthropod species. For example, various ground beetles (Coleoptera: Carabidae); earwigs such as *Labidura riparia* (Pallas) (Dermaptera: Labiduridae), the spined soldier bug, *Podisus maculiventris* (Say) (Hemiptera: Pentatomidae) and the insidious flower bug, *Orius insidiosus* (Say) (Hemiptera: Anthocoridae). There are also vertebrates such as birds, skunks and rodents that consume FAW larvae and pupae (Pair and Gross, 1984). The latter authors reported that predation played an important role in biological control of FAW and that predators may damage or consume 60 to 90% of pupae that occur on crop fields.

Entomopathogens such as viruses, fungi, protozoa, nematodes and certain bacteria have been reported to infect FAW but only a few of these are effective enough to result in a decrease in the pest populations (Capinera, 2017).

1.12. Cannibalism of larvae

Cannibalism is a behavioural characteristic that occurs in a wide range of animal taxa. It is responsible for substantial levels of mortality and may have a significant effect on population structure (Fox, 1975; Richardson *et al*., 2010). Cannibalism is a common characteristic of lepidopteran larvae (Dail and Adler, 1990). According to Bentivenha *et al.* (2017) cannibalism occurs more often when different larval instars interact with each other. They also found that cannibalism mainly occurred when larvae started to compete for resources such as food and space. Previous studies showed that cannibalism in FAW was more frequent when larvae were fed on maize leaves than on artificial diets, leading to the conclusion that this cannibalistic behaviour is related to a lack of food with the required nutritional value (Da Silva and Parra, 2013). There are also other factors besides food availability and food nutrition that influence cannibalism behaviour, for example, insect density, temperature and humidity (Raffa, 1987; Richardson *et al*., 2010).

It is therefore unclear whether there are special adaptations associated with cannibalism that distinguishes it from normal predation (Mayntz and Toft, 2006). There are two possible types of nutritional benefits ascribed to cannibalism for example energy sources and availability of different types of nutrients (Mayntz and Toft, 2006). Energy sources implies the access to an energy source that is not available for non-cannibals, thus the food availability is increased for the cannibals (Fox, 1975). Another benefit that cannibalism may provide is a different composition of nutrients than the normal diet of a particular organism. Cannibalism may therefore provide nutrients in proportions that are more optimal than those in heterospecific diets (Fagan *et al*., 2002).

For arthropod herbivores, occasional cannibalism provides a meal with a higher nitrogen to carbon ratio (N/C ratio) than provided by a normal plant diet (Mayntz and Toft, 2006; Ambrosen and Petersen, 1997). Cannibalism in herbivores may result from a specific need for proteins (Figure 1.13) rather than from hunger (Alzubaidi and Capinera, 1983; Wolcott and Wolcott, 1984).

Figure 1.13: Cannibalistic Fall armyworm, *Spodoptera frugiperda,* feeding on a conspecific larva.

Insect density may affect their growth and development by reducing the availability of food sources due to interference or competition between individuals. Interference and/or competition may increase both the opportunity for cannibalism and the nutritional importance of cannibalism (Joyner and Gould, 1985; Raffa, 1987). Cannibalism may also result in an increase in size, growth and development rates of organisms (Polis, 1981).

In many cases the initiation and control of cannibalism has not been ascribed to a specific factor or obvious reason, but that cannibalism may occur primarily because of the presence of vulnerable individuals (Fox, 1975). Cannibalism rates on eggs and newly hatched larvae may be determined by the size of egg batches and the time span over which they hatch, but there is no cannibalism on eggs or young larvae if thy all hatch before the oldest began to search for prey (Fox, 1975).

1.12.1. Advantages of cannibalism

Cannibalism may be advantageous to a particular individual and may be employed in order to protect and secure itself. Cannibalism can also contribute to the nutritional fitness needed for increased survival, development rate and fecundity (Church and Sherratt, 1996). It can also provide indirect benefits such as the removal of potential competitors and intraspecific predators (Fox, 1975). According to Chapman (1999) another possible benefit of cannibalism is a reduction in the

conspecific population which may result in reducing predation or parasitism that can occur in a denser population.

1.12.2. Issues and limitations of cannibalism

Cannibalism may also provide certain challenges to a particular population or individuals. Cannibals risk injury or death from the defensive responses or mechanisms of the same species (Polis, 1981). The latter author also suggested that cannibalism could have a negative influence on the consuming individual in cases where a pathogen or parasite from an infected individual is carried to the cannibal. Polis (1981) also stated that predation may cause a reduction in the fitness of such individuals, since the cannibalism of kin, or cannibalism on individuals of the next generation may slow down their development rate.

1.13. The importance of the gut microbes in insects

Gut microorganisms are not only important in insect functions such as physiology, evolution, nutrition, reproduction, immune homeostasis, defence, and speciation, but are also relevant to agriculture and ecology (Engel and Moran, 2013).

According to Parmentier *et al.* (2016) knowledge of the microbial communities in the insect's midgut can contribute to understanding the role of these symbionts. The digestion of plant materials that are food for herbivorous insects, and the detoxification of the plant's secondary compounds or the defence mechanism against pathogens are mainly regulated by the gut microbes. Microbial communities dominate in the insect's digestive system and they play an important role in influencing the insect's biology and host plant selection (Engel and Moran, 2013).

1.13.1. Basic structure and purpose of the digestive system (gut) in Lepidoptera

There is a basic structure of the digestive system across all insect species, although they all have different modifications to adapt to their different feeding methods. According to Chapman (1998) the digestive system of insects consists of three primary regions: the foregut, midgut and hindgut (Figure 1.14).

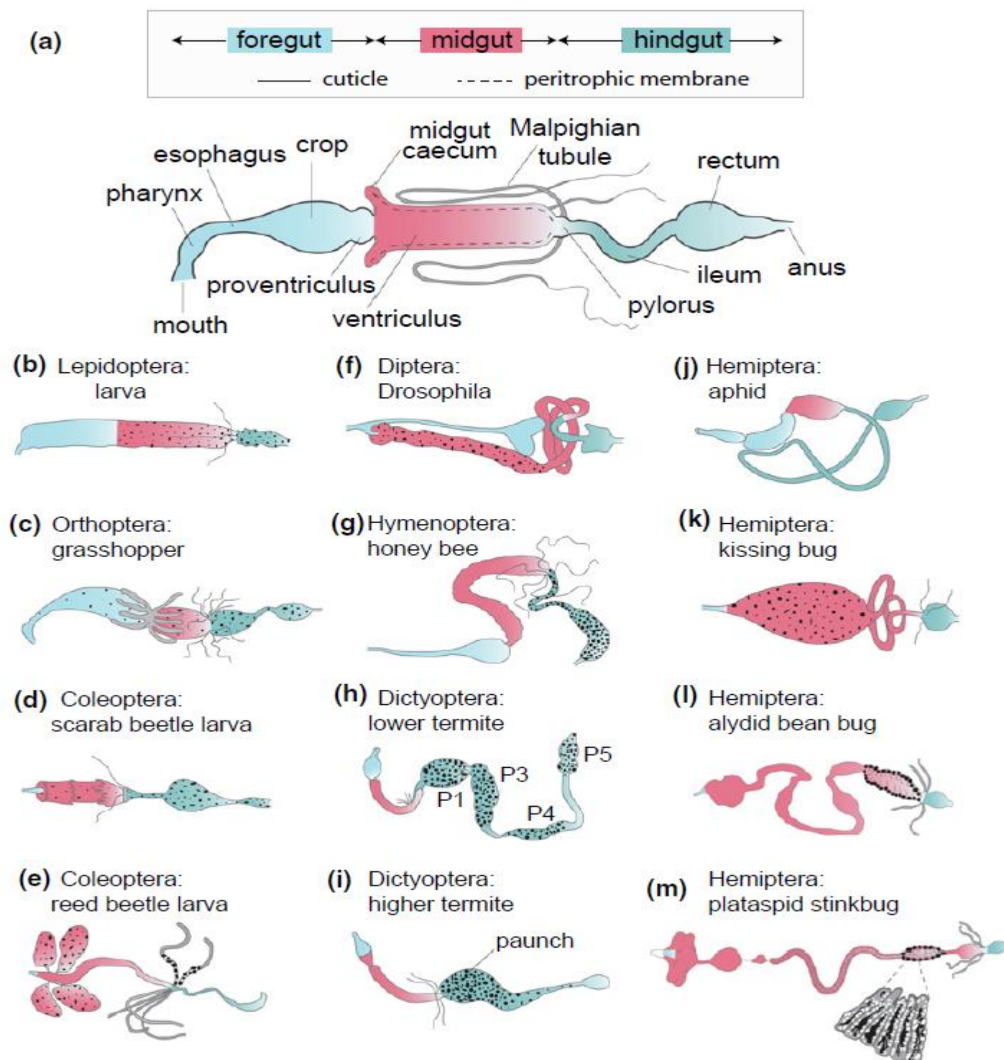

Figure 1.14: (a) Generalized gut structure of insects. The foregut and hindgut are lined by a cuticle layer (thick black line), and the midgut secretes a peritrophic matrix (dashed line). (b – m) Gut structures of insects from different orders (Engel and Moran, 2013).

According to Chapman (1998) the foregut and hindgut originate from the embryonic ectoderm and are lined with the exoskeleton that is made up of chitin and cuticular glycoproteins. The exoskeleton separates the gut lumen from the epidermal cells and is shed at each new instar phase. In some insects the foregut and hindgut are separate subsections of the midgut (Figure 1.14b). The foregut has a separate crop-region or diverticula for temporary food storage, while the hindgut has discrete sections such as fermentation chambers and a separate compartment (rectum) for holding the faeces before defecation.

The midgut is the primary site of digestion and absorption in almost all insect species (Chapman, 1998). The midgut does not have an exoskeletal lining and originates from endodermal cells. The midgut epithelial cells secrete an envelope referred to as the peritrophic matrix or the peritrophic membrane. This matrix is constantly replaced as it is shed when the larvae or insect grows or when

certain food types are ingested. The peritrophic matrix divides the midgut into the endo- and ectoperitrophic space. There are two types of peritrophic matrix, type I and type II (Chapman, 1998).

Peritrophic matrix type I, lines the whole midgut and is actively reproduced when a certain food type is ingested (Chapman, 1998). Peritrophic matrix type II, is produced by a specialized region of the anterior midgut referred to as the cardia and forms a continuous sleeve that is always present (Lehane, 1997). This peritrophic matrix is important and plays a key role in protecting insects from pathogens, mechanical damage done by food particles and destructive digestion enzymes from concentrating food (Shao *et al.,* 2001).

According to Engel and Moran (2013) the exoskeletal lining of the foregut and hindgut starts to shed each time the larvae develops to a bigger instar. It therefore disrupts and eliminates any bacterial population that can be identified. The midgut constantly sustains itself and produces a new peritrophic matrix together with associated microorganisms, most of which do not cross into the space adjacent to midgut epithelial cells.

The microbial colony in the midgut also depends on the physiochemical conditions in the lumen of the different gut compartments, and they play an important role in the extreme variation in the pH values and available oxygen levels in the midgut (Appel and Martin, 1990). The pH value in the lumen is constantly regulated and usually average near a value of 7 (Engel and Moran, 2013). According to Appel and Martin (1990) the lepidopteran larvae's midgut is highly alkaline with a pH value of between 11-12. The authors also stated that the digestive enzymes of insects are adapted to the function in these high alkaline conditions. The pH values in lepidopteran midguts are correlated with its tannin contents (Berenbaum, 1980). Tannin occurs in the leaves of plants and has been interpreted as a defence adaptation by plants to insects that reduces the binding of the proteins in plant tissues with ingested tannins, improving the nutrient availability.

The available oxygen in the gut of insects can range from anaerobic to aerobic conditions. Larger insects have bigger gut compartments and more diverse midgut microbial communities, resulting in anaerobic conditions (Engel and Moran, 2013). Johnson and Barbehenn (2000) studied nine Lepidoptera species and found that they have relatively higher oxygen levels within the foregut than midgut. The oxygen enters the gut while the larva feeds and is depleted as the food moves along the gut system. The conclusion of this finding was that gut microbes reduce the oxygen levels during an oxidation process as they digest or degrade the plant tissues (Johnson and Barbehenn, 2000).

1.13.2. The functions of different microorganisms within the midgut

Typical microorganisms that exist in the gut include the following: protists, fungi, archaea and bacteria. It is known that the insect gut micro-environment influences or can even determine the structure of the gut microbial community and the structure and diversity of the gut microbiota (Xia *et*

al., 2018). These microbial communities are very large in abundance, size, composition, locations and functions within the gut (Engel and Moran, 2013). Microbes contribute to defining insect metabolic traits since these microbial groups play a role in the following cycles: carbon metabolism, nitrogen recycling, methano- and acetogenesis (Brennan *et al.*, 2004). However, the emphasis of most studies assessing the digestive role of insect gut bacteria is on carbohydrate (and sometimes lipid) degradation, rather than protein degradation (Visôtto *et al.*, 2009).Microbes are important for plant-feeding insects whose diet is generally either low in nutrients, high in chemical defences, or both (Acevedo *et al.*, 2016). Symbionts associated with phytophagous insects provide the important and required amino acids (Douglas, 2015), aid in digestion (Visôtto *et al.*, 2009), and detoxify secondary plant metabolites such as terpenes and phenolics (Hammer and Bowers, 2015). Gut microbes in particular have been hypothesized to shape host use and diet breadth by allowing herbivorous insects to detoxify specific plant allelochemicals (Chaturvedi *et al.*, 2017).

Symbionts are considered as primary or secondary, depending on whether they are needed by the host to survive or provide non-essential benefits (Douglas, 2015). Obligatory symbionts are commonly harboured in specialized cells (bacteriocytes) and play important roles for nutrition in certain insect groups (Paniagua Voirol *et al.*, 2018). For example, intracellular Buchnera bacteria associated with aphids provide essential amino acids and vitamins (Hansen and Moran, 2014).

The benefits provided by secondary symbionts are often context-dependent. In aphids, for example, secondary symbionts can provide a range of ecological benefits including resistance to pathogens, parasitoids, and heat tolerance (Oliver *et al.*, 2010).

The gut microbes form a community because one bacterial colony cannot degrade, help with the uptake of nutrients and help detoxifying harmful proteins or pathogens. Therefore, the different gut microbes form a strong symbiotic relationship with each other (Moran and Baumann, 2000). These symbiotic relationships can be divided into a primary relationship (specialised cells called bacteriocytes that are advantageous to their insect host) (Lundgren *et al.*, 2007) and a secondary relationship (live extracellular in the gut of the insect) (Lundgren *et al.*, 2007).

1.13.2.a. Protists

According to Hongoh (2010) a wood-feeding cockroach, *Cryptocercus* sp. (Blattodea: Cryptocercidae) are the only species to have protists in their gut microbial community. These gut protists play an important role in the survival of the cockroach that live on a lignocellulose diet.

1.13.2.b. Fungi

Fungi that live in insect guts can either occur extra- or intercellular, and they play important roles in the insect's nutrition (Vega and Dowd, 2005) as well as the detoxification of toxic plant metabolites

(Dowd, 1989; Vega and Dowd, 2005). They also help with the production of enzymes that produce important amino acids and vitamins needed by the insect to develop.

1.13.2.c. Archaea

Archaea are only part of a small percentage of the total prokaryotic community in the insect guts (Hongoh, 2010; Ohkuma, 2008). *Methanobrevibacter cuticularis* and *Methanobrevibacter curvatus* are commonly isolated form hindguts of termites (Isoptera) (Hongoh, 2010). They may play an important role in the lignocellulose fermentation in the guts of termites by utilising H_2.

1.13.2.d. Bacteria

A few studies reported the presence of bacteria within Lepidopteran species, but knowledge about their function and role in insect development is limited (Broderick *et al*., 2004). Previous studies suggested that gut microbiota may contribute to mortality in a wide range of Lepidoptera species. The midgut of *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) was described by Snyman *et al.* (2016) to represent an intriguing and unexplored niche for analysing microbial ecology.

There is a large amount of different bacterial species that are present in the gut of insects and each one has a specific role that helps the host insect in digestion, degradation of complex molecules and uptake of nutrients, and also helps in detoxification harmful substances (Engel and Moran, 2013). The microbial colonies may differ between species as well as between individuals within their colony size, composition. The location may also have an influence on the microbial communities of insects (Engel and Moran, 2013), as well as the host plants on which the insects feed on especially in the case of polyphagous.

Some microbial colonies are facultative anaerobic organisms that assist in maintaining the anaerobic conditions in the insect's gut through absorbing oxygen molecules (Lundgren *et al*., 2007). They are in a symbiotic relationship to strict anaerobic microbe organisms that help with the digestion of cellulose. Some bacterial colonies contribute to the growth and development of the larvae. An example of this bacterial colony is *Serratia marcescens* that were isolated in the gut of Diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), but it can be pathogenic to other insects.

In addition, bacterial colonies from the family Enterobacteriaceae (Class: Gammaproteobacteria) also aid the host insects in producing digestive enzymes that contribute to the host nutrient uptake (Lundgren *et al*., 2007; Engel and Moran, 2013). Other bacterial colonies such as *Enterobacter* sp. help degrading chlorpyrifos and organophosphate insecticides and therefore, can be an important detoxification mechanism for the insect to develop resistance against insecticides (Singh *et al*., 2004: Lundgren *et al*., 2007; Almeida *et al*., 2017). According to Almeida *et al.* (2017) Enterococcus mundtii also helps with the detoxification of several insecticides.

Enterobacter, has also been reported as the major gut bacterium in termites belonging to the *Rhinotermitiidae* (Eutick *et al.*, 1978), when aerobic culture techniques were used. *Enterobacter* and *Citrobacter* have also being suggested as important for fixing atmospheric nitrogen to produce a source of fixed nitrogen for the termites (Eutick *et al.*, 1978, Janzen, 1985).

Some bacterial species also assist some insect larvae to survive and be killed by the *Bacillus thuringiensis* toxin that needs a high pH environment in the insect gut to act. In the lepidopteran species for example, the high pH value is decreased through some *Enterococcus* sp. that produce acetate and makes the gut environment less alkaline (Broderick *et al.*, 2004; Dillon and Dillon, 2004; Xiang *et al.*, 2006). *Bt* toxins produce midgut lesions that are the entry sites for *B. thuringiensis* spores and enteric microbes into the hemocoel, where they are hindered by immune barriers (Caccia *et al.*, 2016).

1.14. General objectives

The main objective of this study was to evaluate the effects that FAW feeding on *R. communis* may have on its larval behaviour and cannibalism, and to investigate the possible influence that feeding on this plant species may have on the midgut microbe communities of FAW larvae.

1.15. Specific objectives

- To determine and compare the midgut microbial community of *S. frugiperda* larvae that feed on either maize or castor oil plant tissue.
- To assess the behavioural differences between *S. frugiperda* larvae that feed on maize and castor oil plant tissue.
- To determine the feeding effect of *S. frugiperda* larvae on castor plant tissue may have on their cannibalistic behaviour.

1.16. References

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Chapter 2: The effect of maize and castor oil host plants on the midgut microbial community of *Spodoptera frugiperda*

2.1. Abstract

The Fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith), is one of the major pests of maize and other crops in the Americas, and recently in Africa and Asia. Extracts of the Castor oil plant, *Ricinus communis* has been used to control insect pests on several crops, and it has been reported that cannibalism occurs less when FAW larvae feed on castor oil plant material. This study aimed to compare midgut bacteria community composition and diversity in FAW larvae that feed on maize and castor oil plant tissue. To identify FAW larval midgut bacteria diversity, 16S rRNA gene of isolated bacteria was sequenced. Molecular phylogenetic analyses revealed the bacteria affiliated to the Proteobacteria, Actinobacteria and Firmicutes phyla. Seventeen bacterial species were identified in the maize reared larvae and 12 bacterial species in the larvae reared on castor oil plant tissue. *Acidovorax temperans, Acinetobacter calcoaceticus, Acinetobacter pittii, Staphylococcus xylosus* and *Staphylococcus saprophyticus* were the common bacteria species identified in both FAW midguts larvae reared on maize and *R. communis.* The following of the isolated bacterial species were unique to the specific reared larvae: *Pseudomonas peli, Pseudomonas anguilliseptica, Pseudomonas fluorescens, Stenotrophomonas pavanii, Stenotrophomonas maltophilia, Enterobacter asburiae, Luteimonas terrae, Luteimonas aestuarii, Xanthomonas citri, Novosphingobium* sp.*, Sphingomonas melonis, Enterococcus mundtii, Staphylococcus succinus, Bacillus thuringiensis, Bacillus cereus, Bacillus toyonensis, Arthrobacter protophormiae, Glutamicibacter* sp.*, Microbacterium sorbitolivorans*. The Shannon diversity of bacteria in the midgut of larvae reared on maize and castor oil plants, was low (0.0398 - 0.9982) compared to *Helicoverpa armigera* (Lepidoptera: Noctuidae).

2.2. Introduction

Gut microorganisms are important in insect functions and are also relevant to agriculture and ecology (Engel and Moran, 2013). It is known that the insect gut micro-environment influences or can even determine the structure of the gut microbial community and the structure and diversity of the gut microbiota (Xia *et al*., 2018). According to Parmentier *et al.* (2016) the microbial communities in the insect's midgut can contribute to the understanding of the interactions of these symbionts with the host. The potential favourable functionalities for the digestion of plant material, the detoxification of plant secondary compounds or defence mechanisms against pathogens are possible because of the gut microbes. Microbial communities dominate in the insect's digestive system and they play an important role in influencing the insect's biology and host plant selection (Engel and Moran, 2013). The midgut microbial community structure differs between individuals from the same species as well as from different insect species (Engel and Moran, 2013). The midgut microbes form a symbiotic

relationship with their insect host and assist in the enhancement of nutrition, digestion, detoxification of plant secondary compounds, developing the immune system, protection against pathogens and parasites and control of the reproductive systems inside the insect's body (Dillon *et al.*, 2005; Azambuja *et al.*, 2004; Broderick *et al.*, 2004; Rajagopal, 2009; Engel and Moran, 2013; Gimonneau *et al.*, 2014; Tagliavia *et al.,* 2014).

FAW is highly polyphagous and 353 larval host plants have been reported (Montezano *et al.*, 2018). However, the most preferred host plant species is maize (Capinera, 2017). Many non-crop species are also wild hosts for FAW and are known to support larval development (CABI, 2018). *Ricinus communis* which has been used in the management of several insect pests, is also reported as one of the host plants of FAW (CABI, 2018).

The different host plants with their specific nutritional diets and compounds have an influence on the gut microbial community structure and composition (Broderick *et al.*, 2004; Robinson *et al.*, 2010). The variation in host plants or plant composition may cause midgut bacteria adaptation (changing in composition or diversity) to digest the plant tissue to the benefit of the host insect as much as possible (Santo Domingo *et al.*, 1998; Anand *et al.*, 2009). For this reason, the two plant materials used in this study were maize (the most preferred host by FAW) and castor oil plants.

Therefore, this study aimed to determine and compare the midgut bacterial community composition and diversity of FAW larvae that feed on maize and castor oil plant tissue.

2.3. Materials and Methods

2.3.1. Sampling

Larvae of FAW were collected at Groblersdal (25°12'24.0"S, 29°13'21.4"E), South Africa from infested maize plants. Plant whorls that exhibited damage symptoms were removed from plants in the field and opened to retrieve the larvae.

The larvae were subsequently reared in the laboratory on maize leaves until pupation. The plastic rearing containers (100 ml) had steel-infused lids to allow air-flow. The rearing was done at 26 ± 1°C, 65 ± 5% RH and 14L:10D photoperiod in an insect rearing chamber.

Emerged moths were transferred to oviposition chambers (2 l) to mate and lay eggs. The chambers and method used were similar to that described by Kruger *et al.* (2012). The larvae that hatched from these eggs were used in the preference bioassays. The 1st instar larvae that hatched from the eggs were divided into two groups and reared (F1 generation) on maize and castor oil plant leaves respectively. Each larva was reared in a separate rearing container (to avoid cannibalism) and two cuttings of fresh leaf tissue (5 x 5 cm) of either maize or castor oil plants were provided every two

days until the larvae reached the fourth instar to be used in the bioassays. The reason for usig 4th instar larvae is that they are big enough to be dissected.

2.3.2. Sample preparation

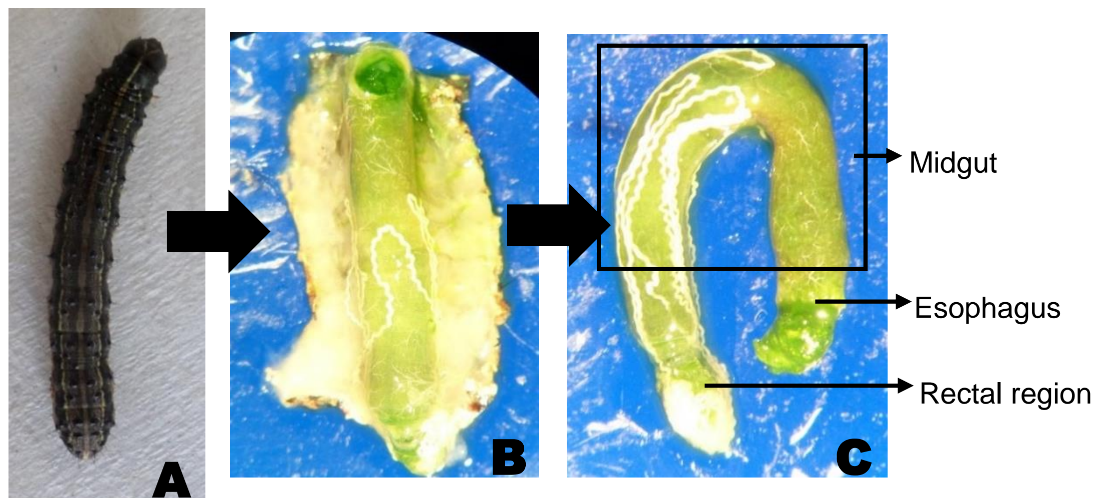

Figure 2.1 A-C: Photos indicating the aseptic dissection process of *Spodoptera frugiperda* larvae to expose the midgut.

The larvae reared on maize and castor oil plants were dissected in order to remove the midgut. Seventy percent ethanol was used to sterilize the exterior of the larvae, prior to dissection. This surface sterilization ensured that their outer surface bacterial contaminations were prevented (Schloss *et al.*, 2006). After dissections, conducted under aseptic conditions, complete guts were removed (Figure 2.1 A-C) and placed in a sterile Petri dish (Peyronnet *et al.*, 1997; Figure 2.1 A). Dissections were done from the last abdominal segment to the first thoracic segment to expose the gut (Figure 2.1 B). Three midguts of the larvae from the same experimental set up were pooled and placed in single 1.5 ml sterile microfuge tube which contained 1 ml of distilled water. Each tube was sonicated for two minutes to disrupt the gut material and to release the bacteria (Broderick *et al.*, 2004; Robinson *et al.*, 2010; Pryia *et al.*, 2012). Samples were then centrifuged for 15 seconds at 12 800 rpm to separate suspended bacteria from heavier gut material. Supernatants were immediately used for analyses.

2.3.3. Bacteria isolation

The supernatant obtained containing bacteria was processed as follows: one hundred microliters were used to make a dilution series of up to 10^{-6}. The remainder of the supernatant was frozen at -80 ºC for later use. Each of the respective dilutions, as well as the stock solution, was spread-plated onto culture agar (Figure 2.2). Thus, for each plant reared larvae there were seven spread plates, each with a different concentration. Nutrient agar (15g Agar Bacteriology, 5g NaCl, 5g Peptone and 3g Yeast extract per 1l water) was used as the culture medium.

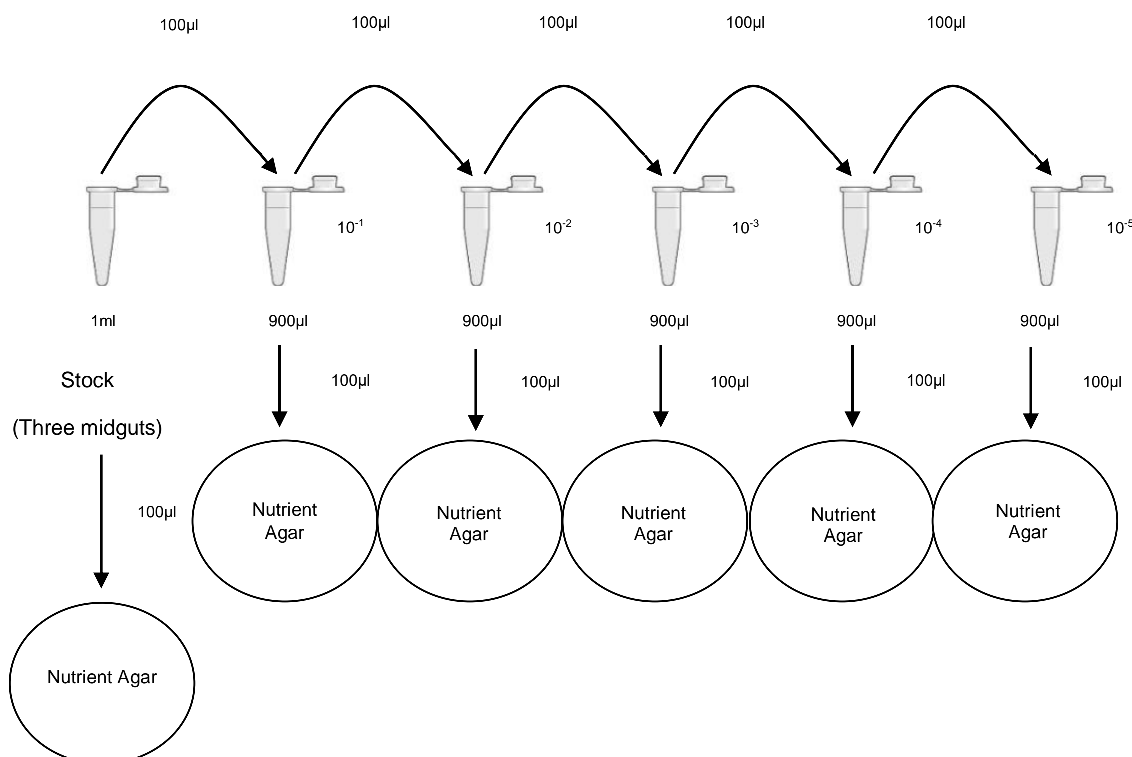

Figure 2.2: Illustration of the dilution series made from the supernatant (Lacey *et al.,* 2007).

Spread plates were incubated at 25°C for a 5-day incubation period, and the number of colonies on each plate was counted. Morphological traits of the different colonies were recorded and the number of colonies with similar traits was counted. Morphotypes were identified based on four characteristics namely, the surface appearance and shape of the colony, the elevation of the colonies, the shape of the colonies' edges and the colour of the colonies (Figure 2.3) (Van der Hoeven *et al*., 2008; Pryia *et al*., 2012). One of each different morphotype on the nutrient agar was collected and streaked onto fresh nutrient agar to obtain pure cultures.

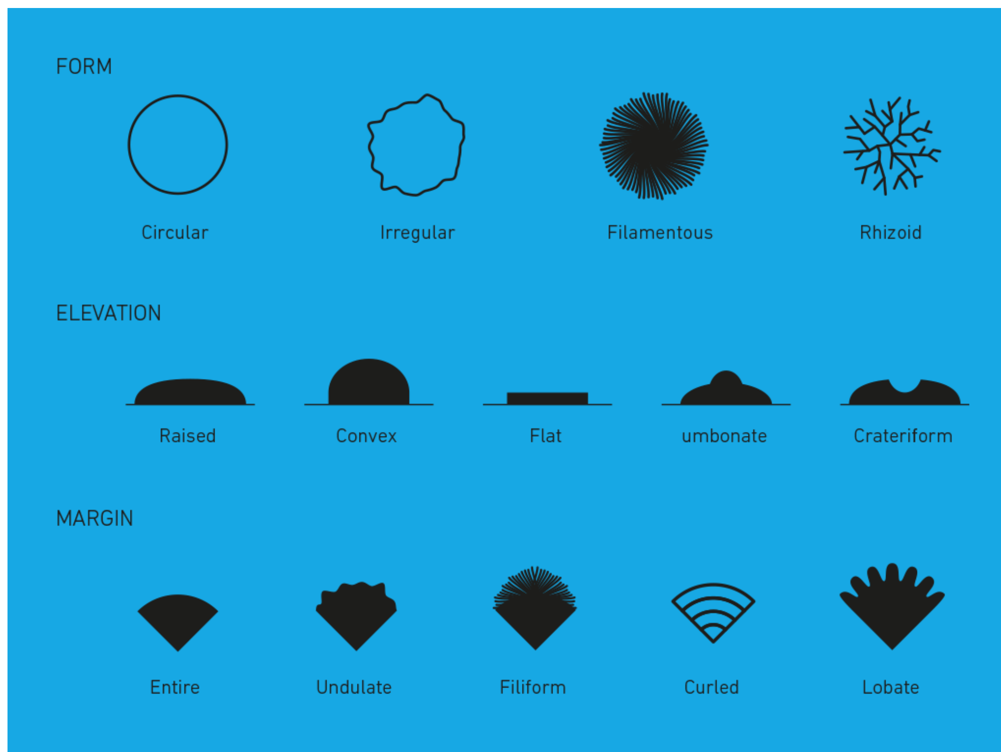

Figure 2.3: Characteristics used to identify morphotypes (Microbiologyonline.org, 2018).

2.3.4. Gram staining

To ensure that the colonies were pure to be used for further down-stream processing analysis Gram staining was performed. The procedure was conducted as described by (Sutton, 2006; Wiley *et al.*, 2008)

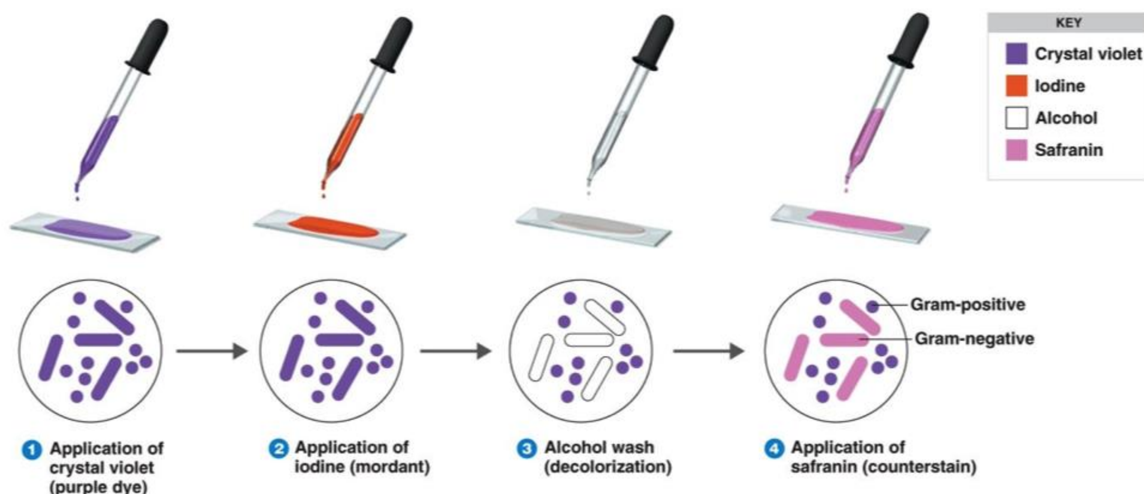

Figure 2.4: Procedure of how Gram-staining is done (Laboratoryinfo.com, 2016).

On a sterile microscope slide, a bacterial colony was mixed with a drop of water using a sterilised inoculation loop. Cultures were allowed to air-dry before heat-fixing. The culture was stained using the steps illustrated in Figure 2.4. Slides were left to dry before cultures were examined under a microscope, using oil immersion. Red or pink colonies are Gram-negative bacteria and purple are a Gram-positive (Sutton, 2006; Wiley *et al.*, 2008).

2.3.5. DNA isolation

The pure isolates were grown in nutrient broth at 25°C for 5 days prior to DNA extraction. DNA was extracted using the Chemagic DNA Bacteria Kit from PerkinElmer (South Africa). This method uses magnetic beads to bind on the DNA to extract it. Two hundred microliters of the overnight culture were placed in a 2 ml centrifuge tube and 400 µl of the Lysis Buffer 1 were added. The latter contained 2 µl of RNase A (10 mg/ml). The rest of the protocol was followed as instructed by the manufacturer.

2.3.6. DNA amplification

The Polymerase Chain Reaction (PCR) was followed to amplify the 16S rDNA gene fragments. Reaction mixtures (25 µl, total volume), consisted of 12.5 µl Master Mix [(0.05 U/µl *Taq* DNA Polymerase in reaction buffer, 0.4 mM of each dNTP, 4 mM $MgCl_2$) (Fermentas Life Science, US)], 9.5 µl nuclease free water. To this mixture 1 µl of forward primer (27F: 5'-AGAGTTTGATCMTGGCTCAG-3'), 1 µl of reverse primer (1492R: 5'-TACGGYTACCTTGTTACGACTT-3'; Allen *et al.*, 2009) as well as 1 µl of DNA were added. The primers set 27F and the 1492R were amplified at the fragment region of 1465 bp. This is nearly the entire length of the gene (Frank *et al.*, 2008). A ProFlex PCR System (ThermoFisher) was used to perform PCR under amplification cycling conditions as described in Snyman *et al.* (2016). The PCR's were performed under the following conditions: an initial step of 95ºC for 5 minutes was followed by 35 cycles consisting of denaturing at 95 ºC for 30 seconds annealing at, 53 ºC for 30 seconds and extension at 72 ºC for 1 minute. This was then followed by a final extension of 72 ºC for 10 minutes after which the reactions were briefly held at 12 ºC, removed from the thermocycler and stored at 4 ºC until further analysis were conducted.

2.3.7. Agarose gel electrophoresis

The DNA integrity and quality were determined with agarose (WhiteSci, USA) gel electrophoresis (Bio-Rad, UK) as well as to confirm if the PCR reactions were successful. A 1% w/v agarose gel were used, and three microliters of each DNA sample were mixed with 3 µl of 6 x Orange Loading Dye (Fermentas Life Science, US) containing GelRed and loaded into a well. A 1Kb and 100bp molecular weight marker was used to determine the success of the genomic DNA amplification and

the PCR amplifications respectively. The electrophoresis was set for 45 minutes at 80 V. A horizontal Power Pac UK gel electrophoresis system (BioRad, US) was used for electrophoresis and a ChemiDoc™ MP Imaging System (Bio-Rad, US) with Image Lab™ software (Version 4.0.1) was used to capture the gel images.

2.3.8. Sequencing of the DNA

Products of the PCR were sequenced by Inqaba Biotech (South Africa) using the 27F primer. Resulting chromatograms were viewed with Geospiza Finch TV (Version 1.4) software and Basic Local Alignment Search Tool (BLAST) searches were performed to compare the sequences obtained to those in the GenBank database (NCBI; http://www.ncbi.nlm.nih.gov/BLAST) web site.

2.3.9. Statistical analyses

In this study the bacterial diversity of the isolates was determined by using the Shannon diversity index, H, calculated as follows:

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

with *S* as the species richness and Pi as the proportion of species (Shannon and Weaver, 1949; Begon *et al.*, 2006). The number of different morphospecies present, represents species richness. The abundance of colonies within these groups represents the P_i values. In the present study the average number of isolated colonies that formed at the 10^{-1}-10^{-5} dilutions were determined and used as the P_i value.

The Shannon diversity index value of the bacteria communities isolated from the reared larvae reared on the different host plants were compared through a T-test.

The DNA sequences received from Inqaba Biotech™ were carefully selected and edited using the MEGA X programe before phylogenetic analysis were perfomed. Sequences were compared to 16S rRNA gene sequences within the GenBank database through BLAST searches. Only those with high sequence similarities (≥ 97 %) were used for phylogenetic analysis. A table with all the ID's were set up and bacterial colonies were grouped according to the appropriate phyla.

2.4. Results and discussion

2.4.1. Morphological identification of the bacterial through media culture

After the midguts were dissected and the various Petri dishes were examined, a total of 34 morphotypes were identified through their unique surface appearance and shape of the colony, the elevation of the colonies, the shape of the colonies edges and the colour (Figure 2.5).

Figure 2.5: An example of different bacterial colonies that occurred in the stock plate after isolation from the gut contents of Fall armyworm.

The different morphotypes that were observed ranged in colour from white to bright yellow. Most of the morphotypes were circular and raised with an entire margin. Other morphotypes were irregular or filamentous that were flat with an undulate or filiform margin. Most observed morphotypes in the maize reared larvae midguts were white, circular and raised with an entire margin as well as light yellow or cream colour, circular and raised with an entire margin. In the case of the castor oil reared larvae's midgut, the most observed morphotypes were also white, circular and raised with an entire margin, a light yellow or cream colour, circular and raised with an entire margin and a bright yellow colour, circular and raised with an entire margin.

2.4.2. Molecular identification of the isolation of symbiotic bacteria

The 34 isolated bacterial morphotypes were purified by at least three repeats of streaking and re-streaking on to appropriate media. The cultures were then prepared for Gram-staining and DNA isolation. The Gram-staining showed that most of the colonies were Gram-positive cocci.

2.4.3. DNA extraction and sequencing

The pure bacterial cultures were incubated in nutrient broth to be used for DNA extraction. The extracted DNA ranged from very high concentrations of 357.4 ng/µl to low concentrations of 21.1 ng/µl. The DNA purity were determined through the ratio of A_{260nm}/A_{280nm} and ranged between 1.58 and 2.01. The ideal ratio should be between 1.7 and 2.0 to ensure that there is no protein or RNA contamination (Thermo Fisher Scientific - NanoDrop products, 2019). Therefore, the DNA samples were considered to be of sufficient quality and quantity for PCR amplification.

After the genomic DNA extraction, they were subjected to agarose (WhiteSci, USA) gel electrophoresis to ensure that the DNA was of high integrity. Similarly, after PCR amplification the amplified fragments were subjected to agarose electrophoresis to ensure that the PCR worked and that the products were of the right size for sequencing. Figure 2.6 is an image of an agarose gel showing the purified PCR products that were send to Inqaba Biotech^{TM} for sequencing. There was a 100 bp molecular weight marker in the gels and all the amplification products were >1000 bp (the largest band in the marker).

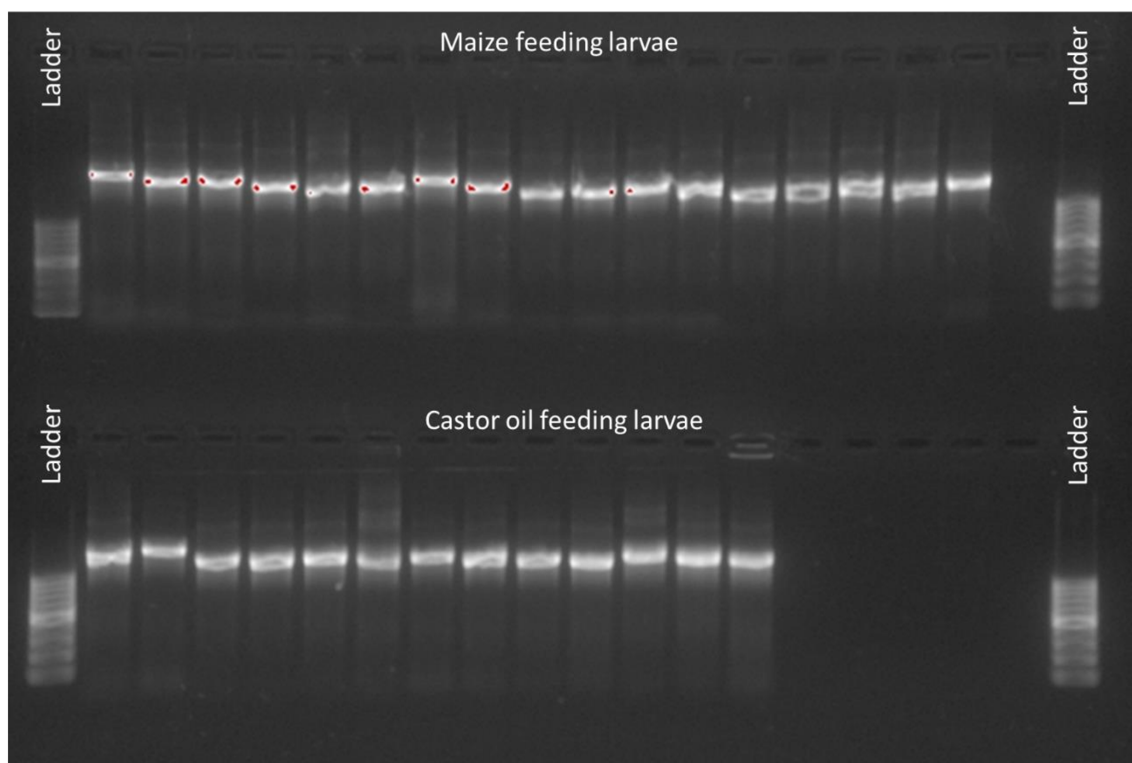

Figure 2.6: Electrophoresis gel of samples after PCR amplification. The top row indicates bacteria isolated from maize-feeding larvae while the bottom row indicates bacteria isolated from the Castor plant-feeding larvae.

Sequencing of the 16S fragments revealed that there were three phyla and a total of 29 bacterial species isolated. Of these 17 different bacterial species were isolated from the larvae that fed on maize plant tissues and 12 different bacterial species in the midguts of castor oil plant fed larvae.

There were three phyla of bacteria found in the midguts of larvae that fed on both maize and castor oil. These were the Proteobacteria, Firmicutes and Actinobacteria. Figure 2.7 shows the percentage or proportion of the different bacteria phyla isolated from the midgut of larvae that fed on maize (Figure 2.7A) and from larvae that fed on castor oil (Figure 2.7B).

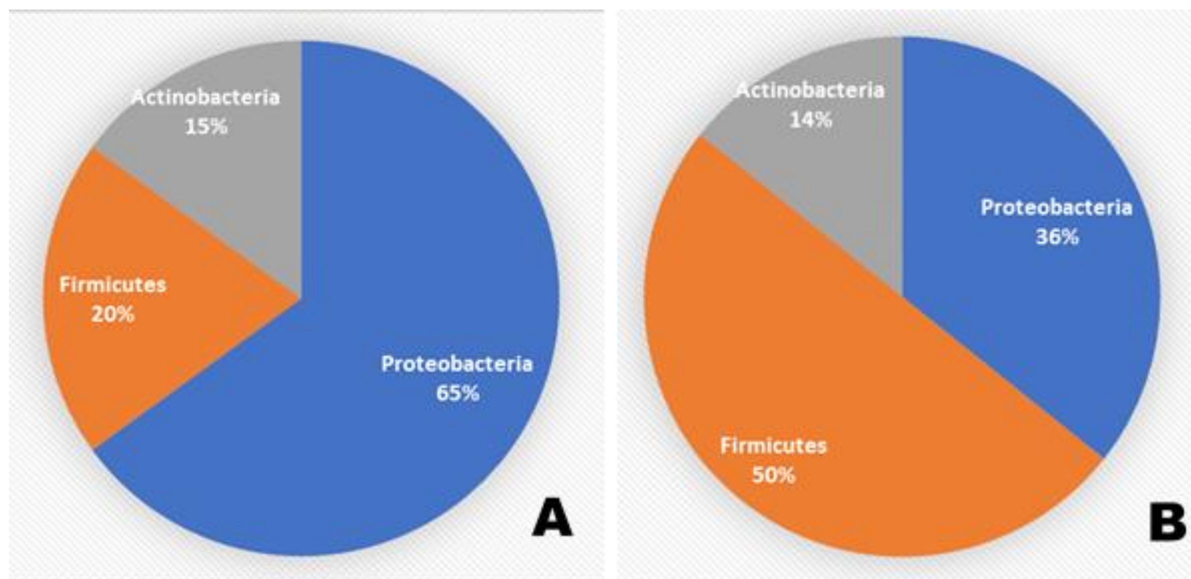

Figure 2.7: Proportional representation of the three different groups of bacteria in the midguts of FAW larvae that were reared on maize leaf tissue (A) and on castor oil leaf tissue (B).

Although the same three phyla were present in the midguts of FAW larvae reared on maize and castor oil leaf tissue, *viz*. Proteobacteria, Firmicutes and Actinobacteria (Figure 2.7), they were however represented in different proportions depending on the food source of the larvae. For example, Proteobacteria were dominant in the midguts of FAW larvae reared on maize leaf tissue (Figure 2.7A), while the Firmicutes dominated in the castor oil fed larvae (Figure 2.7B). The percentage of Actinobacteria present in the midguts of the FAW larvae remained constant regardless of being fed on maize or castor oil leaf tissue. Priya *et al.* (2012) reported similar bacteria phyla structures from the midgut of *H. armigera* larvae (Lepidoptera: Noctuidae) when they fed on maize plants.

The Shannon diversity index values showed that there was a low diversity of midgut bacteria in both the larvae that fed on maize (H= 0.0398) and castor oil plant leaves (H = 0.9982). Typical diversity values are usually between 1.5 and 3.5 in most ecological studies (Kerkhoff, 2010). The Shannon index increases as both the richness and the evenness of the community increase. The bacterial diversity in the midguts of other lepidopteran species that have been studied varied. The bacterial diversity indices in *H. armigera* was between 1.47 and 2.61 (Xiang *et al.*, 2006). Snyman *et al.* (2016) reported indices of 0.57 to 2.36 for *Busseola fusca* (Lepidoptera: Noctuidae), and 0.7 to 2.0 were reported for *Pieris rapae* (Lepidoptera: Pieridae) by Robinson *et al.* (2010). The bacterial diversity

ranges in other insect families were reported to be narrow, with 2.75 to 3.49 for *Anopheles stephensi* (Diptera: Culicidae) (Rani *et al.,* 2009), 2.37 to 2.72 for *Melolontha hippocastani* (Coleoptera: Scarabaeidae) (Arias-Cordero *et al.,* 2012) and 1.63 to 1.91 for *Dastarcus helophoroides* (Coleoptera: Bothrideridae) (Wang *et al*., 2014). The possible reason for the low diversity is because of the low number of all the different bacterial colonies in contrast to the high number of one or two colonies that were present through all the spread plate dilutions of both the maize and castor oil reared larval midguts. This can also be because only the midgut section was used to extract bacteria and not the whole gut section.

Different host plants and nutritional diets with different nutritional compositions have been shown to influence the composition of the gut microbial community (Broderick *et al*., 2004, Robinson *et al*., 2010). The variation in host plants or plant composition may cause midgut bacteria to adapt (changing the composition) in digesting the plant tissue to benefit the insect as much as possible (Santo Domingo *et al*., 1998; Anand *et al*., 2009). Microbial variation may also be as a result of natural environment changes such as temperature, soil type, agricultural practices (such as irrigation), host plant and other environmental influences (Horner-Devine *et al.,* 2004). As can be seen in the results found, the two different host plants do affect the midgut microbial community and structure. Therefore, the midgut microbial community might adapt to the different host plants in order for them to produce the most nutritional benefits for the Fall armyworm (FAW) larvae.

The phylum Proteobacteria consists of three classes, *viz*. Gammaproteobacteria, Alfaproteobacteria and Betaproteobacteria. The Proteobacteria present in the midguts of larvae reared on maize leaf tissue could therefore be further subdivided into 99% Betaproteobacteria and 1% to the other two classes. From the Betaproteobacteria, 99% were *Acidovorax temperans* and 1% was *Pseudomonas* spp. Forty seven percent of the Proteobacteria in the castor oil reared larvae belonged to the class Betaproteobacteria, 47% for Alfaproteobacteria, and 6% to the class Gammaproteobacteria.

Acidovorax temperans and *Novosphingobium* sp. were the most abundant species present in the midgut of the castor oil reared larvae with 48% and 47% respectively. The rest of the Proteobacteria species were *Acinetobacter* spp. (4%) and 1% *Sphingomonas melonis*.

The phylum Firmicutes represented only 20% of the isolates from the maize reared larvae and 50% of the isolates from the castor oil plant reared larvae. From the midguts of larvae reared on maize, 100% of the isolated Firmicutes belonged to the genus *Staphylococcus* (Table 2.1). The Firmicutes isolated from larvae reared on castor oil, consisted of *Enterococcus mundtii* and *Bacillus* spp. that were not found in larvae reared on maize leaves (Table 2.1). Less than 1% were *Bacillus* spp. and *Staphylococcus* spp. *Bacillus thuringiensis* were also one of the isolated species found in *Busseola fusca* by Snyman *et al.* (2016).

From this study no evidence could be found that Actinobacteria were affected at phylum level by the two food sources provided since their presence in the microbial community remained constant. This finding is in accordance with that of Snyman *et al.* (2016) for *B. fusca* larvae that were reared on maize plants. There were, however, differences at genus level of Actinobacteria in the maize and castor oil reared larvae. The Actinobacteria in the maize reared larvae were *Arthrobacter protophormiae* and *Glutamicibacter* sp., while only *Microbacterium sorbitolivorans* was present in the midguts of castor oil-reared larvae.The reason is still unknown.

The three phyla present in the midgut of FAW have also been reported to be associated with many other insect species. It has also been isolated from *Aedes albopictus* (Diptera: Culicidae) (Moro *et al*., 2013), *Apis mellifera* (Hymenoptera: Apidae) (Hendriksma *et al.,* 2013), *H. armigera* (Priya *et al*., 2012), *Melolontha hippocastani* (Coleoptera: Scarabaeidae) (Arias-Cordero *et al.,* 2012), *Culex quinquefasciatus* (Diptera: Culicidae) (Chandel *et al.,* 2013), *Manduca sexta* (Lepidoptera: Sphingidae) (Brinkmann *et al.,* 2008) and *B. fusca* (Lepidoptera: Noctuidae) (Snyman *et al*., 2016).

Table 2.1: Bacterial composition in the midguts of *Spodoptera frugiperda* larvae reared on maize and castor oil leaf tissue, respectively.

Bacteria isolated from FAW midguts	
Maize -fed larvae bacteria species	**Castor oil fed larvae bacteria species**
Phylum: Proteobacteria	
Acidovorax temperans *Acinetobacter calcoaceticus* *Acinetobacter pittii* *Pseudomonas peli* *Pseudomonas anguilliseptica* *Pseudomonas fluorescens* *Stenotrophomonas pavanii* *Stenotrophomonas maltophilia* *Enterobacter asburiae* *Luteimonas terrae* *Luteimonas aestuarii* *Xanthomonas citri*	*Acidovorax temperans* *Acinetobacter calcoaceticus* *Acinetobacter pittii* *Novosphingobium* sp. *Sphingomonas melonis*
Phylum: Firmicutes	
Staphylococcus xylosus *Staphylococcus saprophyticus* *Staphylococcus succinus*	*Enterococcus mundtii* *Staphylococcus xylosus* *Staphylococcus saprophyticus* *Bacillus thuringiensis* *Bacillus cereus* *Bacillus toyonensis*
Phylum: Actinobacteria	
Arthrobacter protophormiae *Glutamicibacter* sp.	*Microbacterium sorbitolivorans*

The abundance of microbial symbionts was generally low in the larval guts. According to Hammer *et al*., (2017) it is expected for herbivores to consume microbe-rich leaf tissue, because diet-derived microbes are transiently present in caterpillar guts, wherein they may be dead or inactive. The following isolated bacteria identified in this study could have been from the leaves and not the larval midguts: *Staphylococcus, Escherichia, Methylobacterium, Klebsiella/Enterobacter, Enterococcus*, and *Sphingomonas* (Hammer *et al*., 2017).

More bacterial species were present in the midgut of larvae reared on maize compared to larvae reared on castor oil. Every bacterial species is responsible for a certain mechanism or used for a

certain function. The different functions for different bacterial species isolated, as well as how they interact with their environment are discussed below.

The genus *Acidovorax* has eight recognized species that are normally found in soil and water (Willems *et al.,* 1990). These bacteria denitrify through the removal of nitrogen in wastewater treatments (Gumaelius *et al.*, 2001). According to Hernández *et al.* (2014) the main functions of these bacteria (also found in the dung beetle, *Thorectes lusitanicus*) (Coleoptera: Geotrupidae) would include nitrogen fixation, denitrification, detoxification, and diverse defensive roles against pathogens.

Acinetobacter sp., *Pseudomonas* sp. and *Stenotrophomonas* sp. have the ability to degrade and detoxify insecticides and other harmful compounds and substances for example phytotoxins and pesiticides (Itoh *et al.*, 2018). These bacterial species could also be responsible for resistance development against insecticides. These bacterial species were also isolated from the following insect species: *Samia Cynthia pryeri* (Lepidoptera: Saturniidae) (Roy *et al.*, 2003), *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae), *M. sexta* (Brinkmann *et al.,* 2008), *Xylosandrus germanus* (Coleoptera: Curculionidae) (Kati and Kati, 2013) and *Spodoptera littoralis* (Lepidoptera Noctuidae) (Özkan-Çakici *et al.*, 2014). Ramya *et al.* (2016) found that *Bacillus cereus* isolated from guts of diamondback moth (*P. xylostella*) showed high degradation and assimilation activities of indoxacarb [methyl-7-chloro-2,5-dihydro-2-[[(methoxycarbonyl) [4-(tri-uoromethoxy) phenyl]amino] carbonyl] indeno [1,2-e][1,3,4]oxadiazine-4a(3H)-carboxylate] which is an oxadiazine pesticide commonly used to control lepidopteran pests. Along with *B. cereus* that were isolated, *Enterobacter asburiae* and *Pantoea agglomerans* were also isolated. These bacteria have been reported to degrade acephate, an organophosphorus compound that inhibits acetylcholine esterase (Ramya *et al.*, 2016).

Enterobacter sp. are normally not insect pathogens but acts as a symbiont assisting in the survival of insect hosts (Özkan-Çakici *et al.*, 2014). *Enterobacter* sp. is known to degrade organophosphates such as chlorpyrifos and can be an important mechanism for the insect to develop resistance to insecticides (Singh *et al.*, 2004: Lundgren *et al.*, 2007). *Enterobacter* sp. is also involved in the degradation of cellulose (Anand *et al.*, 2009). This bacteria species was also isolated from insects such as *Rhynchophorus ferrugineus* (Tagliavia *et al.*, 2014), *S. littoralis* (Özkan-Çakici *et al.*, 2014), *Lymantria dispar* (Lepidoptera: Erebidae) (Broderick *et al.*, 2006), *Harpalus pensylvanicu*s and *Anisodactylus sanctaecrucis* (Coleoptera: Carabidae) (Lundgren *et al.*, 2007).

Novosphingobium sp. is a Gram-negative bacterium that includes *N. taihuense*, which can degrade aromatic compounds such as phenol, aniline, nitrobenzene, phenanthrene, 4-chlorobenzene, pyrene, carbofuran, dibenzofuran and estrogen (Yuan *et al.*, 2009; Yan *et al.*, 2007; Tiirola *et al.*, 2002; Tiirola *et al.*, 2005; Sohn *et al.*, 2004; Notomista *et al.*, 2011; Liu *et al.*, 2005; Hashimoto *et al.,* 2010; Fujii *et al.*, 2003). *Novosphingobium* sp. is also a strong naringenin-biodegrading bacterium

that has been isolated from red turpentine beetle *Dendroctonus valens* and *Leptographium procerum* (Cheng *et al.*, 2016).

Sphingomonas melonis are known to degrade environmental pollutants such as chlorophenols, organophosphate compounds, p-nitrophenol (PNP), polycyclic aromatic hydrocarbons (PAHs), and xenobiotics (Singh *et al.*, 2004; Kallimanis *et al.*, 2007; Liu *et al.*, 2007; Sahoo *et al.*, 2011; Busse *et al.*, 2012). *Sphingomonadaceae* is the most represented proteobacterial family in the microbiomes of arthropods. However, S*phingomonadaceae* are ubiquitous in soil, plant and aquatic environments, consequently constituting common contaminants in the kits and reagents used for DNA extraction and amplification (Glassing *et al.*, 2016; Adams *et al.*, 2015).

The *Staphylococcus* sp. are Gram-positive, non-pathogenic bacteria and are often found in the gut of insects (He *et al.*, 2013). They have a close relationship with insect nutrition and the digestive system. This bacterial species was also isolated from the Mulberry longhorn beetle, *Apriona germari* (Coleoptera: Cerambycidae) (Zhang *et al.,* 2004), from *Hepialus gonggaensis* (Lepidoptera: Hepialidae) (Zhuo *et al.*, 2005) and silkworm, *Bombyx mori* (Lepidoptera: Bombycidae) (Xiang *et al.,* 2006).

Enterococcus sp. including *E. avium* and *E. mundtii* produces acetate which decreases the pH level in the insect's gut so that toxic *Bt* proteins produced by *B. thuringiensis* could not be activated to kill the host insect pest. Therefore, this bacterial species assists in resistance development against *Bt* crops (Broderick *et al.*, 2004; Dillon and Dillon, 2004). *Enterococcus* species are vertically transferred from *M. sexta* moths to the eggs and therefore also to the larvae (Brinkmann *et al.*, 2008). *Enterococcus* sp. were also isolated from other lepidopteran species such as *B. mori* (Inglis *et al.*, 2000) *S. litoralis* (Chung *et al.*, 2017) *and H. armigera* (Priya *et al.*, 2012).

Bacillus thuringiensis and *B. cereus* were among the isolated *Bacillus* sp. only found in larvae reared on castor oil plant. This bacterial species was also isolated from insects such as lepidopteran species and *Solenopsis invicta* (Hymenoptera: Formicidae) (Gunawan *et al.*, 2008). The *Bacillus* sp., *Arthrobacter* sp. and *Microbacterium* sp. are known to produce *N-Acyl*homoserine lactones (AHL) (a class of signalling molecules involved in bacterial quorum sensing) lactonase (Morohoshi *et al.*, 2012). Therefore, these bacterial species disrupts the quorum sensing (QS) signals through the inhibiting of the expression of virulence (Wang *et al.*, 2010). *Bacillus thuringiensis* could have been ingested by the larvae from the environment. This bacterium can produce insecticidal toxins when nutrients are not available, but for as long as the larvae feed, the bacterium in the gut will not produce insecticidal toxins (Knowles, 1994).

Microbial communities in insects differ greatly among the host species and the microbial communities react differently to their hosts and their host's environment (Corby-Harris *et al.*, 2007).

This might be why there were such a large difference found in this study between the midgut microbial communities of the maize and castor oil reared larvae, and further studies are recommended for more clarification.

The selected isolates that were used for the phylogenetic analysis and their respective percentage similarity are provided in Table 2.2.

Table 2.2: Selected isolates for phylogenetic analysis.

Isolate identification according to GenBank	**% Similarity**
C1 *Acidovorax temperans*	98
C2 *Enterococcus mundtii*	98
C3 *Acinetobacter* sp.	98
C4 *Novosphingobium* sp.	98
C7 *Sphingomonas melonis*	97
C13 *Bacillus* sp.	98
C14 *Microbacterium* sp.	97
M3 *Pseudomonas* sp.	97
M4 *Staphylococcus* sp.	99
M5 *Enterobacter* sp.	98
M8 *Stenotrophomonas* sp.	98
M9 *Staphylococcus succinus*	99
M13 *Arthrobacter* sp.	97
M16 *Luteimonas* sp.	98

The sequences that were used for the phylogenetic analysis were obtained from GenBank database (http://www.ncbi.nlm.nih.gov/).

Evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model (Kimura, 1980) through the MEGA X program. The tree with the highest log likelihood (-4054.17) is shown (Figure 2.8). The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (10 categories (+G, parameter = 0.4378)). The rate of variation model allowed for some sites to be evolutionarily invariable ([+I], 33.67% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis

involved 37 nucleotide sequences. There was a total of 1145 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al.*, 2018).

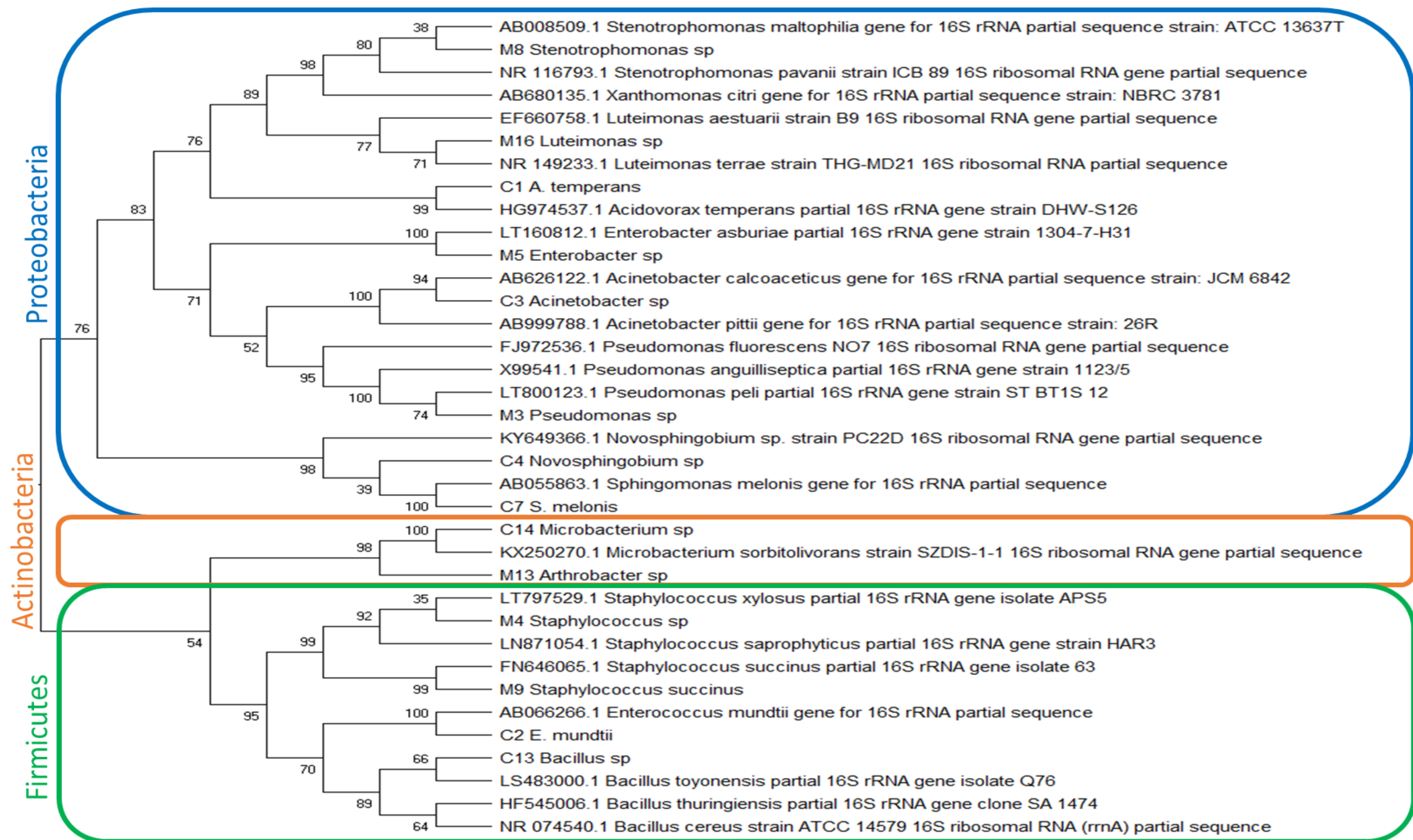

Figure 2.8: Neighbor-Joining tree constructed from partial 16S rRNA gene sequences collected from Fall armyworm (FAW) larvae. Obtained Bootstrap confidence values (400 replicates) are given at the branch point. Entries include the selected bacterial groups (Table 2.2) together with species names and accession numbers obtained from the GenBank database.

All the selected isolates in Figure 2.8 show exactly how they are divided into the three phyla and what their bootstrap support is according to known sequences from GenBank. There is an 80% bootstrap support between the M8 *Stenotrophomonas* sp., isolated in the study, to the known sequence of *Stenotrophomonas pavanii*. A 70% bootstrap support between the M16 *Luteimonas* sp., isolated in the study, to the known sequence of *Luteimonas aestuarii.* A 99% bootstrap support between the C1 *A. temperans*, isolated in the study, to the known sequence of *Acidovorax temperans.* A 100% bootstrap support between the M5 *Enterobacter* sp., isolated in the study, to the known sequence of *Enterobacter asburiae.* A 94% bootstrap support between the C3 *Acinetobacter* sp., isolated in the study, to the known sequence of *Acinetobacter calcoaceticus* and 100% bootstrap support to *Acinetobacter pittii.* A 100% bootstrap support between the M3 *Pseudomonas* sp., isolated in the study, to the known sequence of *Pseudomonas anguiliseptica.* A 100% bootstrap support between the C14 *Microbacterium* sp., isolated in the study, to the known sequence of *Microbacterium sorbitolivorans.* Similarly, 100% bootstrap support between the C7 *S. melonis* sp., isolated in the study, to the known sequence of *Sphingomonas melonis.* A 92% bootstrap support between the M4 *Staphylococcus* sp., isolated in the study, to the known sequence of *Staphylococcus saprophyticus* and 99% to *Staphylococcus succinus.* In addition, a 100% bootstrap support between the C2 *E. mundtii*, isolated in the study, to the known sequence of *Enterococcus mundtii.* Furthermore, 89% bootstrap support between the C13 *Bacillus* sp., isolated in the study, to the known sequence of *Bacillus thuringiensis* or *B. cereus.* The only outgroup found was M13 *Artrobacter* sp.

2.5. Conclusion

Gut microorganisms are extremely important in insect function and are relevant to agriculture and ecology (Engel and Moran, 2013). Microbial communities dominate in the insect's digestive system play an important role in influencing the insect's biology and host plant selection (Engel and Moran, 2013; Acevedo *et al.*, 2016) while the insect gut micro-environment can even determine the structure of the gut microbial community and the structure and diversity of the gut microbiota (Xia *et al.*, 2018). There is large variety in different bacterial species present in the midgut of insects and each one has a specific role that helps the insect with digestion, degrading of complex molecules and uptake of nutrients and also help with the degrading of harmful substances such as insecticides or secondary plant metabolites (Engel and Moran, 2013; Visôtto *et al.*, 2009; Hammer and Bowers, 2015; Chaturvedi *et al.*, 2017). There were more different bacterial individuals isolated and identified in the maize reared larvae than in the castor oil reared larvae in the phyla Proteobacteria and more identified bacteria in the phyla Firmicutes in the castor oil reared larvae than in the maize reared larvae.

The variation in host plants or plant composition may cause midgut bacteria to adapt through changing the bacterial composition or the different bacterial colonies present to digest the plant

tissue to benefit the insect as much as possible (Santo Domingo *et al.*, 1998; Anand *et al.*, 2009). Results indicte that the larval host plants of FAW affect the midgut microbial composition, enabling the bacterial colonies to digest these different host plant materials, so that the insect could get the most benefit from the digested plant material and molecules. It could aslo be affected through the different bacteria present on the host plant leaves.

For future studies it is recommended that bacteria must first be identified that occurs on the leaves of the host plants. Determining how these bacteria influence the biology of the Fall armyworm, from the deposited eggs to the moths emerging.

2.6. References

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Chapter 3: The effect of food source and larval density on cannibalism behavioural responses of *Spodoptera frugiperda* larvae reared on maize and castor oil plants

3.1. Abstract

Fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith), is one of the main and most invasive pests of maize and other crops in the Americas, Africa as well as Asia. Cannibalism is a common characteristic of Lepidoptera larvae and larval densities are usually reduced to one or two per plant due to cannibalism. Although extracts of *Ricinus communis* has been used to control insect pests in several crops, it has been reported that cannibalism in FAW is reduced when larvae feed on castor oil plant material. This study aimed to determine if the cannibalistic behaviour changes when the larvae are under stressed conditions such as high larval densities and when they feed on different host plants such as maize and castor oil. Y-tube tests were also conducted to determine if the larvae emit possible chemical compounds that either could attract or repel conspecific larvae and which could in turn enhance or suppress cannibalistic behaviour. The study showed that feeding on castor oil plant tissue affects larval behaviour, but larvae were still cannibalistic at higher densities. The Y-tube tests also showed that larvae do not emit any chemical compounds that attracted or repelled conspecific larvae.

3.2. Introduction

Fall Armyworm (FAW), is a highly polyphagous insect pest. Montezano *et al.* (2018) recently stated that there are 353 different host plants recorded for FAW. However, the most preferred hosts are in the Poaceae, for example maize, sweet corn, sorghum, Bermudagrass and crabgrass (Sparks, 1979; Múrua *et al.*, 2009; Capinera, 2017). *Ricinus communis* (Euphorbiaceae) is one of the reported host plants of FAW (CABI, 2018). This is an evidence that, it is not only crop species that are attacked by FAW. Many non-crop species such as *Taraxacum officinale* (dandelion) (Asteraceae), *Carex* (sedges) (Cyperaceae), *Convolvulus* (morning glory) (Convolvulaceae) and *Atropa belladonna* (deadly nightshade), *Solanum* (nightshade) (Solanaceae) are also wild hosts for the FAW and are known to support larval development (CABI, 2018).

Two strains of FAW exist based primarily on their host plant preference (Pashley, 1988; Dumas *et al.*, 2015). There is one strain feeds primarily on maize, but it also feeds on sorghum and cotton. The other strain feeds primarily on rice, Bermuda grass and Johnson grass They may be host races in the initial stages of speciation in which interbreeding is reduced due to host preference differences (Diehl and Bush 1984; Prowell *et al.*, 2004; Meagher *et al.,* 2004). Nagoshi *et al.* (2008) did, however, speculate that FAW population could be more genetically complex than that described by the two strains. Both the maize and rice strain occur in mixed populations in South Africa (Jacobs *et al.*, 2017).

Cannibalism is not only a common characteristic of Lepidoptera larvae (Dail and Adler, 1990), but also a behavioural characteristic that occurs in a wide range of animal taxa. It is responsible for substantial levels of mortality and may have a significant effect on population structure (Fox, 1975). Exploring this phenomenon could contribute to a specific pest population management stategy for pests, including FAW.

The Y-tube is very appropriate to determine the behavioural responses of larvae since it allows the study of the effects of host plants on larvae, which, depending on host plant perception, disperse by means of trailing or ballooning, make the selection between different host plants possible (Berger, 1993). The stage during which insects search for suitable host plants can be divided into three sub-stages: perceiving, responding and finding the host plant (Schoonhoven *et al.*, 1998).

The objective of this study was to determine if the cannibalism behaviour of FAW larvae are affected by the host plants they feed on, identify at which larval density does cannibalism occur, and assess if the larvae are able to distinguish between maize and castor oil plant material through a choice bioassay.

3.3. Materials and methods

3.3.1. Sampling and insect rearing

Larvae of FAW were collected at Groblersdal (25°12'24.0"S, 29°13'21.4"E), South Africa from infested maize plants. Plant whorls that exhibited damage symptoms were removed from plants in the field and opened to retrieve the larvae. The larvae were subsequently reared in the laboratory on maize leaves until pupation. The plastic rearing containers (100 ml) had steel-infused lids to allow air-flow. The rearing was done at 26 ± 1°C, 65 ± 5% RH and 14L:10D photoperiod in an insect rearing chamber.

Moths that emerged were transferred to oviposition chambers (2 L) to mate and lay eggs. The chambers and method used were similar to that described by Kruger *et al.* (2012). The larvae that hatched from these eggs (F3 generation) were used in the preference bioassays. The first instar larvae that hatched from the eggs were divided into two groups and reared on maize and castor oil plant leaves respectively. Each larva was reared in a separate rearing container (to avoid cannibalism) and two cuttings of fresh leaves (5 x 5 cm) of either maize or castor oil plants were provided every two days until larvae reached the desired instar to be used in the bioassays. Second instar larvae were used in the cannibalism bioassays (to examine cannibalism as the larvae develop through their larval stages) while fourth instar larvae (they are very active and mobile) were used in the larval density bioassays and Y-tube bioassays.

3.3.2. Cannibalism bioassay

Three hundred second instar larvae were randomly selected from the two rearing colonies, divided into three groups (food types) and fed on either maize or castor oil leaves or maize leaves sprayed with castor oil (commercial formulation found in pharmacies). The maize leaves were sprayed with a 1:10 castor oil/water solution. The leaves were sprayed until they were completely covered with the castor oil solution and left to dry off completely before they were fed to the larvae. This was done to determine if compounds found in castor oil have an effect on the cannibalism rate. These larvae were used five days after hatching (second instar) in bioassays to determine the effect of food type on cannibalism behaviour. Larvae were placed in groups of four per Petri dish and replicated 25 times for food type (Figure 3.1) with enough food for two days (four pieces of leaf material of 5 x 5 cm). The number of surviving larvae per Petri dish was recorded at 2, 5, 7, 9, 11 and 13 days after being put together into the Petri dishes (Chapman, 1999). After two days, the Petri dishes were cleaned, and the missing larvae set were replaced with a new set of four larvae that were the same size, so that different larval instars did not affect the cannibalism behaviour (Bentivenha *et al.*, 2017).

Figure 3.1: Four FAW larvae (4th instar) per Petri dish were added before new leaves were added for the cannibalism experiment.

3.3.3. Larval density bioassay

The experiment consisted of five treatments, each replicated three times. The five different treatments were used for each of the maize and castor oil plants. These treatments (different larval densities) were: 2, 4, 8, 16 and 32 larvae per Petri dish. After two and four days, the numbers of surviving larvae per Petri dish were recorded. The experiment was done using fourth instar larvae.

3.3.4. Y-Tube setup and treatments

A Y-tube experiment was developed to evaluate larval response to the different treatment combinations. This experiment consisted of four treatment combinations: (1) maize leaf and blank control, (2) maize leaf and castor oil leaf, (3) maize leaf tissue as well as a larva (4th instar) that was reared on maize leaf tissue, and (4) maize leaf tissue as well as a larva that was reared on castor leaves. Each treatment was replicated 30 times with one larva per replicate. The Y-tube was cleaned after every 5 replicates. Only maize-reared larvae were used for the choice tests and sevred as a control larva.

Fourth instar larvae (seven days old) were starved for 18 hours before they were used in the bioassays. Pieces of cut leaf material (5 x 5 cm) of maize and castor oil plants were used as potential stimulus in the respective olfactometer arms. In the first two treatments plant leaf tissue was cut to the same size for each of the two plant treatments. The maize plants were young (V3-V4) and the largest greenest leaves of the castor oil plants were used. However, for treatments 3 and 4, less leaf material (2 x 2 cm) was used to limit the likelihood that leaf volatiles could mask the possible chemical volatiles associated with the larvae.

The Y-tube was placed directly under a light source (to make light distribution homogeneous). This was done to ensure that the direction of dispersal of the larvae was not influenced by other light sources, since larvae are phototactic (Briscoe and Chittka, 2001). The glass tubes with plant material were rotated between the Y-tube arms after five replicates have been done to prevent the effect that potential external factors could have influenced the insect behaviour.

The two arms of the Y-tube (11 cm long and 2.5 cm in diameter) were connected to two glass tubes which contained the plant material (Figure. 3.2). A ventilation system softly blew air (1900 ml/min) through the tubes, into the Y-tube. The Y-tube was positioned vertically with the base-arm vertically downwards, to resemble a plant stalk on which the larvae could climb upwards (Figure 3.2). A cotton string (1.5 mm diameter) was hanged in the centre of the arms of the Y-tube and attached close to the centre of the rubber plugs at the end of each olfactometer arm. This positioning of the string ensured that the string was in the centre of the three arms and that the larvae did not made contact with the sides of the tubes. The use of the string in such a way also improved exposure of larvae to a more representative mixture of volatiles in the olfactometer arms since the larvae would be positioned in the centre of the air-stream.

With a soft camel hair brush, a larva was introduced by putting it onto the cotton string to proceed with an upwards movement in the main olfactometer arm. The larva therefore reached the split of the main arm of the Y-tube, by climbing upwards on the thread, until it was exposed to the two different treatment arms. Each replicate lasted for 15 minutes. If a larva did not walk upwards in the

base arm of the olfactometer after 15 minutes to choose between the two different treatment arms, the larva was removed and replaced with another larva.

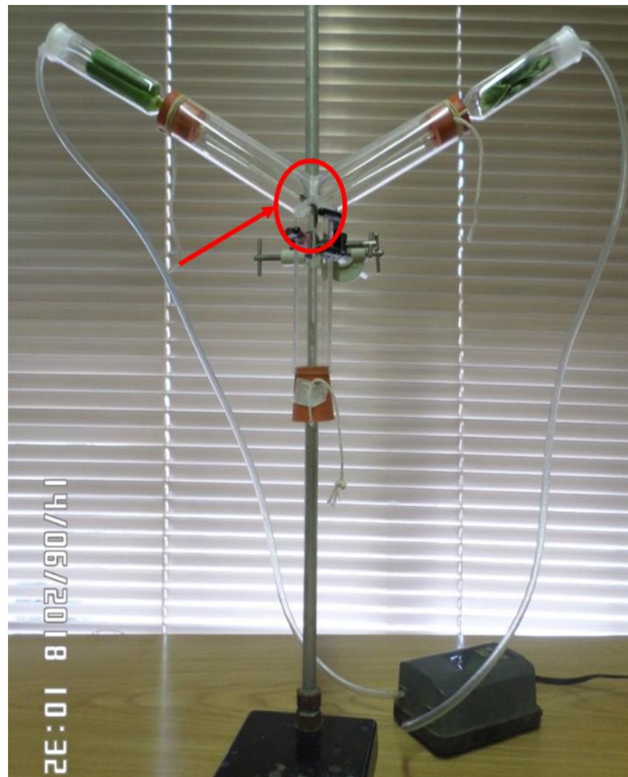


Figure 3.2: The Y-tube bioassay setup. The left arm contained maize leaf material and the right arm contained castor leaf material. The larva is at the intersection where it is about to choose the maize leaf treatment.

3.3.5. Statistical analyses

For the cannibalism data sets, the percentage survival was calculated after 2 days for each treatment. Repeated measures ANOVA was used to analyse the effect of larval density on cannibalism of FAW over time followed by Tukey's HSD test using STATISTICA 13 (Statsoft, Inc., 2016). For the larval density experiment, the data were logtransformed. T-test analysis was performed to determine if cannibalism differed at the different larval densities as well as if there was a significant difference between the larval density and host plant interactions using STATISTICA 13 (Statsoft, Inc., 2016). The proportion of larvae that chose either of the two Y-tube arms, was calculated. The data were analysed by means of a binomial distribution test in STATISTICA 13 (Statsoft, Inc., 2016).

3.4. Results and discussion

3.4.1. Cannibalism bioassay

There was a significant days * treatment interaction effect for the cannibalistic behaviour of FAW ($F_{8,288} = 3.9$; $P < 0.001$). The level of cannibalism by FAW when fed on maize leaf tissue and maize leaf tissue sprayed with castor oil were similar, but significantly more larvae survived when fed on castor oil plant tissue, indicating reduced cannibalism (Table 3.1).

Table 3.1: The number of surviving larvae on different plant treatments after 13 days

Treatment	Mean percentage (%) of larval survivors ± SE
Maize	85.75 ± 0.05 a
Maize and Castor oil	86.50 ± 0.05 a
Castor oil	94.25 ± 0.05 b

Larvae that were fed on maize leaf tissue exhibited cannibalism from seven days onwards after hatching (Figure 3.3).

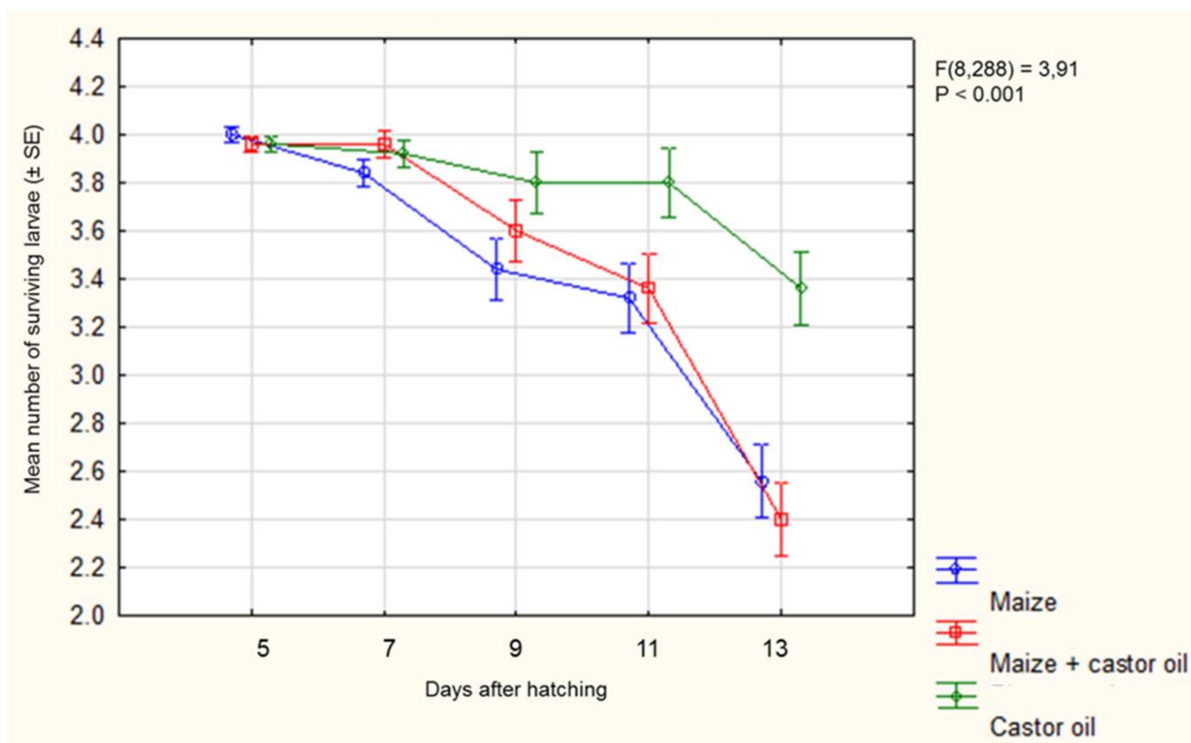


Figure 3.3: Number of larvae that survived cannibalism (out of four) at different days after hatching.

Third instar larvae did not show any cannibalistic behaviour five days after hatching when they were feeding on maize leaf tissue. On day seven after hatching with most larvae in the fourth instar, 96%

survived. The percentage survival decreased to 86%, nine days after hatching and being together for four days (Figure 3.3). After 11 days, the percentage survival decreased to 83%, where most of the larvae were in the sixth instar. When all larvae were in the sixth instar 13 days after emergence, the survival rate dropped to 64%. This six instar was then observed to be the most cannibalistic with only 64% of larvae that survived (Figure 3.3). There was no significant difference in survival of the larvae fed with maize, maize sprayed with castor oil and castor oil plant tissue on the different days after initiation of the experiment (day 5 to day 11). There was also no difference in cannibalism among FAW larvae 13 days after emergence in the larvae fed with maize and maize treated with castor oil (Figure 3.3). Cannibalism of these two groups was significantly higher than in the group fed with castor oil plant tissue only. Castor oil sprayed onto maize leaves did therefore not affect the cannibalism of FAW larvae. The percentage survival of larvae fed on castor oil plant remained high (84%) on day 13, while only 59 and 64% of larvae fed with maize and maize treated with castor oil survived respectively. This finding therefore indicates that feeding on castor oil plant tissue lowered the rate of cannibalism in FAW larvae, while the cannibalism rates increased on maize and maize treated with castor oil.

A possible reason of these variations might be that castor oil plant leaves are more nutritional than maize leaves or it may contain substances which alter the bacterial community within the digestive system of FAW larvae which could in turn affect the feeding status of the larvae. Cannibalism that occurred in this case might possibly be ascribed to limited space (Bentivenha *et al.*, 2017). The experiment was terminated on day 13 since the larvae entered the pre-pupal stage.

Cannibalism behaviour by FAW larvae reared on maize was observed from the fourth instar onwards and the larvae remained cannibalistic until the final instar and is in colaberation with the findings of Chapman *et al.,* (1999). A possible reason for no cannibalism by larvae fed with maize until they reached the fourth instar, could be because a lack of stress in terms of food and space. Possible reasons may be that as the larvae developed and grew, they ate more and became overpopulated in terms of space (Figure 3.4) (Fox, 1975; Richardson *et al.*, 2010; Joyner and Gould, 1985; Raffa, 1987; Polis, 1981).

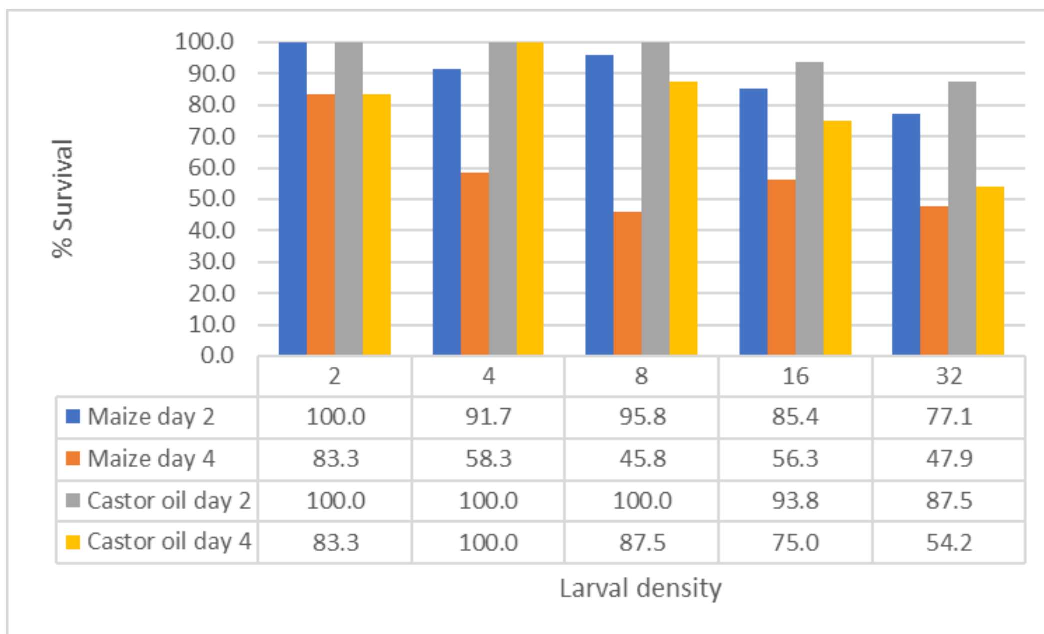
This could therefore result in stressed larvae which could lead to cannibalistic behaviour. From the fourth instar onwards, they continuously had to compete for food and space.

Figure 3.4: Dissected midgut and contents of a Fall armyworm larva that was cannibalistic. The midgut (yellow) contains head capsules of several other FAW larvae (dark granular material).

The survival of larvae that were fed on maize leaves sprayed with castor oil was similar to those that fed on maize leaf tissue only and a possible reason might be that the castor oil sprayed onto the leaves did not provide the same nutritional value or did not contain the components/ compounds present in castor oil plant that affected larval nutrition (Maheshwari and Kovalchuk, 2016; Dyer *et al.*, 2008) and possibly effecting the microbiome.

3.4.2. Effects of larval densities on cannibalism rates

The host plant species * larval density interaction was not significant ($F4_{,40}$ = 1.02; P = 0.41) indicating that cannibalism of larvae fed with maize was not differently affected by larval density compared to larvae fed on castor oil. However, significantly higher numbers of larvae survived when they were fed with castor oil compared to maize as a food source ($F_{1,40}$ = 7.3; P< 0.01). The plant species * time (day 2 vs day 4) interaction was not significant ($F_{1,40}$ = 3.26; P = 0.08). Cannibalism of larvae fed with maize was therefore not differently affected by the exposure time of four days compared to larvae fed on castor oil over the same time period. The survival of the larvae was, however, significantly affected by the number of larvae that were kept together in a Petri dish ($F_{4,40}$= 116.1; P< 0.0001). In the experiment where larvae were fed with maize leaf tissue, the highest percentage cannibalism occurred at 32 larvae per treatment and the least at two larvae per Petri dish (Figure 3.5).



	2	4	8	16	32
Maize day 2	100.0	91.7	95.8	85.4	77.1
Maize day 4	83.3	58.3	45.8	56.3	47.9
Castor oil day 2	100.0	100.0	100.0	93.8	87.5
Castor oil day 4	83.3	100.0	87.5	75.0	54.2

Figure 3.5: Percentage survival of FAW larvae at different larval densities (2 – 32 larvae) after two and four days on maize and castor oil leaves.

Similar cannibalism rates occurred for the castor oil leaf tissue experiment (Figure 3.5). The larval density at which the most cannibalism occurred were at the density of 32 larvae per treatment. After two days on castor oil the percentage survival was 100% for 2, 4 and 8 larval densities. At a larval density of 16 per Petri dish the percentage survival was 94% and decreased slightly to 88% survival at the density of 32 larvae. After four days on the castor oil leaf tissue the percentage survival at density of 2 larvae was 83%. At density 4 the survival increased to 100% and decreased to 88% at density of 8 larvae. At the density of 16 larvae the percentage survival decreased to 75% and at density of 32 larvae the survival decreased to just above the half mark at 54% (Figure 3.5).

Fewer larvae survived at an increased larval density of FAW when kept in a confined space. The lowest number of larvae survived at a density of 32, compared to all the other densities while most of the larvae survived when they were only two larvae placed in a Petri dish.

Fox (1975) stated that herbivorous insects may cannibalize even when their food sources are still available in abundance. This was also observed in the results where cannibalism occurred at the larval densities of 2 and 4 larvae per Petri dish. But there is still less cannibalism found on the castor oil plant tissues than on the maize plant tissues (Lynn, 2017).

3.4.3. Y-tube bioassay and FAW preference

Significantly more larvae chose maize leaf tissue when offered a choice between maize leaf tissue and a blank control treatment (Figure 3.6). Larvae did not show any significant preference for maize when provided a choice between castor oil leaf tissue and maize leaf tissue (P = 0.12) (Figure 3.6).

Capinera (2017) reported that FAW primarily feeds on maize plants or other hosts that are in the Poaceae family. Although FAW larvae can survive on the castor oil plants and get more nutrients from the plant (Lynn, 2017), they would most likely prefer to feed on maize.

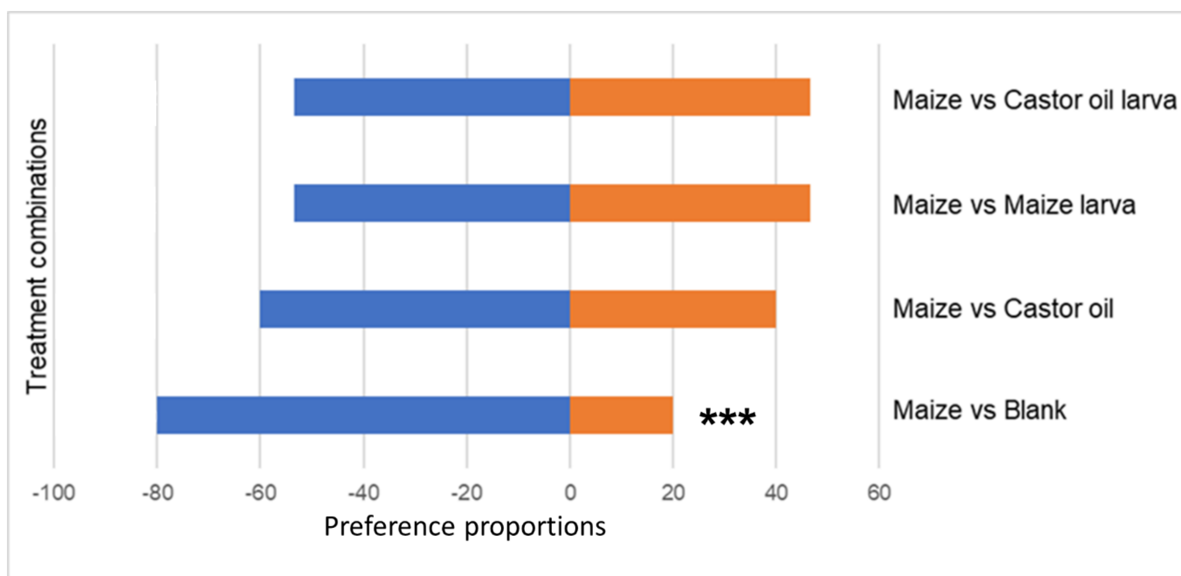


Figure 3.6: Percentage preference by FAW larvae for different treatments in Y-tube bioassay. Significance indicated by *** $P < 0.001$.

No preference for any treatment was exhibited in any of the choice combinations with maize leaves without any larval damage and a larva that fed either on maize or castor oil leaves (Figure 3.6). This indicated that larvae that fed on the two different plant species most likely did not emit any different volatile chemical compounds that suppressed or enhanced the need to be cannibalistic. However, further studies are warranted to elucidate the chemistry behind this larval behaviour.

3.5. Conclusion

This study indicated that the larval instar which is most cannibalistic through all the treatments, was the sixth instar. Therefore, the larger the larvae, the more plant material they digest, the more they have to compete for limited food and space, and consequently the more cannibalistic they are. The larvae the fed on maize were also more cannibalistic than the larvae that fed castor oil. The reason is still unknown but further studies have to be conducted to determine both maize and castor oil nutritional values.

As noted in the larval density experiment, the higher the density of the larvae present in a single contained environment or space, the more does cannibalism occur, because of the competition for limited food sources and space.

FAW larvae only tend to be cannibalistic when they are stressed to compete for limited food sources and space and not necessarily because of possible volatile chemical compounds that they emit that may lure them to be cannibalistic. Therefore, the larvae must get in direct contact with each other before they become cannibalistic (Bentivenha *et al.*, 2017). If the larvae get enough nutrients from their food sources (Da Silva and Parra, 2013; Raffa, 1987), they seemingly do not have the need to constantly feed, thereby reducing the risk of competition and cannibalism.

Recommendations for future studies with volatile chemical compounds are to determine if there is a contact chemical difference between the larvae that were reared on castor oil and maize that can influence the need to be more or less cannibalistic.

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Chapter 4: General discussion, Conclusions and Recommendations

4.1. Conclutions

Cannibalism by the Fall armyworm (FAW) can be affected by larval density, available space and nutrient status of their food source. According to Raffa (1987) higher larval densities result in increased cannibalism rates, which may also be a mechanism for population regulation (Raffa, 1987). Similarly, the pest midgut bacterial communities are related to the food source, where significant variations as regard to the bacteria diversity were observed in FAW larvae reared on maize compared to castor oil plants.

Based on the objectives set for this study, the key subsequent findings are provided below:

- Midgut bacterial diversity (Chapter 2): there were 3 different phyla bacteria extracted and a total of 29 bacterial species were identified, 17 bacterial species in larvae that fed on maize and 12 species in FAW larvae that fed on castor oil plant tissue. Therefore, the different food sources did affect the midgut bacterial composition.
- Different food sources (Chapter 3): there was a significant difference in the cannibalism rates when larvae fed on the two different plant tissues, maize and castor oil. There was also a significant difference in the cannibalism rates between the different larval instars with later instars being more cannibalistic. Therefore, the different food sources and developmental stage did have effects on FAW cannibalism rates.
- Larval density (Chapter 3): cannibalism was observed to occur even at the lowest density of 2 larvae per Petri dish. There was a positive relationship between larval density and the rate of cannibalism. Therefore, larval density was found to have significant effect on the cannibalism rates of the pest.
- Choice tests of chemical compounds and larval behaviour (Chapter 3): the Y-tube olfactometer experiments did not show the presence of volatile chemical compounds that attracted larvae to treatments that contained other larvae. Therefore, larval odours or scents do not have any effect on larval behaviour to become more or less cannibalistic.

Gut microorganisms are important in insect functions and are relevant to agriculture and ecology (Engel and Moran, 2013). Microbial communities dominate in the insect's digestive system and they play an important role in influencing the insect's biology and host plant selection (Engel and Moran, 2013; Acevedo *et al*., 2016; Douglas, 2015; Visôtto *et al*., 2009; Hammer and Bowers, 2015; Chaturvedi *et al*., 2017). It is also known that the insect gut micro-environment influences or can even determine the structure of the gut microbial community and the structure and diversity of gut

microbiota (Xia *et al.*, 2018). As the results of Chapter 2 indicated, the microbial species composition differed between larvae that fed on the two host plants as well as their behaviour (Chapter 3).

The midgut microbes contribute to defining insect metabolic traits since these different microbial groups play a role in carbon metabolism, nitrogen recycling, methano- and acetogenesis cycles (Brennan *et al.*, 2004). The three different microbial phyla found in the midgut of the FAW were Proteobacteria, Firmicutes and Actinobacteria. These three phyla were also found in *Helicoverpa armigera* (Priya *et al.*, 2012), *Manduca sexta* (Lepidoptera: Sphingidae) (Brinkmann *et al.,* 2008) and *Busseola fusca* (Lepidoptera: Noctuidae) (Snyman *et al.*, 2016).

There was a large number of different bacterial species that can be found in the gut of insects and each one of them has a specific role that helps the host insect digestion, degrading of complex molecules and uptake of nutrients. The bacteria also help host insect in degrading harmful substances such as insecticides or secondary plant metabolites (Engel and Moran, 2013; Acevedo *et al.*, 2016; Douglas, 2015; Visôtto *et al.*, 2009; Hammer and Bowers, 2015; Chaturvedi *et al.*, 2017). Chung *et al.* (2013) also found that *Stenotrophomonas*, *Pseudomonas*, and *Enterobacter* are responsible for defense suppression against harmful substances. The microbial colonies may differ between species as well as between individuals within their colony size and composition as found in Chapter 2 between the two different hosts reared larvae. Chung *et al.* (2017) determined that host plant species determines the microbiome that suppresses plant defences. Some of the species identified could also be from possible contamination, for example: *Sphingomonadaceae*. These bacteria are ubiquitous in soil, plant and aquatic environments, consequently constituting common contaminants in the kits and reagents used for DNA extraction and amplification (Glassing *et al.*, 2016; Adams *et al.,*2015).

Cannibalism is a common characteristic of lepidopteran larvae (Dail and Adler, 1990). According to Bentivenha *et al.* (2017) cannibalism occurs more often when different larval instars interact with each other and when the larvae started to compete for resources such as food and space. Previous studies showed that cannibalism in FAW was more frequent when larvae were fed on maize leaves than on artificial diets, leading to the conclusion that this cannibalistic behaviour is related to a lack of food with the required nutritional value (Da Silva and Parra, 2013). The results of Chapter 3 clearly support this cannibalism behaviour when the larvae were reared on the two different plant materials, maize and castor oil plants. There was more cannibalism that occurred when the larvae fed on maize leaves than when they were fed on the castor oil leaves.

There are two possible types of nutritional benefits ascribed to cannibalism for example energy sources and availability of different types of nutrients (Mayntz and Toft, 2006). Energy sources implies the access to an energy source that is not available for non-cannibals, thus the food availability is increased for the cannibals (Fox, 1975). Another benefit that cannibalism may provide

is a different composition of nutrients than the normal diet of a particular organism. Cannibalism may therefore provide nutrients in proportions that are more optimal than those in hetero-specific diets (Fagan *et al.*, 2002). Consequently, cannibalism in herbivores may result from a specific need for proteins or other nutriens (Xiao *et al.*, 2010) rather than from hunger (Alzubaidi and Capinera, 1983; Wolcott and Wolcott, 1984). If the larvae get enough nutrients from their food sources, they seemingly do not have the need to constantly feed, thereby reducing the risk of competition and cannibalism (Da Silva and Parra, 2013; Raffa, 1987).

In addition, cannibalism is also enhanced by insect density (Raffa, 1987). As demonstrated in Chapter 3, the higher the insect density is, the more the cannibalism rate increased. Insect density may affect their growth and development by reducing the availability of food sources due to interference or competition between individuals. Interference and/or competition may increase both the opportunity for cannibalism and therefore, the nutritional importance of cannibalism (Joyner and Gould, 1985; Raffa, 1987).

FAW larvae only tend to be cannibalistic when they are stressed to compete with limited food and space, and not necessarily because of possible chemical signals they emit that may lure them to conspecifics where they exhibit cannibalistic behaviour. As the results of Chapter 3 indicated, the chemical compounds associated to larvae did not influence behaviour of conspecific larvae. Therefore, the larvae might possibly get into direct contact with each other before they exhibit cannibalism behaviour (Bentivenha *et al.*, 2017).

4.2. Recommendations

From the above results, it is suggested that the following research aspects need to be investigated in future:

- further investigation regarding community composition of midgut bacteria of the larvae to identify bacteria that might not be identified in this current study through culture methods need to be taken, especially using a next generation sequencing approach.
- determine if there are bacteria present on the host plant leaves that possibly have an effect on gut microbiota.
- Microbiome evolution/engineering: feed larvae on host plant leaves; select the best grown larvae; paint their gut bacteria onto surface sterilised leaves; inoculate fresh larvae; select best grown larvae; repeat to determine when the microbiome stabilise.
- the effects of nutrient value of different host plant species on FAW larval development to determine the relationships between proteins or nutrients and behavioural traits such as cannibalism need further attention.

- the effect of physical contact on cannibalism behaviour in the FAW could also be assessed for more clarification.
- further studies are also warranted to elucidate the chemistry behind FAW larval behaviour.
- assessment of specific rearing space as regard to the larval density for limited cannibalism rates need to be further investigated.

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