METAGENOMIC ANALYSIS OF MICROBIOME IN BAGWORM, *Metisa plana* WALKER (LEPIDOPTERA: PSYCHIDAE)

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by

ANDREW TING CHUNG JIE

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LIST OF SYMBOLS AND ABBREVIATIONS

μm	Micrometer
m	Meter
mm	Millimeter
μL	Microliter
°C	Degree Celsius
g	Force
%	Percent
min	Minutes
mL	Milliliter
sec	Seconds
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
sp.	Species
PE	Paired-end

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ANALISIS METAGENOMIK MIKROBIOM DALAM ULAT BUNGKUS, *Metisa plana* WALKER (LEPIDOPTERA: PSYCHIDAE)

ABSTRAK

Ubat bungkus *Metisa plana* merupakan salah satu perosak utama di ladang kelapa sawit di Malaysia, dengan serangan yang boleh mengakibatkan kerugian ekonomi yang besar. Walau bagaimanapun, biologi ulat bungkus seperti mikrobiom juga masih belum dikenal pasti. Kajian tentang mikrobiom *M. plana* dapat memberi gambaran mengenai masalah ini kerana bakteria yang berkaitan dengan serangga sering memberi banyak faedah kepada serangga itu sendiri. Dengan menggunakan penjujukan 16S rRNA, kajian ini dilakukan untuk membandingkan komposisi komuniti bakteria daripada dua peringkat larva (peringkat instar awal dan peringkat instar akhir) daripada kawasan wabak, serta membandingkan larva peringkat instar akhir daripada kawasan bukan wabak dan kawasan wabak. Secara amnya, komuniti bakteria didominasi oleh filum *Proteobacteria* dan *Actinobacteria* sementara *Enterobacteriaceae* adalah keluarga bakteria yang dominan. Filum *Proteobacteria* didapati lebih banyak pada peringkat instar lewat (82.45%) berbanding dengan peringkat instar awal (82.28%). Pada peringkat famili bakteria, terdapat lebih sedikit *Enterobacteriaceae* pada peringkat instar lewat (75.46%) berbanding dengan peringkat instar awal (75.29%). Peringkat instar didapati tidak mempunyai kesan yang signifikan terhadap variabiliti bakteria dan menunjukkan struktur komuniti bakteria yang serupa. *Proteobacteria* jauh lebih banyak di kawasan wabak (82.02%) berbanding dengan kawasan bukan wabak (20.57%). Walau bagaimanapun, *Actinobacteria* jauh lebih banyak di kawasan bukan wabak (76.29%) berbanding

dengan kawasan wabak (14.16%). Pada peringkat famili bakteria, *Enterobacteriaceae* lebih banyak terdapat di kawasan wabak (75.41%) berbanding dengan di kawasan bukan wabak (11.67%). *Microbacteriaceae* didapati lebih banyak di kawasan bukan-wabak (70.87%) berbanding dengan di kawasan wabak (12.47%). Walaupun dapatan tidak menunjukkan perbezaan yang bererti dalam kebolehubahan bakteria antara kawasan yang berbeza, struktur komuniti bakteria berbeza secara signifikan. Kajian ini merupakan kajian pertama tentang mikrobiom ulat bungkus *M. plana*. Hasil daripada kajian juga memberi gambaran bahawa persekitaran seperti jumlah hujan atau kelembapan, dan mikrobiom tanah mungkin mempengaruhi mikrobiom larva, seterusnya mungkin menyumbang kepada wabak ulat bungkus, justeru membantu memahami faktor-faktor di sebalik wabak ulat bungkus.

METAGENOMIC ANALYSIS OF MICROBIOME IN BAGWORM, *Metisa plana* WALKER (LEPIDOPTERA: PSYCHIDAE)

ABSTRACT

The bagworm *Metisa plana* is one of the major pests in the oil palm plantation in Malaysia, with infestation that results in huge economical loss. However, the biology of the bagworm such as the microbiome has yet to be identified. Studying the microbiome of *M. plana* could provide insight on the problem as the bacteria associated with insects often provide numerous benefits to the insect itself. Using 16S rRNA sequencing, the study was conducted to compare the composition of the bacterial communities of two larval stages (early instar stage and late instar stage) from outbreak area, as well as comparing the late instar stage larvae from non-outbreak and outbreak areas. Generally, the bacterial community was dominated by *Proteobacteria* and *Actinobacteria* phyla while the *Enterobacteriaceae* was found to be the dominant family. The *Proteobacteria* phylum was found to be more abundant in the late instar stage (82.45%) than in the early instar stage (82.28%). At the family level, the *Enterobacteriaceae* was slightly more abundant in late instar stage (75.46%) than in early instar stage (75.29%). The instar stage was observed to have no significant impact on the bacterial variability and showed similar bacterial community structure. *Proteobacteria* was significantly more abundant in the outbreak area (82.02%) than in the non-outbreak area (20.57%). However, *Actinobacteria* was significantly more abundant in the non-outbreak area (76.29%) than in the outbreak area (14.16%). At the family level, *Enterobacteriaceae* was more abundant in outbreak area (75.41%) than in non-outbreak area (11.67%). *Microbacteriaceae* was observed to be more abundant

in the non-outbreak area (70.87%) than in the outbreak area (12.47%). Although the result showed no significant difference in bacterial variability between different areas, it the bacterial community structure was significantly different. This study provides a first study into the microbiome of the bagworm *M. plana*. The result of the study also hinted that the environments such as the amount of rainfall or moisture, and the soil microbiome might be influencing the microbiome of the larvae, which in turn could contribute to the outbreak of the bagworm, hence helping us to understand the factors behind the outbreak of bagworm.

CHAPTER 1 INTRODUCTION

The agriculture sector contributed 7.1 % or RM101.5 billion to Malaysia's Gross Domestic Product (GDP) in 2019. Of that 7.1 %, the oil palm industry was the major contributor with 37.7 % (Department of Statistics Malaysia, 2020). However, the oil palm like other agriculture is prone to infestation of pests such as rhinoceros beetles, nestle caterpillars and bagworms (Halim *et al*., 2018). Currently, bagworm is the most critical oil palm pest (Sahari *et al*., 2019) and the outbreak of bagworms in oil palm plantations had resulted in losses of up to millions of ringgit (Ahmad Ali *et al*., 2013). The leaf eating bagworms are characterized by the possession of bag built from silk and dried plant materials (Cheong *et al*., 2010; Sugiura, 2016). Common major species of bagworms such as *Metisa plana* Walker, *Pteroma pendula* Joannis and *Mahasena corbetti* Tams can be found in the Malaysia's oil palm plantation (Ahmad Ali *et al*., 2013; Cheong *et al*., 2010; Sankaran, 1970), and *M. plana* is the most dominant among them (Cheong *et al*., 2010; Kamarudin & Wahid, 2007). Numerous approaches had been recommended for managing *M. plana*, of which the fastest and most effective approach would be the use of chemicals (Salim *et al*., 2015; Yap, 2000). In addition, timely management of the bagworm in its early stages of growth can substantially lower the cost of managing and lowering crop loss (Salim & Hamid, 2012). Good understanding of the bagworm biology is also crucial in managing the outbreak of bagworm (Kok *et al*., 2011). Currently, there is limited information on the microbiota of *M. plana.* The microbiota affects a wide array of behavioural and physiological features in the animal host (Dillon & Dillon, 2004; Morimoto *et al*., 2019), such as providing nutrient, protection against enemies and detoxification of toxins (Douglas, 2015). As the diversity of microbiota in insects is generally less compared to mammals, the microbial functions can be linked to

individual microbial species (Douglas, 2011). This allows us to further understand how the microbiota affects the host. Here in this study, it is hypothesized that there is a difference in microbial community: 1) between different developmental stages of the bagworm; and 2) between two different oil palm plantation areas.

1.1 Objectives

1. To identify the microbial communities of early and late instar stage larvae of *M. plana* from the outbreak area and non-outbreak areas.
2. To compare the microbial community of *M. plana* larvae between early instar stage and late instar stage larvae.
3. To compare the microbial community of *M. plana* larvae between non-outbreak area and outbreak area.

CHAPTER 2 LITERATURE REVIEW

2.1 Bagworm *M. plana* and Their Economic Importance

The *Psychidae* family includes about 1,000 species of bagworms worldwide, and several of the species are important pests of cultivated crops (Rhainds *et al*., 2009). Some examples these economically important bagworm pests include *Oiketikus kirbyi* which has been an increasing pest in the Peruvian avocado orchards (Rhainds & Cabrera – La Rosa, 2010), *Thyridopteryx ephemeraeformis* which is an important pest of ornamental trees and shrubs in the eastern United States (Ellis *et al*., 2005), and *Eumeta variegate* in which the larvae causes economic losses in China (Chen *et al*., 2021). Bagworms are leaf-eating larvae that lives in individual bag (hence the name bagworm). In the oil palm plantations of Malaysia, there are a few recorded species of bagworm, namely, *Amatissa cuprea*, *Brachcyttarus*, *Cryptohelia cardiophaga*, *Dapula* (*Clania*) *tertia*, *Mahasena corbetti*, *Manatha albipes*, *Metisa plana*, *Pteroma pendula* and a few other species (Sankaran, 1970; Wood & Kamarudin, 2019). However, among the three species that commonly reached outbreak status (*M. plana*, *P. pendula* and *M. corbetti*), the *M. plana* (Figure 1) is the most serious among them (Chung, 2012; Kamarudin *et al*., 2017; Kamarudin & Wahid, 2007).

Figure 2.1 *Metisa plana* larva removed from its bag.

The bagworm *M. plana* in the oil palm plantation feeds on the leaf of oil palm tree as their diet during their larvae stages (Kok *et al*., 2011; Mohd Basri & Kevan, 1995). At the early stage, small holes can usually be found on the upper portion or epidermis of fronds of the oil palm tree. However, the entirety of the frond would be damaged and dried up when there are many bagworms. Severe incident by the bagworms could result in a yield loss of 33 % to 47 % in the following years after infection (Basri, 1993). Fresh fruit bunch (FFB) yield loss of up to 43 % could be the result of a damage of 50 % to the leaf surface area (Mohd Basri & Kevan, 1995), which could be translated into millions of ringgit loss (Ahmad Ali *et al*., 2013).

Figure 2.2. Outbreak of *M. plana*. The number of *M. plana* larvae on each frond exceeded the economic threshold (more than five larvae per frond).

Figure 2.3. Effect of bagworm infestation on oil palm tree. Damaged leaves due to bagworm outbreak causes the leaves to look “burnt”.

2.1.1 Life Cycle of *M. plana*

Similar to other holometabolous insect, *M. plana* undergoes a few lifecycles; egg, larva, pupa and adult (Kok *et al*., 2011). The newly laid egg of *M. plana* are about 300 µm to 500 µm in length, yellowish-coloured, oblong-shaped and are laid in clusters of 200 to 300 eggs. The surface of the egg also has mucilaginous materials covering the surface. At five to eight days post-incubation, the egg will turn translucent and contains developing neonate with dark-brown body as well as distinct black and round spot in the middle. It will take approximately 12 to 15 days for freshly laid eggs to hatch. The hatching rate of *M. plana*'s eggs was reported to be around 70 % (Kok *et al*., 2011).

At roughly 1 mm in length, the newly hatched neonate termed first instar larva will begin feeding as soon as it plots on the surface of leaf. It will also begin building a case from plant materials such as lichens or twigs, in which the larva will carry the case as they feed. The case at first instar is cone-shaped, with closed distal end as well as an open base for the neonate to feed and to release waste (Kok *et al*., 2011). The case of first instar has a smooth surface with a length of 1.6 mm at average. After around nine to 16 days, the first instar larva will mould in into second instar stage larva.

At second instar, the larva has an average length of 2.0 mm. As the larva continues to grow, it will constantly enlarge its bag by adding more plant materials (Davis, 1964; Kaufmann, 1968). The case of the second instar larva with an average length of 4.6 mm, will have two to three small, rounded lead pieces that are loosely fastened to the basal end of the case. After 14 to 17 days, the second instar larva will mould into the third instar, and another 16 to 18 days to mould into fourth instar larva. The average length of third instar larva is 3.3 mm while the fourth instar larva has an

average length of 5.7 mm. The case of third instar with an average length of 5.9 mm has four to six rectangular leaf pieces attached to the proximal half of the case. The distal half of the case has a smooth surface, while the posterior end of the case has a disarranged surface. On the other hand, there are many loosely attached, big and round or rectangular leaf pieces on the surface of the case of the fourth instar. The case at the fourth instar stage has an average length of 9.5 mm. The fourth instar larva will take another 10 to 15 days to develop into fifth instar stage and 12 to 16 days to develop from fifth instar to sixth instar. The fifth instar larva has an average length of 7.1 mm, while the sixth instar larva has an average length of 8.5 mm. Most of the loose-leaf pieces are plastered onto the case of fifth instar, forming a smooth surface, with a few semi-rounded leaf pieces glued to the proximal base. The case of the fifth instar has an average length of 11.3 mm. On the other hand, the surface of the sixth instar case is smooth with no loose-leaf pieces attached and the leaf pieces are whitish grey in colour. The case has an average length of 13.0 mm. It will take approximately 71.5 days for the *M. plana* larva to develop from first instar to sixth instar (Kok *et al*., 2011).

During pupation, the larva is enclosed in the cage. The proximal opening of the case is sealed, and the case will remain affixed to the abaxial surface. It will take approximately eight to 12 days for the male adults to emerge from the case (Kok *et al*., 2011). On the other hand, the female will remain inside the case. In the case of successful mating, the adult male *M. plana* will remain active for three or four days before dying (Kok *et al*., 2011). The female *M. plana* will lay eggs in the cocoon, before leaving the case and die after a few hours (Kok *et al*., 2011; Mohd Basri & Kevan, 1995).

2.1.2 Management of Bagworms

In order to control the outbreak of bagworms, numerous control methods have been introduced. These control methods include trunk injection of synthetic insecticides and spraying biological insecticides *Bacillus thuringiensis* (Bt) pesticides and pyrethroids. The trunk injection method has been the preferred choice since mid-1970s and has been used effectively in various areas of oil palm plantations (Wood & Kamarudin, 2019). This method is completely selective as the pesticides used in trunk injection only contain systemic insecticide that act as stomach poison. The chemical residue may also linger in the leaves for some time, and sometimes have been shown to exterminate future generations of pest (Wood & Kamarudin, 2019). After the full emergence of young larvae, the application of trunk injection is at its most optimum. Although there might be some mortality to the parasitoids that are in developing larvae, it would not significantly reduce the natural enemies of the bagworm to the point of disrupting the natural balance (Wood & Kamarudin, 2019; Yap, 2000, 2005).

The Bt used in the control of *M. plana* produces toxin that works as stomach poison and is known to be useful against leaf-eating caterpillars. Bt-based products were described as perfectly selective and their potentials in oil palm were also tested. Although Bt products often give good result in the drop of bagworm numbers, there has been vague results observed (Hoong & Ho, 1992; Wood & Kamarudin, 2019) in which the larval population remained above the economic threshold level (Salim *et al*., 2015). At times, the commercial product Thuricide® and Dipel® showed little regulatory effect (Basri *et al*., 1988), with disparity in effect also similarly found in Sumatera (Cahyasiwi & Wood, 2016). A Bt strain (Ecobac-1®) which is appropriate for spraying and aerial use gives a good control during outbreak of *M. plana* (N. Kamarudin *et al*., 2017; Wood & Kamarudin, 2019).

Synthetic pyrethrum was considered a selective prospect when the outbreak of bagworms caused from the disruptive organochlorine pesticides affecting the oil palm plantations back in 1950s and 1960s (Conway & Wood, 1964). Development of synthetic analogues such as cypermethrin and deltamethrin were observed to be selective substitutes for oil palms. The residues were alleged to be fast fading and were proposed that their selectivity may be enhanced by the using the lowest dosage that is effective in exterminating the pests. It was reported that the cypermethrin was commonly used on estates and was a common practice in oil palm plantation (G F Chung *et al*., 1994; Norman & Basri, 1992) . However, the use of synthetic pyrethrum insecticides should be use with caution as they kill natural enemies too (Teh, 1996; Wood & Kamarudin, 2019).

The use of trichlorfon (also known as triclorphon) has been the standard practice for spraying operations ever since the first serious outbreaks of bagworms back in the early 1960s. It was used for its selective attributes and showed great kill of *M. plana* and *P. pendula* (Wood, 1968; Wood & Kamarudin, 2019). The use of trichlorfon resulted in no severe resurgences, but the cost for its usage has increased (Wood & Kamarudin, 2019; Yap, 2005).

2.2 Microbiome

At times, “microbiome” is used interchangeably with “microbiota”. However, they have both have subtle differences. The term “microbiome” refers to the collection of genomes from the microorganisms of the environment while the “microbiota” refers to the microorganisms of the environment (Valdes *et al*., 2018). The field of microbiome has quickly progressed over the past few decades, mainly due to the improvements in the Deoxyribonucleic Acid (DNA) sequencing, and since became a field with huge scientific and public interest (Cullen *et al*., 2020; Gonzalez & Knight,

2012; Knight, 2016). Such studies have revealed a multitude of data, resulting in a vast understanding into the nature of the microbial studies, which includes the interactions and the impacts of the microorganisms within host as well as in an external environment. Understanding the functions of these microorganisms could also be beneficial in various fields, from ecology, agriculture to forensics and medicine (Cullen *et al*., 2020; Huttenhower *et al*., 2014).

At present, microbiome studies tend to take either from top to bottom approach or from bottom to top approach. The first approach investigates the entirety of microbial communities, which observes the microbial communities at a bigger perspective. For example, Byerley *et al*., (2017) reported that the addition of walnuts to diet of Fischer 344 rats changes their gut microbial communities. Their study suggested that the walnuts may confer advantageous health gains through a new mechanism. In another study by Henderson *et al*., (2015), they investigated the microbial community composition of ruminant livestock from a wide geographical range. Their study provides an insight on the dominant methanogens which could be used to develop strategies for mitigating methane emissions. On the other hand, the second approach focuses on mechanistic studies or the roles of the individual microorganisms, metabolites, or genes, which is a more focused observation (Huttenhower *et al*., 2014). This approach was used by Caesar *et al*., (2016) in their study to investigate how does the interaction between the gut microbiota in mice and dietary lipids controls the lipid composition in the liver and plasma as well as the gene expression in the liver.

2.2.1 Lepidopteran Microbiota and Their Roles

Insect is one of the most diverse organisms on Earth and can be found in huge range of ecological niches, with Lepidoptera being the second most diverse in the insect order (Krishnan *et al*., 2014; Voirol *et al*., 2018). The diversification and

evolutionary success of insects can be attributed to their relationship with beneficial microbes. However, the microbes are mainly found in the digestive tract of their hosts where they act as vital modulators in the host' lifestyles (Gupta & Nair, 2020). It was estimated that the gut of insect contains more microbes than the total cells of the insect (Rajagopal, 2009). Voirol *et al*., (2018) stated that the most common bacteria across 30 different lepidopteran species are from the *Proteobacteria* phylum, and from the *Enterobacteriaceae* family although the gut microbiome also varies greatly across and even within lepidopteran species (Voirol *et al*., 2018). However, a study reported that caterpillars contain few to no resident bacteria when compared to the other insect order, due to their peculiarly alkaline gut, with fast food passage as well as well as them being holometabolous (Hammer *et al*., 2017).

Nonetheless, studies have shown that essential physiological functions in Lepidoptera are affected by the bacteria. The microbiota in insect provides numerous benefits to their insect host (Gupta & Nair, 2020; Krishnan *et al*., 2014; Voirol *et al*., 2018). The gut bacteria have a crucial role in nutrient acquisition in their insect host (Engel & Moran, 2013). For example, the high carbon to nitrogen ratio in leaves implies that the chewing insect would need to cope with their limited nitrogen in their diet. Some species of bacteria complimented their insect hosts limited dietary nitrogen by fixing and converting nitrogen into relevant compounds (Voirol *et al*., 2018; Waldbauer & Friedman, 1991). Indiragandhi *et al*., (2008) observed that bacteria isolated from the *Plutella xylostella* gut was able to fix nitrogen *in vitro*. On the other hand, the resident gut bacteria could also provide protection against pathogens through several means. Some gut bacteria in certain insect could produce bactericidal elements which selectively aim for foreign bacteria (Voirol *et al*., 2018). For example, *Micrococcus* sp. which was involved in the synthesis of antimicrobial peptides was

observed in the gut of *Helocverpa armigera* which protect the host from invading bacteria (Bulet *et al*., 1999; Ranjith *et al*., 2016).

2.2.2 Factors Affecting Microbial Communities

The high variability of lepidopteran microbiome could be affected by various factors, which may act independently or jointly. First of all, the environment where the insects live could affect the microbial community of insects. Ng *et al*., (2018) in their study found that the environment significantly altered the structure of the gut bacterial community in field crickets. Yun *et al*., (2014) observed a significant difference in the relative abundance of the anaerobes in insects from different environment. Besides that, the diet of insect could have a great influence on the variability of bacterial community (Ng *et al*., 2018; Voirol *et al*., 2018). For example, Leite-Mondin *et al*., (2021) observed a significantly different microbial community structure in *Trichoplusia* after feeding the insect with different diets. In another study, it was observed that diet was the main determinant in the gut bacterial community composition in two *Cerambycidae* species (Kim *et al*., 2017). In addition to the previous two factors, the developmental stage of an insect could also affect the microbial community. Andongma *et al*., (2019) observed a shift in the dominant operational taxonomical units (OTUs) from early developmental stages to the late developmental stages as well as the adult stages in the *Bactrocera minax*. In a study done by Wang *et al*., (2020), they observed a significant shift in the gut bacterial community structure in different developmental stages of the rainbow stage beetle *Phalacrognathus muelleri*.

2.2.3 Potentials of microorganisms in pest management

The contribution of microbiomes towards the insect various insect invasiveness-related characteristics could set forth numerous resources available for pest

management. A straightforward method would be to eliminate or interrupt the insect symbiosis (Baumann, 2005; Qadri *et al*., 2020). For example, antibiotics such as tetracycline and penicillin haven been proven to sterilise the tsetse flies when ingested by influencing the obligate mutualist *Wigglesworthia*, which disrupt the development of immature ticks and reduce the reproduction of adult ticks (Baumann, 2005). Aside from that, it was shown that substantial genome degradation makes the obligate symbionts of the pests more susceptible to the environmental fluctuations compared to the host itself (Gupta & Nair, 2020). For example, *Nezara viridula,* the southern green stinkbug depends on particular Gammaproteobacterial symbionts with a greatly diminished genome for the stinkbug's normal growth as well as survival. However, the gene loss has made the symbiont extremely susceptible to temperature fluctuations, which could kill the symbionts with even little changes in temperature, ultimately killing the host (Kikuchi *et al*., 2016). In such circumstances, the constraints imposed by the obligatory symbionts may aid in the control of pests (Gupta & Nair, 2020).

2.3 Metagenomics

Microorganisms are found in a huge range of environments and most of the knowledge of microbial life is based on the organisms that were raised in pure culture. However, many of them cannot be or have not been cultured in laboratories, making identification by traditional methods a very big obstacle. Cultural methods could only account for less than 1 % of the total microbial diversity of an environment (Edet *et al*., 2017; Streit & Schmitz, 2004; Tyson & Banfield, 2005). Metagenomics provides a way to overcome this obstacle by analysing the DNA obtained from environmental samples without the need to culture the organisms. The term "metagenomics" was coined by Handelsman *et al*., (1998), and it refers to the study of genomes of the

members of microbial community. This field of study enables researchers to tap, characterise and understand the vast unknown microbiome (Ghosh *et al*., 2019).

2.3.1 Brief History

In the early years, pioneer scientists started using solid phase nutrients to isolate microorganisms for counting and visualization. This technique helped them to comprehend the physiologies of the microbes (Blevins & Bronze, 2010; Escobar-Zepeda *et al*., 2015). Then, advancement of staining methods greatly improved the resolution of microscopy techniques which was the primary tool in exploring the microbes as well as their interaction (Beveridge, 2001; Blevins & Bronze, 2010; Escobar-Zepeda *et al*., 2015). However, it was found that microbes need specific conditions to grow, which lead to making culture media that resembled the microbes' natural environment. This idea and contribution by Winogradsky changed the world of microbiology and a new concept was born. This concept was named "microbial ecology", which means the study of microorganisms and their roles in the environment (Escobar-Zepeda *et al*., 2015; McFall-Ngai, 2008; Prayogo *et al*., 2020).

Carl Woese in the late 1970s suggested the idea of using ribosomal RNA genes as markers for classification (Woese & Fox, 1977) and combining with Sanger automated sequencing technique, made a huge impact on the research and classification of microbes. Numerous techniques popped out after several decades later with the advancement in molecular techniques. These techniques include the famous polymerase chain reaction (PCR) technique developed by Kary B. Mullis and Fred A Faloona (Mullis & Faloona, 1987). Moving forward, the development of a system which uses microorganisms to test gene functions and roles in the microbial community allows the discovery of new genes, functions as well as metabolic products brought forth biotechnology (Escobar-Zepeda *et al*., 2015). These advancements later

set the foundation to the era of metagenomic analysis. Even now, these approaches led to the discovery of new microbial community members, molecules and even microbial functions (Escobar-Zepeda *et al*., 2015; Jünemann *et al*., 2017).

The first Next Generation Sequencing (NGS) instrument which was the GS20 sequencing-by-synthesis (SBS) pyrosequencing was developed by 454 Life Science in 2004 (Kulski, 2016; Slatko *et al*., 2018). This platform was later succeeded by 454 GS GLX which offers read length of 100 - 150 base pairs (bp) and throughput of 20 Megabyte (Mb) per run. The platform was constantly improved, leading to the 454 GX FLX+ in 2009 which was able to produce a read length of up to 1 kbp and more than 600 Mb in a single run (Kulski, 2016; Slatko *et al*., 2018). Another platform that was a competitor in the market was the Genome Analyzer developed in 2006 by Solexa (Kulski, 2016; Slatko *et al*., 2018). In 2007, Illumina bought over Solexa and developed huge improvements and instruments such as the HiSeq and Miseq family in the years to follow. There are a few other platforms in the market but to this day, the Illumina's sequencing platform is the most broadly used due to their exceptional per-base cost efficiency as well as their high sequencing accuracy (Goodwin *et al*., 2016; Hodkinson & Grice, 2015; Jünemann *et al*., 2017; Kulski, 2016; Slatko *et al*., 2018).

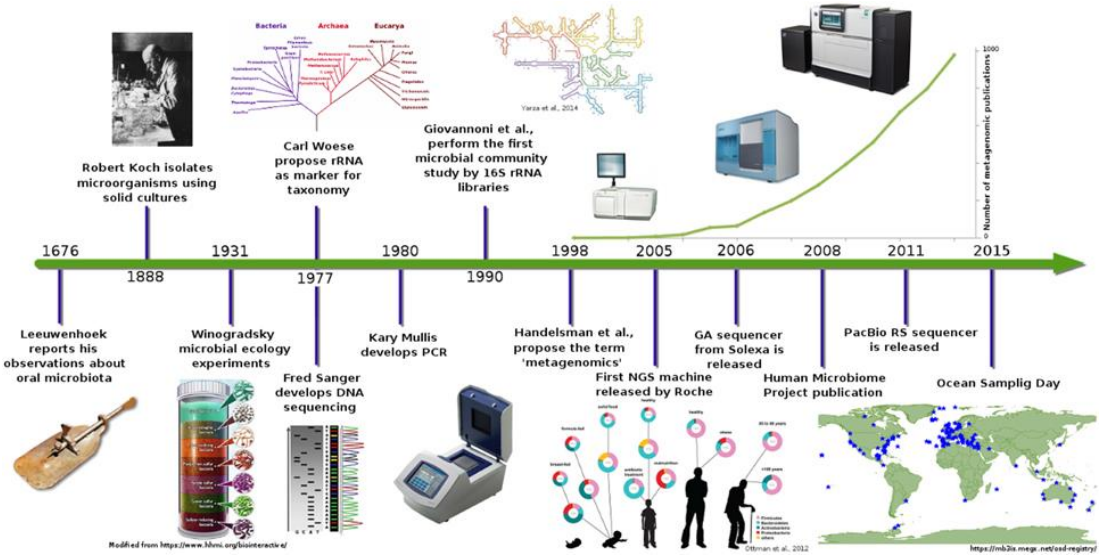

Figure 2.4 . The timeline of advancement in microbial studies. Figure taken from Escobar-Zepeda *et al*., (2015)

2.3.2 Amplicon Sequencing

In this study, amplicon sequencing was used and will be described here. The amplicon sequencing approach is commonly utilized for its handiness in executing taxonomical and phylogenetic classification of large complex samples (Clarridge, 2004; Oulas *et al*., 2015). It is a rapid and cheaper way to identify the bacterial community or taxonomic distribution profile (Clarridge, 2004; Oulas *et al*., 2015). This approach uses DNA as template for polymerase chain reaction (PCR) to amplify a marker or gene of interest such as the 16S ribosomal RNA gene of bacteria, internal transcribed spacer (ITS) for fungi and 18S rRNA gene for eukaryotes (Clarridge, 2004; Mitra, 2019; Oulas *et al*., 2015).

In bacterial community studies, the DNA would first be extracted from environmental samples. Then, the conserved regions of the bacterial 16S rRNA gene are amplified using universal primers that are complimentary to that specific conserved region of interest. PCR product will then be purified before sequencing. The sequencing method that is commonly used is the Illumina MiSeq platform as it offers high number of reads with high accuracy (Lawley & Tannock, 2017). After sequencing, there are a few software that packages that are accessible to analyse the data, such as Mothur (https://mothur.org/) and QIIME2 (https://qiime2.org/). Although there is numerous software available, there is seldom a "best" software to use. A simple analysis pipeline for the amplicon data based on Mothur pipeline will be described here. After receiving the raw sequence data, the sequence data will undergo pre-processing whether the quality of the sequence will be checked, and adapters as well as primers will be removed. The sequences that meet the quality

requirement will be merged to form contigs before aligning to a reference database of choice such as SILVA (Quast *et al*., 2013), Greengenes (DeSantis *et al*., 2006) or Ribosomal Database Project (RDP) (Cole *et al*., 2007; Maidak, 1996). Any chimeric sequences searched for and removed before clustered and picked into operational taxonomic units (OTUs). For bacteria, the OTUs that have similarity of over 97% are usually grouped into the same species (Fox *et al*., 1992; Lozupone & Knight, 2008; Martin, 2002). The OTUs are then classified into taxonomy based on the previous selected reference database. The α-diversity and β-diversity can then be obtained and visualized with any visualization software of choice such as RStudio (https://rstudio.com/). The α-diversity is a gauge of diversity within a community and also a measure of species richness (the number of species). On the other hand, β-diversity offers insight the differences in the composition of species in different groups (Escobar-Zepeda *et al*., 2015; Lawley & Tannock, 2017; Lozupone & Knight, 2008; Mitra, 2019; Oulas *et al*., 2015; Whittaker, 1972).

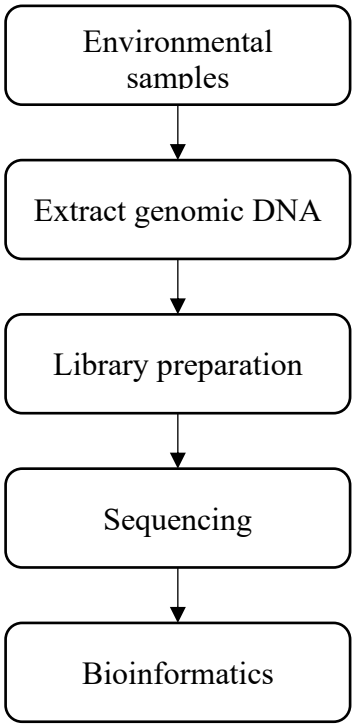

Figure 2.5. General workflow of the amplicon sequencing.

2.3.3 Application of amplicon sequencing in agriculture

The use of amplicon sequencing in agriculture should be highlighted as it provides a robust instrument for uncovering domestication genes or species in crop plants as well as in their wild relatives (M. Perez-de-Castro *et al*., 2012). Kharabian-Masouleh *et al*., (2011), demonstrated the use of amplicon sequencing for screening germplasm to discover variants in starch-related genes in germplasm of rice (*Oryza sativa* L.) On the other hand, Sexton *et al*., (2010) characterised the genetic diversity that exist within candidate genes in *Eucalyptus pilularis* Smith tree as an effort to breed for high-value word products through amplicon sequencing. Cordeiro *et al*., (2003) also used amplicon sequencing to identify genetic constitution of present-day sugarcane cultivars of interspecific origin as modern-day sugarcane varieties are of complex hybrids. As such, the use of amplicon sequencing in agriculture provides plant breeders the chance to identify and introduce diversity into their varieties, at the same time preserving or improving plant performance and product quality (Henry, 2012).

At times, amplicon sequencing was also used to identify the association of microbial communities with plants. Higo *et al*., (2020) used this technique in their study to shed light on how arbuscular mycorrhizal fungi (AMF) communities and the diversity in maize roots differ under different cover cropping systems as well as two types of tillage (rotary and no tillage). They found that the tillage system significantly changed the AMF communities. Jang *et al*., (2020) analysed 16S amplicon sequencing data and found that the *Proteobacteria* phylum appears to play the most crucial role in the survival of rice under drought condition. In another example, Dagher *et al*., (2019) studied whether repeated bioaugmentation with *Proteobacteria* affect plant productivity as well as the microbial communities related with the rhizosphere of four plant species growing in sediments that were contaminated with petroleum

hydrocarbon (PHC). By the use of amplicon sequencing, they observed that the presence of plant and their species identity were more influential on the structure of the microbiome in PHCs contaminated sediments (Dagher *et al*., 2019). Their results imparted the knowledge on the diversity as well as the behaviour of rhizosphere microbes linked to indigenous plants following continual bioaugmentation, which in turns emphasized the importance of plant selection in order to hasten land reclamation (Dagher *et al*., 2019).

2.3.4 Application of amplicon sequencing in agricultural insect pests

Amplicon sequencing has also been used as a tool to identify microbial communities in insect hosts, especially in insect pests. Zepeda-Paulo *et al*., (2018) managed to study the bacterial communities of two introduced aphid pests (*Sitobion avenae* and *Rhopalosiphum padi*). In their study, they observed a difference in endosymbionts between the two aphids, as well as detected the presence of a previously unidentified bacterial species that is closely related to phytopathogenic *Pseudomonad* species. In another study, Dematheis *et al*., (2012) investigated the fungal and bacterial communities from the eggs and gut of western corn rootworm (pest of maize) larvae. They found that the composition of the microbial communities in the gut of the larvae were soil type-independent with dominance of only a few microbial populations, the dominance of *Fusarium* species in the gut which suggested the larvae as vector of mycotoxin-producing fungi and postnatal procurement of *Herbaspirillum* sp. from the environment. Hadapad *et al*., (2019) on the other hand profiled the gut bacterial communities of wild and mass-reared and newly emerged melon fly *Zeugodacus cucurbitae* (Coquillett) and Oriental fruit fly *Bactrocera dorsalis* (Hendel), both of which are insect pests. They found diverse bacterial composition with varied relative abundance in the gut of wild and mass-reared *Z.*

cucurbitae as well as *B. dorsalis*. Their results could be useful in developing efficient mass-rearing protocols for fruitful execution of sterile insect technique.

With more people becoming aware of the dangers of synthetic insecticides of non-target organisms, the demand for sustainable and eco-friendly pest management approaches is becoming more pressing (Kesho, 2020). As such, the information on the microbial communities of insect pests could be used in pest managements. For example, Hosokawa *et al*., (2007) swapped *Ishikawaella* symbionts between the stink bug *Megacopta punctatissima* (a pest of soybean and other legumes) and the *Megacopta cribraria* (a closely related non-pest species), and they observed a poor *M. punctatissima* egg hatching. On the other hand, Moran & Yun, (2015) showed that by experimentally replaced the primary symbiont *Buchnera* with different genotype in pea aphid, the thermal tolerance of the aphid was altered. Both of the studies described above requires prior knowledge of the microbial communities.

CHAPTER 3 MATERIALS AND METHODS

3.1 Sampling Sites

The bagworms were collected from two different sites: outbreak area and non-outbreak area. The outbreak area is categorised by the population of bagworm which exceed the economic threshold of five larvae per frond (Salim *et al*., 2015). The bagworms from the outbreak area were collected from Felda Gunung Besout 2/3, Sungkai, Perak while bagworms from non-outbreak area were collected from Felda Jengka 7, Pahang. The bagworms were brought back to the laboratory and separated into early instar stage and late instar stage.

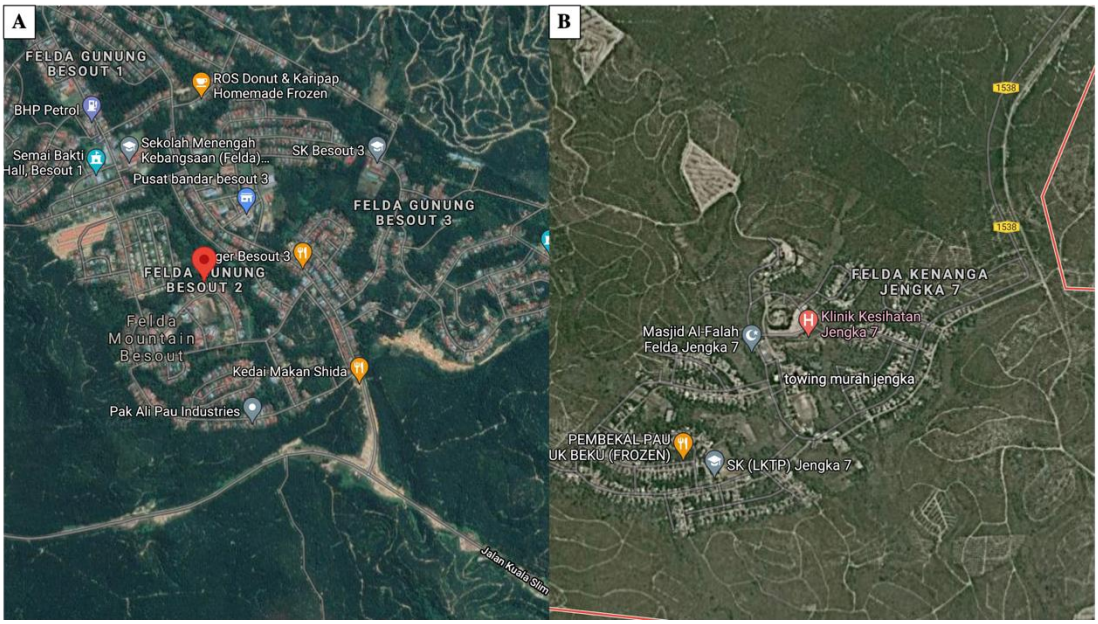

Figure 3.1. Sampling sites of bagworm *M. plana*. A: Bagworm from outbreak area were collected from Felda Gunung Besout 2/3, Sungkai, Perak. **B**: Bagworms from non-outbreak area were collected from Felda Jengka 7, Pahang.

3.2 Genomic DNA (gDNA) Extraction

Genomic DNA (gDNA) was extracted in four replicates for each group (late instar stage larvae from non-outbreak area, early instar stage and late instar stage larvae from outbreak area) using Qiagen DNeasy Blood and Tissue Kit (Cat No./ID: 69506, purchased from Qiagen) with some modifications. For each replicate, 20 bagworms

were removed from their bags and placed in 1.5 mL microcentrifuge tube before adding 180 µL of ATL buffer. The samples were then kept in -20 °C for 30 min before homogenized using micropipette tips. Subsequently, 20 mL of proteinase K was added to the sample and mixed by vortexing before incubating the samples at 56 °C for 10 min. The samples were then vortexed for 15 sec before adding 200 µL of AL buffer. The samples were then mixed by vortexing and incubated at 56 °C for 10 min. Ice-cold absolute ethanol of 200 µL was added to the samples and mixed. The samples were centrifuged at 6, 000 × g for 1 min and the supernatant were transferred to DNeasy Mini spin column. The spin columns were then centrifuged at 6,000 × g for 1 min. The spin columns were placed in a new 2 mL collection tubes and 500 µL of Buffer AW1 was added before centrifuging for 1 min at 6, 000 × g. The spin columns were again placed in new 2 mL collection tubes and added with 500 µL of Buffer AW2 before centrifuging at 13, 200 × g for 8 min. The spin columns were placed in new 1.5 mL microcentrifuge tubes and 50 µL of Buffer AE was added directly to the spin columns' membranes. They were then incubated for 3 min at room temperature before centrifuging at 6, 000 × g for 1 min. The eluates were pipetted back into the spin column's membrane and incubated for another 3 min before centrifuging at 6, 000 × g for 1 min. After that, 1 mL of RNase A was added to the eluate and incubated for 5 mins at room temperature. Gel electrophoresis was performed, and the gDNA were visualized under ultraviolet light.

3.3 Polymerase Chain Reaction (PCR) of V3-V4 region

PCR was performed to confirm the presence of the V3-V4 region of the 16S ribosomal RNA (rRNA) from the extracted gDNA. The components of the 20 µL reaction for the amplification of the V3-V4 region of bacteria are shown in Table 3.1 while the parameter for the PCR is shown in Table 3.2. The PCR product of

approximately 450 base pair (bp) was visualized with gel electrophoresis using 1.5 % agarose gel.

Table 3.1. Components of the 20 µL reaction mix for the amplification of V3-V4 region of bacteria.

Components	Volume (µL)
Autoclaved distilled water	14.8
10X *Taq* buffer	2.0
dNTP	1.0
DNA template	1.0
341F primer	0.5
806R primer	0.5
Taq polymerase	0.2

Table 3.2. PCR parameter for the amplification of V3-V4 region

Stage	Temperature (°C)	Duration	Number of cycle(s)
Initial denaturation	95	3 min	1
Denaturation	95	30 sec	
Annealing	55	30 sec	30
Extension	72	30 sec	
Final extension	72	5 min	1

3.4 Library Preparation and Sequencing

The gDNA were sent to the sequencing service provider, Apical Scientific Sdn Bhd (https://apicalscientific.com/). The quality of the gDNA was checked on 1 % TAE agarose gel while the concentration of the gDNA was measured using spectrophotometer (Implen NanoPhotometer® N60/N50) and fluorometric quantification using iQuant™ Broad Range dsDNA Quantification Kit. The V3-V4 region of the 16S rRNA were amplified using the bacterial 16S V3V4 primers (Table 3.3) (Sinclair *et al*., 2015) during the quality check of Amplicon PCR. In the first part of the library construction, the V3-V4 region of the bacterial 16S rRNA were amplified using V3-V4 primers with overhang adapters (Table 3.4). All the PCR reactions were carried out with Q5® Hot Start High-Fidelity 2X Master Mix Dual indices were

attached to the amplicon PCR using Illumina Nextera XT Index Kit v2 following manufacturer's protocols. The quality of the libraries was measured using Agilent Bioanalyzer 2100 System by Agilent DNA 1000 Kit and fluorometric quantification by Helixyte GreenTM Quantifying Reagent. The libraries were normalized and pooled according to the protocol recommended by Illumina and proceed to sequencing using MiSeq platform using 300 PE.

Table 3.3. Bacterial 16S V3-V4 primers (341F and 805R)

Primers	Sequences (5' to 3')
16S V3-V4 Forward (341F)	CCTACGGGNGGCWGCAG
16S V3-V4 Reverse (805R)	GACTACHVGGGTATCTAATCC

Table 3.4. Overhang adapters

Adapters	Sequences
Forward overhang	5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-[locus- specific sequence]
Reverse overhang	5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-[locus- specific sequence]

3.5 Analysis of Microbial Community using Mothur

Sequence analysis was done using Mothur software (v.1.44.3) (Schloss *et al*., 2009) with adaptation from MiSeq standard operating procedure (SOP) (https://mothur.org/wiki/miseq_sop/) (Kozich *et al*., 2013).

3.5.1 Creating customized refence alignment

Before proceeding with the data analysis, a customized database was created. This is done by using an *Escherichia coli*'s 16S rRNA gene sequence (Appendix A) and the sequence was trimmed until the sequence starts after the forward primer and ends before reverse primer. The trimmed sequence was then aligned (align.seqs) to SILVA seed refence v132 file (https://mothur.org/wiki/silva_reference_files/) and the (summary.seq) command was used to identify the starting and ending position of the sequence. After this, (pcr.seqs) command was used make the customised refence file

using the starting and ending position, using the SILVA seed reference v132 file as the reference and the keepdots was set to default.

3.5.2 Sequence analysis

A stability file was created using mothur with the (make.file) command. The two sets of reads (forward reads and reverse reads) were combined using the (make.contig) command and the primers were removed. After contig assembly, the command (summary.seq) was used to look at the details of the assembled contigs. Sequences that failed to achieve any one of the criteria were excluded using (screen.seqs) command: maximum length of 440 base pair (bp), minimum length of 406 bp, and the occurrence of any ambiguities. Duplicates sequences were also removed by using (unique.seqs) command.

The (count.seqs) command was used to obtain a table of names of the unique sequences, names of the groups as well as the number of times each unique sequence shows up in each group. The sequences were then aligned to the customized refence using the (align.seqs) before running (summary.seqs). The (screen.seqs) command was used again to remove the sequences that did not start at position two and ends at position 17,012, with maximum homology of eight as well as minimum length of 406 bp. The (summary.seqs) command was used again to check the details of the sequences after the (screen.seqs). The (filter.seqs) command was used to remove the gap characters "-" , with the perimeters: vertical=T and trump= "." . The (unique.seqs) command was used again as due the possible presence of redundancy after the (filter.seqs) command. The sequences were pre-clustered using the (pre.cluster) command with the diffs set at four. Chimeras were removed using (chimera.vsearch) command with the dereplicate=T, followed by (remove.seqs) command. The sequences were classified using Bayesian classifier with the (classify.seqs) command,

with cutoff=80. The reference taxonomy used in this command was from SILVA seed v132. After classifying the sequences, the unwanted classification such as “chloroplast”, “mitochondria”, “unknown”, “archaea” and “eukaryote” were removed using (remove.lineage) command.

A taxonomy summary file was created using (summary.tax) command. The sequences were then clustered into operational taxonomical units (OTU) by using (dist.seqs) command with cutoff = 0.03, followed the (cluster) command. A make.shared file was created using (make.shared) command with label=0.03. The consensus taxonomy for each OTUs were done using (classify.otu) command with label=0.03. The count, tree, shared and consensus taxonomy files were renamed using (rename.file) command. The number of sequences in each samples were checked using the (count.groups) command, and subsampled to the smallest number of sequences found from the samples using (sub.sample) command.

3.5.3 Bacterial community analysis

Rarefaction curves were obtained using the (rarefaction.single) command with calc=sobs and freq=100. The Shannon diversity index, number of OTUs and Shannon evenness index were calculated using the (summary.single) command with subsample=T. A simple T-test with significance at p-value less than 0.05 was performed to see whether the alpha diversity was significantly different. The shared community membership and community structure were analysed using Jaccard and Theta index through (dist.shared) command. The distance matrices were visualised sing the Principal Coordinates (PCoA) using the (pcoa) command. Analysis of molecular variance (AMOVA) and homogeneity of molecular variance (HOMOVA) were performed using Mothur software.

CHAPTER 4 RESULTS

4.1 Genomic DNA (gDNA) Extraction

The gDNA extracted from the bagworms using Qiagen DNeasy Blood and Tissue Kit were visualized on 1 % TAE agarose gel. The result of the visualization (Figure 4.1) showed that most of the gDNA were of good quality with very little degradation.

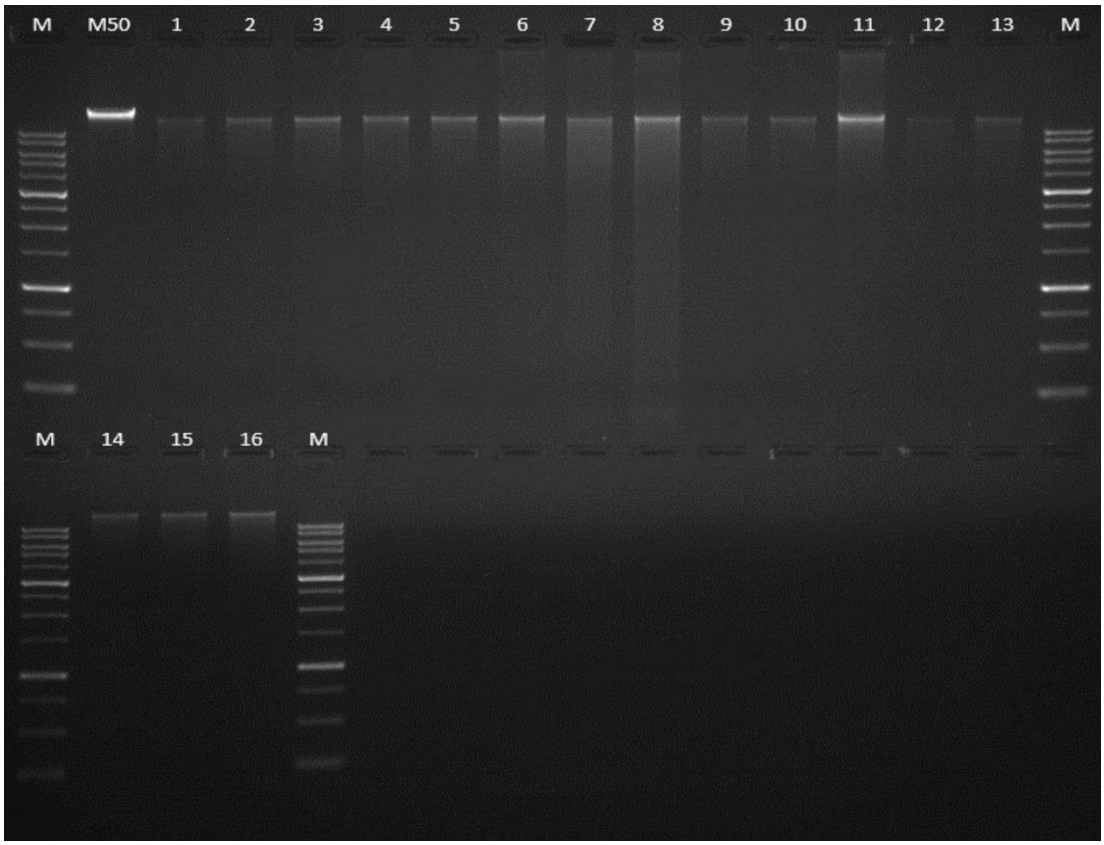

Figure 4.1. Genomic DNA (gDNA) extracted from the bagworms. M: ExactMark 1kb DNA Ladder; **M50**: Bacterial culture (Positive control); **1-5**: Late instar stage *M. plana* from outbreak area; **6-11**: Late instar stage *M. plana* from non-outbreak area; **12-16**: Late instar stage *M. plana* from outbreak

4.2 PCR of V3-V4 region

The V3-V4 region of the bacterial 16S rRNA was amplified and visualized on 1.5 % TAE agarose gel (Figure 4.2). The PCR products were of the correct size of approximately 450 bp.

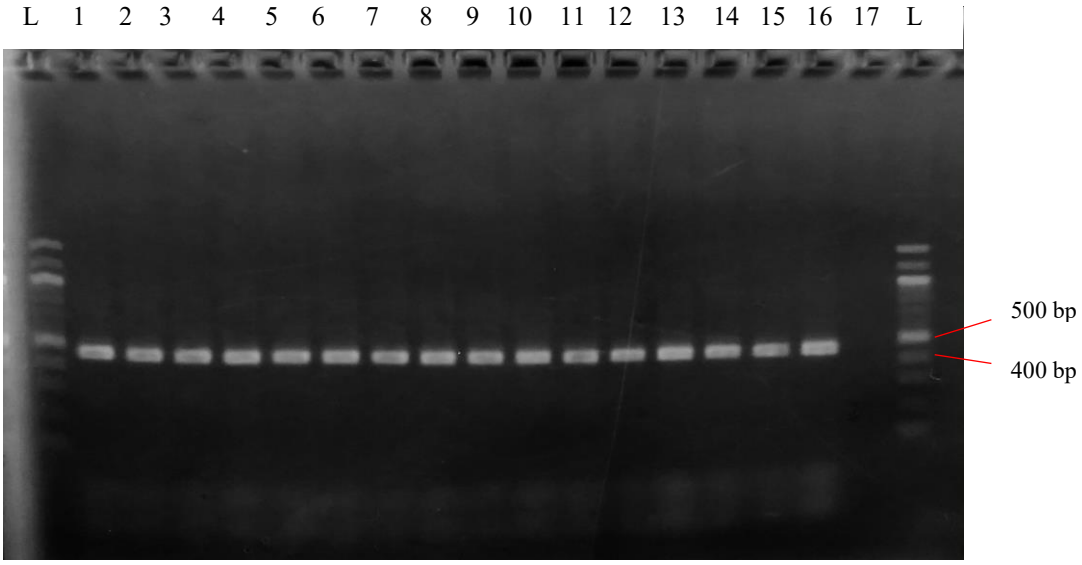

Figure 4.2. PCR product of the amplification of V3-V4 region of bacterial 16S rRNA. L: NEB 100 bp ladder; **1-5**: Late instar stage *M. plana* from outbreak area; **6-11**: Late instar stage *M. plana* from non-outbreak area; **12-16**: Late instar stage *M. plana* from outbreak; **17:** Negative control

4.3 Amplicon PCR Library Quality Check (QC)

The PCR product from library construction was visualized on 1.7 % TAE agarose gel (Figure 4.3) and showed the correct size of approximately 500 bp.

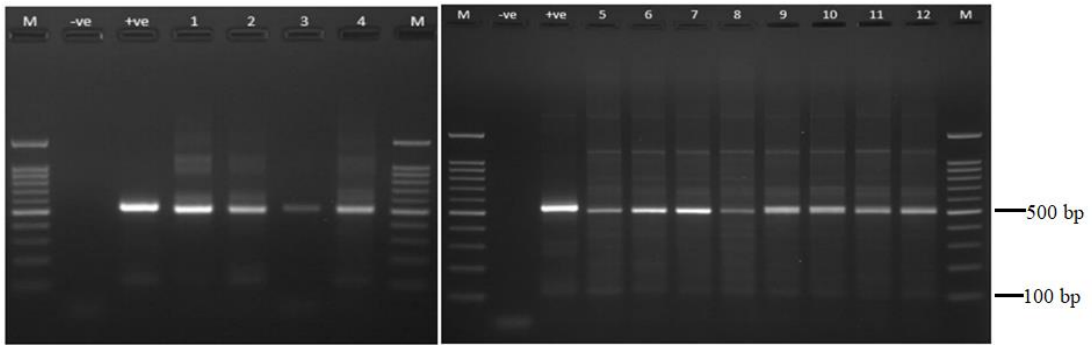

Figure 4.3. PCR amplicon library quality check. M: ExactMark 100 bp DNA Ladder; **-ve**: negative control (water replacing DNA template); **+ve**: positive control (DNA template from bacterial culture); **1-4**: Late instar stage *M. plana* from outbreak area; **5-8**: Late instar stage *M. plana* from outbreak area; **9-12**: Early instar stage *M. plana* from non-outbreak

4.4 Bacterial Community of *M. plana* larvae

4.4.1 Overview of the Bacterial Community in *M. plana* larvae

From the overall results of this study, it was observed that the bacterial community of *M. plana* larvae was dominated by bacteria from the phyla *Proteobacteria* and *Actinobacteria* although it defers between comparisons. At the family level, the bacterial community were generally dominated by *Enterobacteriaceae* and *Microbacteriaceae* but they differ between comparisions. A detailed result of the comparison is explained systematically as follows.

4.5 Comparison between early instar stage and late instar stage from outbreak area

In order to access the composition of the bacterial community of the *M. plana* larvae at early instar and late instar stage from outbreak area, the V3-V4 region of the bacterial 16S rRNA gene was amplified and sequenced. After sequencing, a total of 2,738,727 sequences were obtained from eight samples (four early instar stage samples and four late instar stage samples). After performing quality checks and removing unwanted sequences, a total of 385,297 sequences with 3,757 unique sequences were left. The sequences were then clustered at 97 % similarity into 959 Operational Taxonomical Units (OTUs). The rarefaction curve was obtained, and the curve did not completely plateau (Figure 4.4), which suggested that the sequencing depth was insufficient to capture the entire bacterial community.

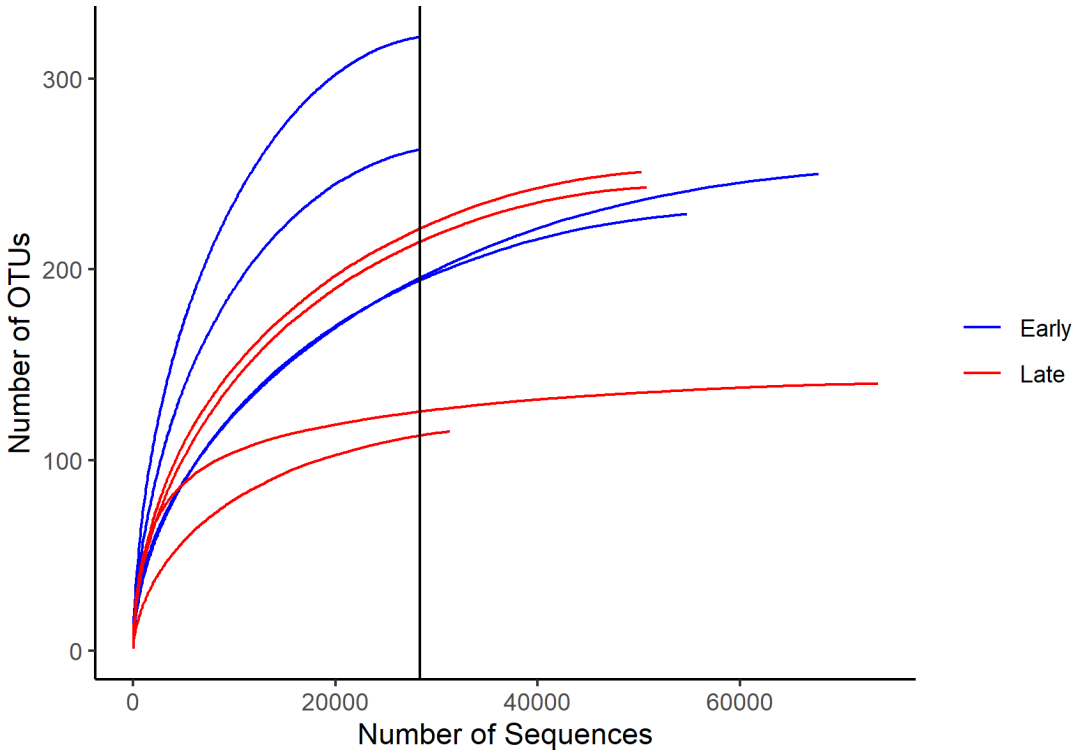

Figure 4.4. Rarefaction curve for the early instar stage and late instar stage bagworm larvae from outbreak area. (x- axis intercept: samples were subsampled to 28,340 sequences). The curves showed that the early instar stage larvae generally have a higher number of OTUs

4.5.1 Variability of bacterial communities between early instar stage and late instar stage

After the OTUs were assigned to their taxonomy, the results showed that the bulk of the bacteria were of *Proteobacteria* (82.36 %), *Actinobacteria* (14.80 %), *Bacteroidetes* (1.48 %), *Firmicutes* (1.01 %) and remaining individual phyla consisting of less than 1% (Figure 4.5 and Table 4.1). A simple T-test was used to determine if there was any significant different in the relative abundance between bacterial phyla and at significance of p-value < 0.05, the results showed no significant difference in relative abundance in any of the phyla.

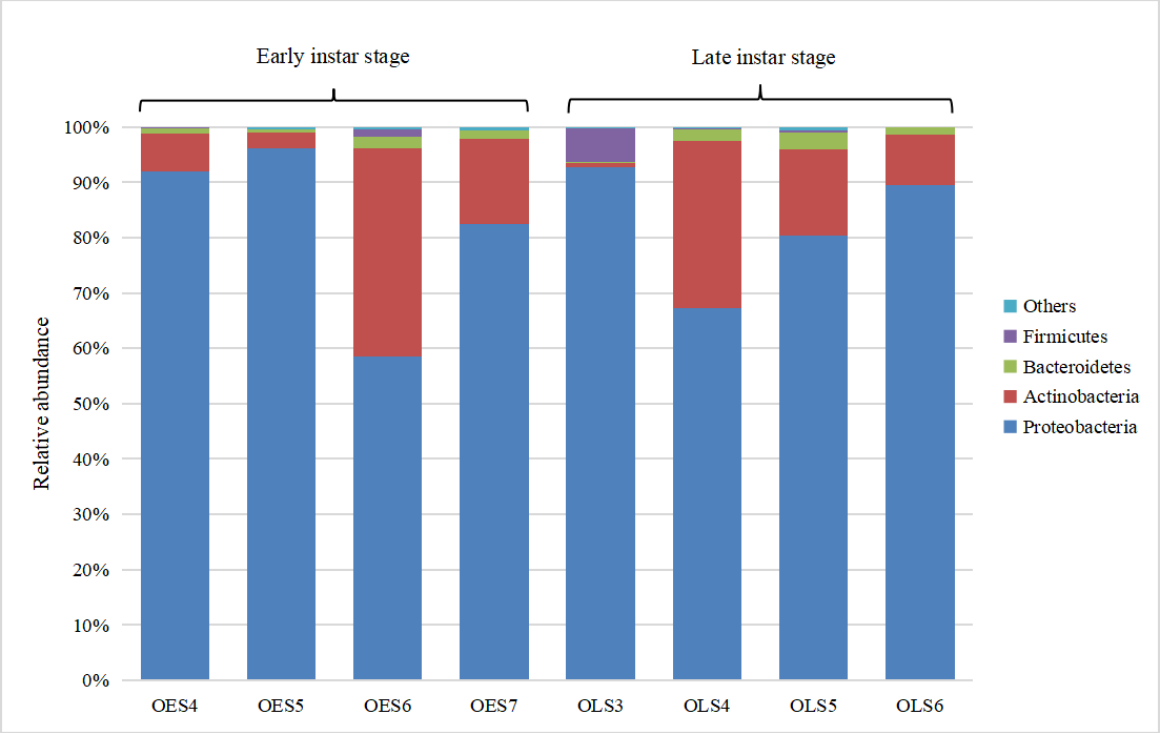

Figure 4.5. Bacterial phyla of early instar stage and late instar stage of *M. plana* larvae from outbreak area. Bacterial phyla with more than 1 % relative abundance are shown while the individual bacterial phyla with less than 1 % are grouped as "Others".

Table 4.1. Bacterial phyla with an overall relative abundance of more than 1 % in the comparison between early instar stage and late instar stage.

Phyla	Early Instar Stage (%)	Late Instar Stage (%)	Overall Presence > 1% (%)
Proteobacteria	82.28	82.45	82.36
Actinobacteria	15.68	13.92	14.80
Bacteriodetes	1.26	1.70	1.48
Firmicutes	0.38	1.65	1.01

The OTUs were also assigned to their taxonomy at the family level. The results showed that at family level, the *Enterobacteriaceae* was the dominant family (75.37 %), followed by *Microbacteriaceae* (13.63 %), *Burkholderiaceae* (3.44 %), *Pseudomonadaceae* (2.56 %), *Sphingobacteriaceae* (1.09%) and the remaining families individually having less than 1% relative abundance (Figure 4.6 and Table 4.2). T-test was also performed on the relative abundance of the bacterial families and

at significance of p-value < 0.05, there was no significant difference in relative abundance between the dominant families, but there were a few minor families that were significantly differently in between the instar stage such as *Flavobacteriaceae*, *Legionellaceae*, *Nocardioidaceae* and *Pseudonocardiaceae.* Looking deeper into the significantly different bacterial families, there were more *Flavobacteriaceae*, *Nocardioidaceae* and *Pseudonocardiaceae* in the late instar stage, but the *Legionellaceae* was more abundant in the early instar stage (Table 4.3).

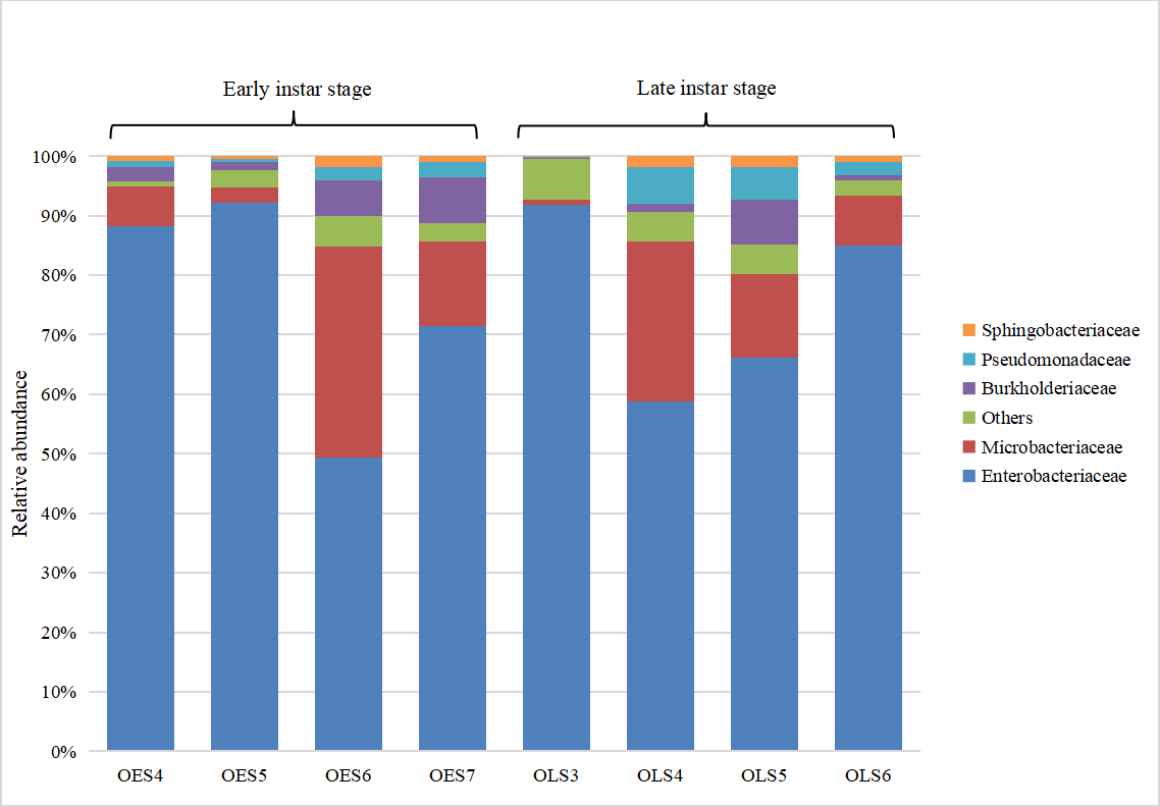

Figure 4.6. Bacterial families of early instar stage and late instar stage of *M. plana* larvae from outbreak area. Bacterial families with more than 1 % relative abundance are shown while the individual bacterial families with less than 1 % are grouped as "Others".

Table 4.2. Bacterial families with an overall relative abundance of more than 1 % in the comparison between early instar stage and late instar stage

Families	Early Instar Stage (%)	Late Instar Stage (%)	Overall Presence >1% (%)
Enterobacteriaceae	75.29	75.46	75.37
Microbacteriaceae	75.29	75.46	13.63
Burkholderiaceae	4.40	2.49	3.44
Pseudomonadaceae	1.59	3.53	2.56
Sphingobacteriaceae	0.99	1.18	1.09

Table 4.3. Bacterial families with significant difference in relative abundance between early instar stage and late instar stage

Families	Early instar (%)	Late instar (%)	Overall Presence (%)
Pseudonocardiaceae	0.04	0.28	0.16
Flavobacteriaceae	0.00	0.06	0.03
Nocardioidaceae	0.00	0.04	0.02
Legionellaceae	0.03	0.00	0.01

4.5.2 Diversity of the Bacterial Community

To investigate the alpha-diversity of the bacterial community between early instar stage and late instar stage, the Shannon diversity, number of OTUs and Shannon evenness were calculated (Figure 4.7 and Table 4.4) . Shannon diversity index were calculated to estimate the diversity of the bacterial community in the early and late instar stage. The index showed that the bacterial community of early instar stage was on average, more diverse than that of the late instar stage. The number of OTUs was calculated to estimate the number of bacterial species present. From the result, the number of OTUs was higher in the early instar stage than the late instar stage, revealing that the early instar stage was richer than the counterpart. Shannon evenness was obtained to observe the evenness of the bacterial community. The result showed that the bacterial community in early instar stage was more even than the late instar stage. After T-test was performed on the alpha-diversity, the shannon diversity index,

number of OTUs and evenness between the early instar stage and late instar stage were all not significantly different at significance at p-value < 0.05.

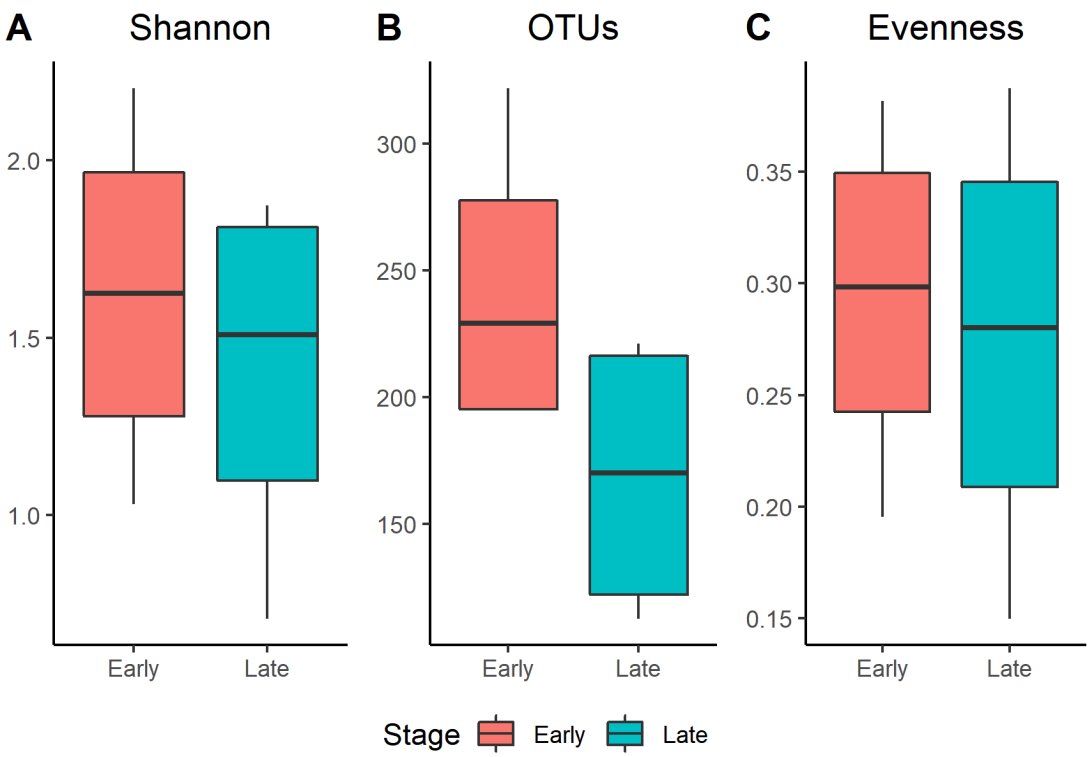

Figure 4.7. Alpha-diversity of the larvae of *M. plana* in comparison between early instar stage and late instar stage. A: Shannon diversity index; **B:** Number of OTUs; **C:** Shannon Evenness

Table 4.4. Shannon diversity index, Number of OTUs and Shannon evenness of bacterial community in the early instar and late instar stage. (Significance at p-value < 0.05)

Stage	Sample	Shannon	OTUs	Evenness
Early	OES4	1.361	194.736	0.258
	OES5	1.030	195.553	0.195
	OES6	2.204	322.000	0.382
	OES7	1.888	262.820	0.339
	Average	**1.621**	**243.777**	**0.294**
Late	OLS3	0.708	112.792	0.150
	OLS4	1.791	221.302	0.332
	OLS5	1.872	125.301	0.388
	OLS6	1.227	214.961	0.228
	Average	**1.400**	**168.589**	**0.274**
T.Test	**p-value**	**0.279**	**0.101**	**0.383**

(OES: Samples are of early instar stage from the outbreak area.; OLS: Samples are of late instar stage from non-outbreak area)

The PCoA was ordinated to visualise the cluster separation of the bacterial community. However, the ordination (Figure 4.8) did not show clear separation between the early instar stage and late instar stage. AMOVA test was done on the samples to test whether the cluster of the early instar and late instar stage was significantly different. The result of AMOVA (Table 4.5) revealed that the observed separation in the early instar and late instar stage was not significantly different. This meant that the bacterial community structure is like one another.

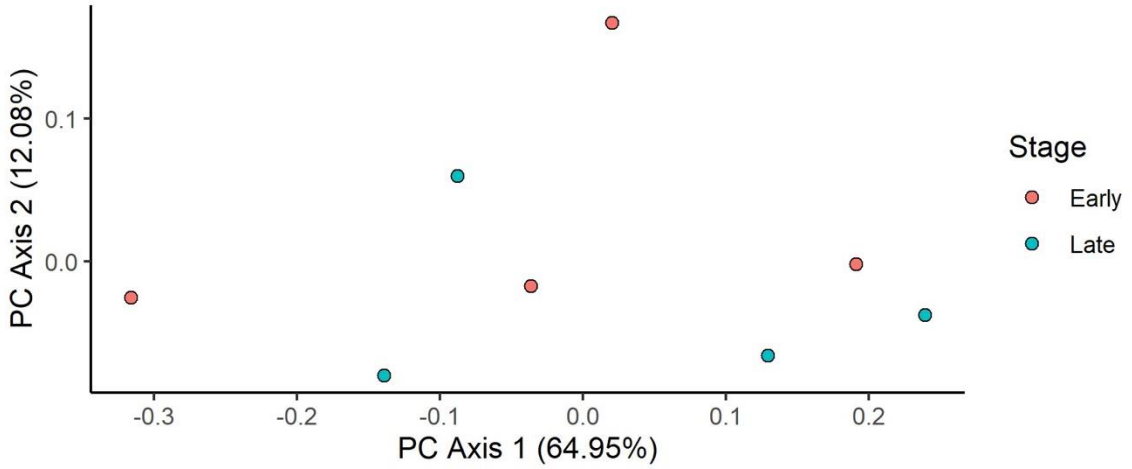

Figure 4.8. Principal Coordinate Analysis (PCoA) plot of bacterial communities of *M. plana* bagworm larvae in the comparison between early instar stage and late instar stage.

Table 4.5. AMOVA test done on samples from early instar stage and late instar stage. (Significance at p-value < 0.05)

Early - Late	Among	Within	Total
Sum-of-square (SS)	0.010	0.191	0.201
Degrees of freedom (df)	1	6	7
Mean squares (MS)	0.010	0.032	
F ratios (Fs)	0.325		
p-value: 0.554			

HOMOVA was performed to know whether the variation of the bacterial community in the early instar stage larvae was significantly different from that of the late instar stage. From the HOMOVA test (Table 4.6), it showed that there was no significant difference in the variation with the early instar stage and late instar stage. Nonetheless, the early instar stage has a higher variation (0.038) compared to the late instar stage (0.026). This showed that bacterial community in the early instar stage was less stable than the late instar stage and has higher chances for variation.

Table 4.6. HOMOVA test done on the samples from early instar stage and late instar stage. (Significance at p-value < 0.05)

HOMOVA	P-Value	SSwithin/(Ni-1) values
Early-Late	0.776	0.038 - 0.026

4.6 Comparison of Late Instar Stage Between Non-Outbreak Area and Outbreak Area

4.6.1 Bacterial community composition of *M. plana* bagworm larvae from non-outbreak area and outbreak area

As spatial difference can affect the microbial community of insect, a comparison between late instar stage of *M. plana* from non-outbreak area and outbreak area was done. The V3 and V4 region of the bacterial 16S rRNA gene was amplified using late instar stage larvae from the non-outbreak area and outbreak area. After sequencing, a total of 2,848,936 sequences were obtained from eight samples (four samples from non-outbreak area and four samples from outbreak area). After performing quality checks and removing unwanted sequences, a total of 271,821 sequences with 2,471 unique sequences were left. The sequences were then clustered at 97 % similarity into 796 OTUs. A rarefaction curve was also obtained, and the rarefaction curve did not plateau (Figure 4.9), suggesting the sequencing depth was insufficient to capture the entire bacterial community, and there could be more bacterial species that were yet to be sequenced.

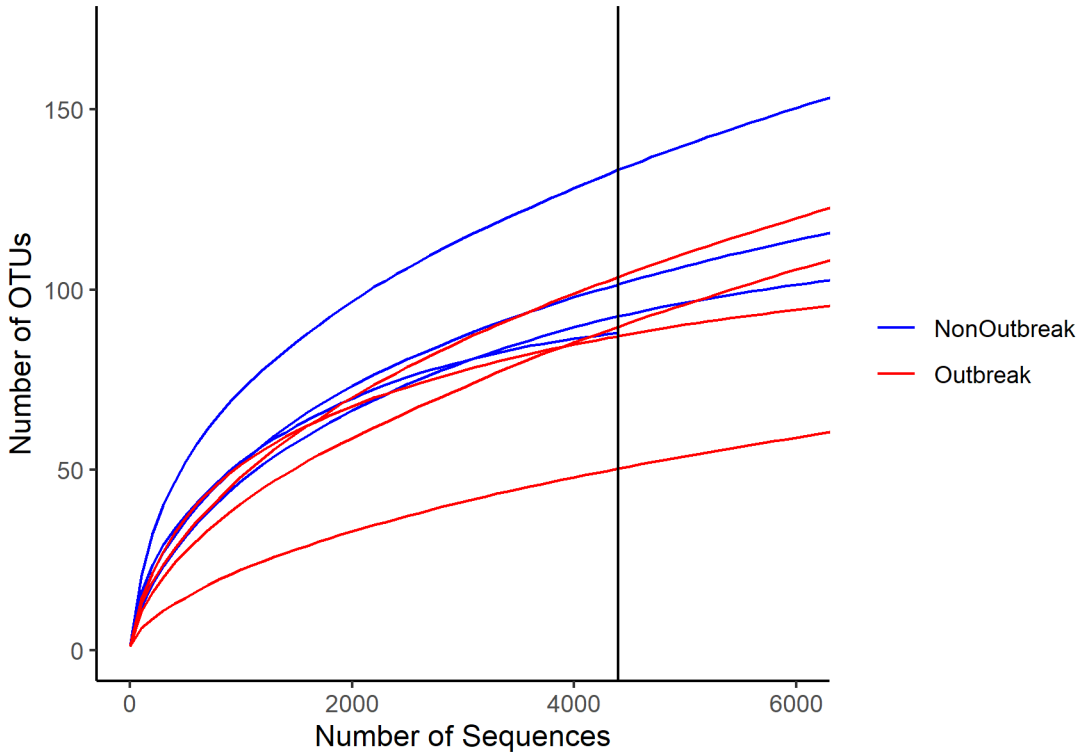

Figure 4.9. Rarefaction curve for the late instar stage bagworm larvae from non-outbreak area and outbreak area. (x- axis intercept: samples were subsampled to 4,399 sequences). The curves showed that the larvae from non-outbreak area generally have a higher number of OTUs.

4.6.2 Variability of bacterial communities between non-outbreak area and outbreak area

The OTUs obtained were assigned to their taxonomy, and the result showed that the most abundant phyla consisted of *Proteobacteria* (51.30 %) followed by *Actinobacteria* (45.22 %), *Bacteroidetes* (1.98 %) and the rest of the phyla individually consisting of less than 1 % in relative abundance (Figure 4.10 and Table 4.7). T-test was done to determine the significantly different phyla in terms of relative abundance. It was observed that there were a few phyla significantly different in relative abundance (Table 4.8). The *Proteobacteria* phylum from the outbreak area (82.02 %) was greater in relative abundance than of non-outbreak area (20.57 %). However, the second most dominant phylum which was the *Actinobacteria* was higher in relative abundance in non-outbreak area (76.29 %) than that of outbreak area (14.16 %). The

unclassified bacteria in non-outbreak area (0.60 %) were also greater than in outbreak area (0.14 %).

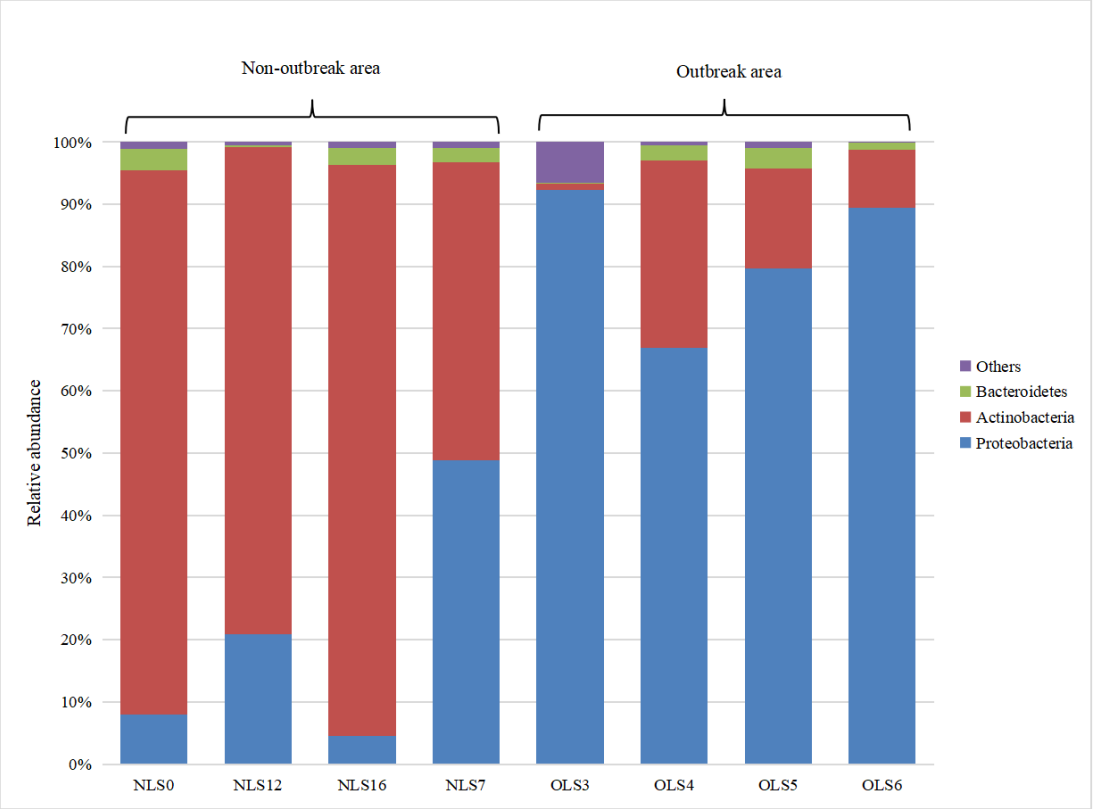

Figure 4.10. Bacterial phyla of the late instar stage of *M. plana* larvae from non-outbreak area and outbreak area. Bacterial phyla with more than 1 % relative abundance are shown while the individual bacterial phyla with less than 1 % are grouped as "Others".

Table 4.7. Bacterial phyla with an overall relative abundance of more than 1% in the comparison between non-outbreak area and outbreak area

Phyla	Non-outbreak area (%)	Outbreak area (%)	Overall Presence >1% (%)
Proteobacteria	20.57	82.02	51.30
Actinobacteria	76.29	14.16	45.22
Bacteriodetes	2.19	1.76	1.98

Table 4.8. Bacterial phyla with significant difference in relative abundance between non-outbreak area and outbreak area

Phyla	Non-outbreak area (%)	Outbreak area (%)	Overall Presence (%)
Proteobacteria	20.57	82.02	51.30
Actinobacteria	76.29	14.16	45.22
Unclassified bacteria	0.60	0.14	0.37

The OTUs were also assigned to their taxonomy at the family level. From the result, the most abundant families consisted of *Enterobacteriaceae*, followed by *Microbacteriaceae*, *Pseudomonadaceae*, *Burkholderiaceae*, *Sphingobacteriaceae*, *Kineosporiaceae* and other families individually having less than 1% relative abundance (Figure 4.11 and Table 4.9). T-test was done to compare the relative abundance of the bacterial families between the 2 areas and found that there were a few families that were significant difference in relative abundance (Table 4.10). *Enterobacteriaceae* was more abundant in the outbreak area (75.41 %) compared to non-outbreak area (11.67 %). However, there were more of *Microbacteriaceae* (70.87 %) and unclassified bacteria (0.60 %) in non-outbreak area compared to outbreak area (12.47 % and 0.14 % respectively. There were presence of *P3OB-42* (0.10 %) and unclassified *Alphaproteobacteria* (0.06 %) in non-outbreak area while there were none of the 2 families in the outbreak area.

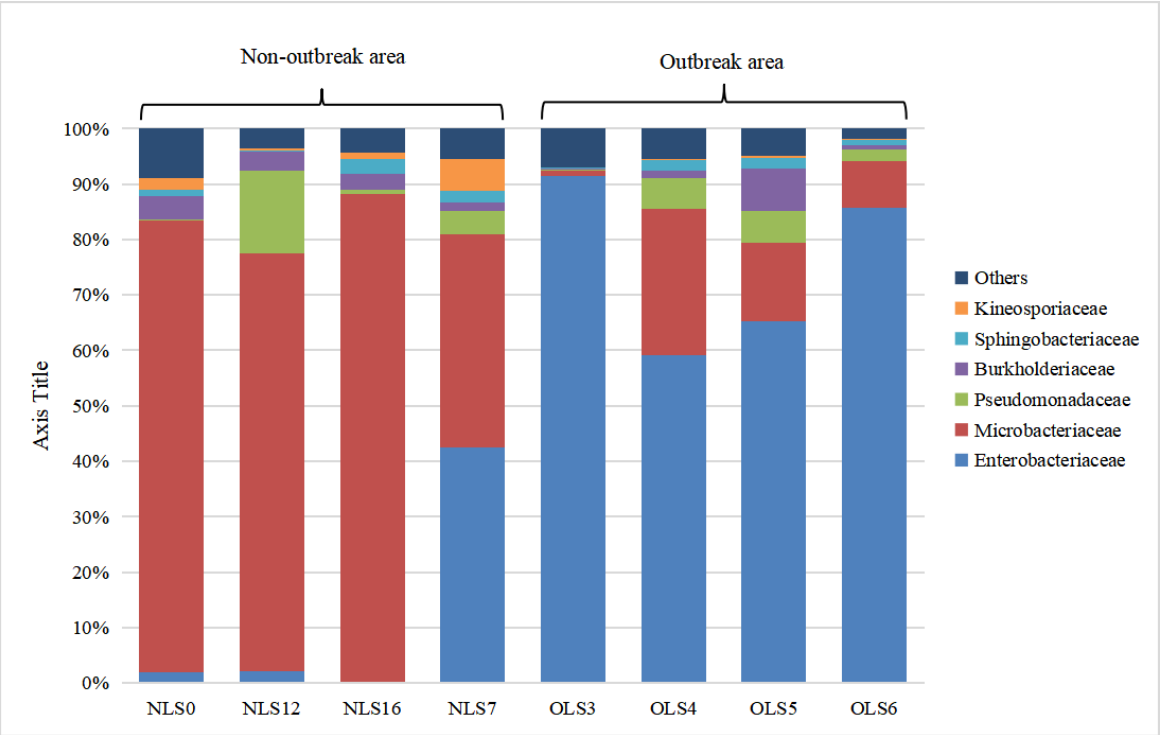

Figure 4.11. Bacterial families of the late instar stage of *M. plana* larvae from non-outbreak area and outbreak area. Bacterial families with more than 1 % relative abundance are shown while the individual bacterial families with less than 1 % are grouped as "Others".

Table 4.9. Bacterial families with an overall abundance of more than 1% in the comparison between non-outbreak area and outbreak area.

Families	Non-outbreak area (%)	Outbreak area (%)	Overall Presence >1% (%)
Enterobacteriaceae	11.67	75.41	43.54
Microbacteriaceae	70.87	12.47	41.67
Pseudomonadaceae	5.03	3.34	4.18
Burkholderiaceae	2.93	2.56	2.74
Sphingobacteriaceae	1.57	1.24	1.40
Kineosporiaceae	2.33	0.15	1.24

Table 4.10. Bacterial families with significant difference in relative abundance between non-outbreak area and outbreak area

Families	Non-outbreak area (%)	Outbreak area (%)	Overall Presence (%)
Enterobacteriaceae	11.67	75.41	43.54
Microbacteriaceae	70.87	12.47	41.67
Unclassified bacteria	0.60	0.14	0.37
Sphingomonadaceae	0.12	0.01	0.07
P3OB-42	0.10	0.00	0.05
Unclassified *α-proteobacteria*	0.06	0.00	0.03

4.6.3 Diversity of the bacterial community

To investigate the alpha-diversity, Shannon diversity, number of OTUs and Shannon evenness was calculated. The Shannon diversity index showed that the bacterial community from the non-outbreak area had a higher diversity than the counterpart (Figure 4.12 and Table 4.11).The number of OTUs was higher in non-outbreak area than outbreak area, revealing that the bacterial community in the non-outbreak area was richer. Shannon evenness was calculated and it showed that the bacterial community in outbreak area was more even compared to the non-outbreak area. T-test was performed but it showed that the diversity, richness and eveness between the non-outbreak area and outbreak area were not significantly different.

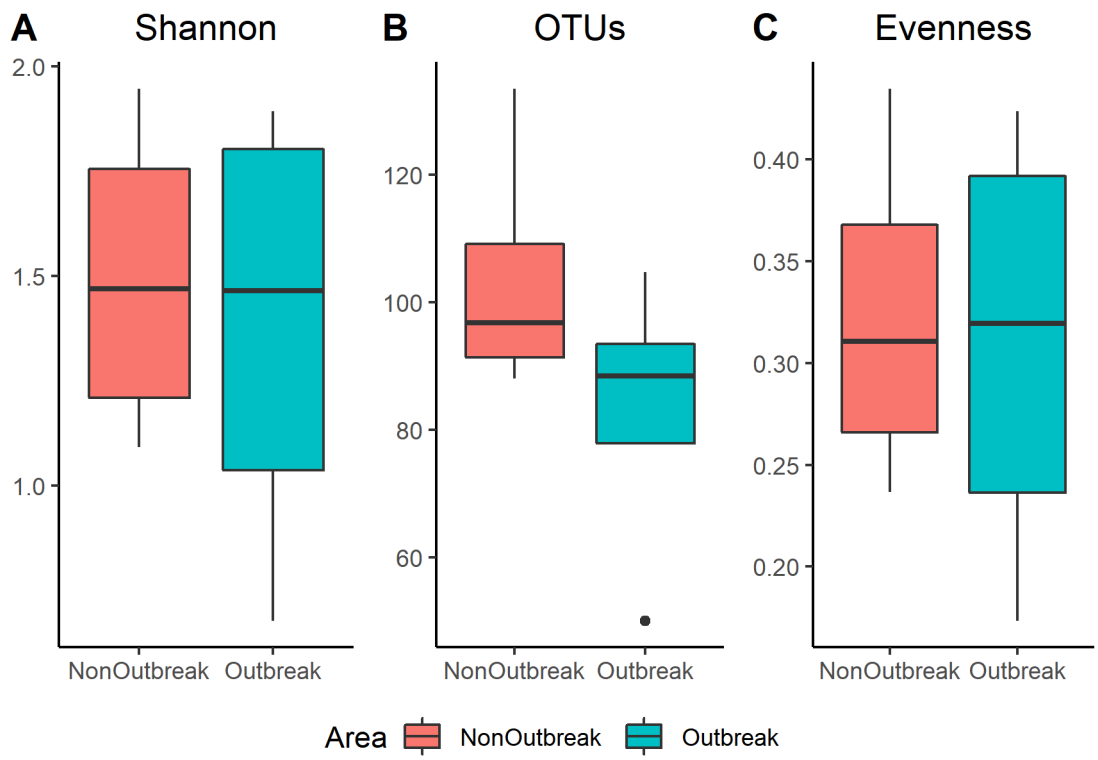

Figure 4.12. Alpha-diversity of larvae of *M. plana* in the comparison between non-outbreak area and outbreak area. A: Shannon diversity index; **B:** Number of OTUs; **C: D:** Shannon Evenness.

Table 4.11. Shannon diversity index, number of OTUs and Shannon evenness of bacterial community in the non-outbreak and outbreak area.

Area	Sample	Shannon	OTUs	Evenness
Non-Outbreak	NLS0	1.691	133.576	0.345
	NLS7	1.947	88.000	0.435
	NLS12	1.248	92.506	0.276
	NLS16	1.093	101.029	0.237
	Average	**1.494**	**103.778**	**0.323**
Outbreak	OLS3	0.678	50.042	0.173
	OLS4	1.773	104.775	0.381
	OLS5	1.893	87.202	0.424
	OLS6	1.156	89.667	0.257
	Average	**1.375**	**82.922**	**0.309**
T.Test	**p-Value**	**0.376**	**0.204**	**0.423**

The PCoA was again ordinated to visualise the cluster separation of the bacterial community. From the PCoA (Figure 4.13), we observed a clear separation between the samples from non-outbreak area and outbreak area. AMOVA test was done on the samples and the result (Table 4.12) showed separation between the two areas was significantly different. This meant that the bacterial community structure was different from one another.

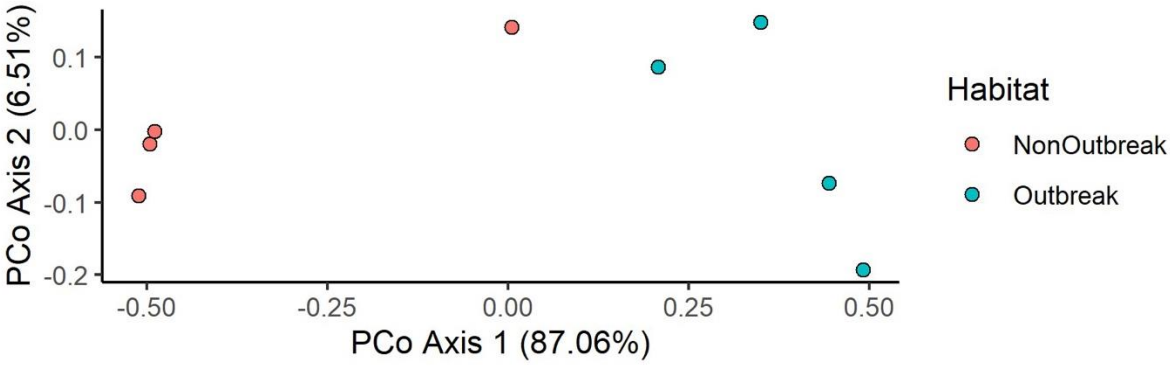

Figure 4.13. PCoA plot of bacterial communities of *M. plana* bagworm larvae in the comparison between non-outbreak area and outbreak area.

Table 4.12. AMOVA test done on samples from non-outbreak and outbreak area. (Significance at p-value < 0.05)

Non-Outbreak - Outbreak	Among	Within	Total
Sum-of-square (SS)	1.087	0.269	1.357
Degrees of freedom (df)	1	6	7
Mean squares (MS)	1.087	0.045	
F ratios (Fs)	24.209		
p-value: 0.034*			

HOMOVA was performed to know whether the variation of the bacterial community in the late instar stage larvae from non-outbreak area was significantly different from that of outbreak area. The HOMOVA test (Table 4.13) showed that there was a significant difference in the variation of bacterial community between the two areas. The non-outbreak area has a higher variation (0.063) compared to the outbreak area

(0.027). This showed that bacterial community in the non-outbreak area was less stable than the outbreak area, hinting that the bacterial community in the non-outbreak was more prone to changes.

Table 4.13. HOMOVA test done on the samples from non-outbreak and outbreak area. (Significance at p-value < 0.05)

HOMOVA	P-Value	SSwithin/(Ni-1) values
NonOutbreak-Outbreak	0.17	0.063 - 0.027

CHAPTER 5 DISCUSSION

5.1 Overview of the Bacterial Community

At present, most studies on lepidopteran microbiota were focused on the microorganisms linked to the larval gut. However, this only provide a smaller but more focused view of the community. In the present study, focus was made on the early instar stage as well as the late instar stage from the outbreak area to see whether there were any differences between them. The bacterial community of the late instar stage larvae of *M. plana* between non-outbreak and outbreak areas were compared to investigate spatial-associated shift in the bacterial community. Generally, the bacterial community was found to be dominated by *Proteobacteria* and *Actinobacteria*. These phyla were commonly found within the Lepidoptera order (Montagna *et al*., 2016; Reetha & Mohan, 2018; Voirol *et al*., 2018). On the family level, there were a few dominant families such as the *Enterobacteriaceae* and *Microbacteriaceae.* Again, these families can also be found in different Lepidopteran species (Jones *et al*., 2019; Voirol *et al*., 2018; Xia *et al*., 2017).

The overall dominance of the mentioned bacterial phyla and families in the present study may have some sort of beneficial roles to the health of the larvae. Although *M. plana* larvae is polyphagous, they were collected from the foliage of the oil palm tree, and might only have that host plant as its diet. However, it could also be that the larvae obtained these bacteria solely from their environment or diet but provided little or no benefit. In a study done by Phalnikar *et al*., (2018), they observed that their most common and abundant OTUs in butterflies were also common in different insect-associated microbiomes. This lead them to hyphothesise that the insect-bacterial co-occurrencce may indicate evolved functional relationships or it could merely act as ecological or dietary roles. The latter hypothesis might be due to

absence or presence of very little resident bacteria found in caterpillar such as reported by Hammer *et al*., (2017). Besides, Phalnikar *et al*., (2018) found a substantial overlap of bacterial communities from larval and dietary resources which indicated that bacterial communities in larval are mainly influenced by passive procurement of bacteria from dietary resources. Nonethelss, *Proteobacteria* linked with insects helps in breaking down of carbohydrate (Delalibera et al., 2005), vitamin B synthesis or essential amino acid (EAA) production (Bennett et al., 2014), and pesticide detoxification (Werren, 2012). *Actinobacteria* on the other hand possessed a vast range of physiological and metabolic capabilities, including the synthesis of extracellular enzymes and the production of a large range of secondary metabolites (Schrempf, 2001). For example in termites, *Actinobacteria* aided in the acquisition of nutrients from a variety of polysaccharides, including cellulose (Pasti & Belli, 1985; Watanabe et al., 2003) as well as hemicellulose (Schäfer et al., 1996). Nevertheless, it is important to note that the microbiome varies greatly across lepidopteran species and even within species (Voirol *et al*., 2018). As the entire larvae were sampled, yet there was no trace as to where exactly these bacteria reside, although some studies had found that the bacterial communities from the whole insect can be similar to the bacterial communities sampled from the gut (Hammer *et al*., 2014; Sabree *et al*., 2012; Sudakaran *et al*., 2012).

5.2 Comparison Between Early Instar Stage and Late Instar Stage

Here in this part, the bacterial community of early instar stage larvae and late instar stage larvae of *M. plana* from outbreak area were compared. It was hypothesised that there was difference bacterial community of the bagworm *M. plana* between the two instar stage as in holometabolous insect such as the bagworm, the bacterial community is affected by their developmental stage (Engel & Moran, 2013; Huang *et*

al., 2019; Voirol *et al*., 2018). Findings from this present study reveals that, the *Proteobacteria* phylum was the most dominant phylum, followed by *Actinobacteria*, *Bacteroidetes, Firmicutes* and other minor phyla. This is in keeping with a review done by Voirol *et al*., (2018) which screened independent studies of 30 different lepidopteran species, and found that *Proteobacteria* phylum was the most common phylum. Another study done on the moth *Brithys crini* at different developmental stages also found the *Proteobacteria* as the most abundant phylum (González-Serrano *et al*., 2020). At family level, the *Enterobacteriaceae* was the dominant family and is also commonly found in other Lepideptoran species (Pandiarajan & Krishnan, 2018; Reetha & Mohan, 2018; Voirol *et al*., 2018). However, the bacteria that were significantly different in relative abundance were all from the minor families. This observation could be attributed to the change in the feeding behaviour of the larvae. In the early instar stage, the larvae scrape on the leaf epidermis using their mandibles but changed to cutting leaves at late instar stage (Cheong & Tey, 2012). The less active feeding behaviour of the larvae at late instar stage could also played a part in this difference in relative abundance of the mentioned families.

In a study done by Kok *et al*., (2011), they observed that the lab-reared *M. plana* larvae reduced their feeding activities and remained in their cases after the fourth instar stage. If the bulk of the bacterial community of the larvae is obtained from their diet, this reduced feeding activity could have impacted the abundance of certain bacterial species. However, it could only be assumed that the wild *M. plana* larvae exhibit the same feeding behaviour as the lab-reared larvae. It was also hypothesised that the difference in relative abundance of the mentioned minor families might be due to the developmental time from one instar to another. The early instar stage larvae require longer time to develop to their next instar stage than in the late instar stage

(Kok *et al*., 2011). With a longer time required to develop and moult, the early instar stage would be exposed to environmental microorganisms for a longer period of time than the late instar stage. Nonetheless, there could be a chance that the difference in relative abundance in the minor families could be due to slight difference in microbial community between individual larvae.

In the same comparison, it was observed that the bacterial diversity, richness, and evenness in the early instar stage were higher than that of the late instar stage. However, the results were not significantly different between the two larval instar stage. It was further shown that the bacterial community structures from early instar stage and late instar stage did not form significantly separated clusters. This observation hinted that the instar stage did not significantly contribute to variability of the bacterial community. In some Lepidopteran species such as *Plodia interpunctella* and *Plutella xylostellai*, their bacterial community did not change across developmental stages (Mereghetti *et al*., 2017; Ng *et al*., 2018; Xia *et al*., 2013). This could also be true to the *M. plana* larvae where its bacterial community structure is not significantly affected by the developmental stage. This similarity could also be attributed to the larvae having the same host plant, as different diet might influence bacterial communities in different ways such as promoting differential bacterial growth (Staudacher *et al*., 2016; Vorholt, 2012; Yang *et al*., 2001). Additionally, the similarity of the bacterial community structure could also be due to the same area where the bagworms were collected as different environments could affect the microbial community in insect (Ng *et al*., 2018; Yun *et al*., 2014).

5.3 Comparison Between Non-Outbreak Area and Outbreak Area

Here the bacterial community of the late instar larvae from non-outbreak area as well as outbreak area were compared as it was hypothesised that there were

difference in the bacterial community in the bagworm from both areas. Results showed that there was indeed a difference when comparing the microbial communities from the non-outbreak area and outbreak area. This difference in microbial communities between locations was also observed by Yu *et al*., (2021) where they observed similar microorganisms when comparing Colorado potato beetle (*Leptinotarsa decemlineata*) collected from different locations but having different relative abundance. On the other hand, Wang *et al*., (2019) observed a varied bacterial community composition and diversity in leafhopper *Psammotettix alienus* across different geographic populations. Varying geographical conditions could divide the insect distributions into distinct populations or ecological types, with differences among the geographical populations include ecological adaptability, resistance as well as physiology. (TU Xiaoyun *et al*., 2015; Xia *et al*., 2018; Yu *et al*., 2021). Similarly, the microbial community of insect could also be affected by different conditions as in many insect, as most if not all of the gut microbes are dominated by far-spread bacteria that colonize the host opportunistically. (Yu *et al*., 2021). At a large scale, there could be a slight difference in the environmental conditions althought both locations are of oil palm plantations. According to the meterology data based on the nearest meteorlogy station to the sampling sites (Malaysian Meterology Department, 2020), the average temperature for the non-outbreak area and outbreak areas were similar at around 27.8 ℃ to 27.9 ℃ at the time of collection. However, there were records of 22.8 mm and 0.3 mm of rainfall on the day prior to and the first day of sampling at the non-outbreak area. It was also recorded that the humidity increased to 80.5 % and 82.0 % after the rainfall. On the other hand, there was no record of rainfall before and on the day of sampling at the outbreak area, with the humidity of 76.3 % on the day of sampling. This could be a contributing factor to the difference in the bacterial community structure and relative

abundance of certain bacteria as seen. In a study done by Allard *et al*., (2020), they observed a shift induced by rain in the epiphytic bacterial communities in cucumber and tomato fruits. They stated that increased moisture as well as relative humidity in the air prior to, during and after rain could promote the fast growth of certain bacterial species at the expense of another. Studies also showed that the soil microbiome are affected by the rainfall (Cregger *et al*., 2012; Cruz-Martínez *et al*., 2012; Kardol *et al*., 2010; Šťovíček *et al*., 2017; Zhang *et al*., 2016), which in turn could affect the microbial composition. However, most of the studies were focused on the effect of precipitation or rain on the microbial community of the soil microbiome and with no studies of the effect of rainfall on the microbial community in insect at the time of writing, hence it could not be certain that the amount of rainfall did indeed directly affect the microbial community in insect. A more detailed and systemic experiment is needed to investigate this hypothesis.

In addition, it was also suspected that the soil microbiome in the two areas might be different in terms of bacterial diversity or abundance of certain bacteria. A study showed that insects that feed on foliar obtained their microbiomes from the soil (Hannula *et al*., 2019). Hannula *et al*., (2019) stated that the microbiome of the caterpillar that fed on intact plant had a more distinct microbiome and the microbiome resembled the soil microbiomes. In another study, Gomes *et al*., (2020) found that the caterpillar's bacterial communities resembled the local soil microbiomes in which the host plant was growing. The microorganisms that originate from the soil can be assimilated into the microbiome of the insect via the host plant when the insect ingest the plant containing those microorganisms (Chi *et al*., 2005; Sugio *et al*., 2015), although recent study showed that the microorganisms do not presist in the gut of caterpillar (Hammer *et al*., 2017). Gomes *et al*., (2020) further hypothesise a

possibility that changes in soil microbiomes could lead to alteration in insect microbiomes, further altering the performance of insects through the microbiome of the plant, or through direct soil-insect association. Their studies provide us with the hypothesis that although the bacterial communities of the bagworm larvae from outbreak area and non-outbreak area generally are similar, the significant difference in abundance of certain bacteria species found in this comparison could be reflected in the difference of local soil microbiota where the bagworms were collected. However, the soil microbiota from the two areas need to be profiled to confirm this hypthoesis.

Lastly, bacteria are not the only microorganisms that can be found in and on the bagworm, hence this study only provides a partial picture of bagworm's microbiota. Fungus (Draganova *et al*., 2013; Gielen *et al*., 2021; Poitevin *et al*., 2018) and viruses (Brooks *et al*., 2002; Myers & Cory, 2016) can also be found on other Lepidopteran species and they could also have a part in the outbreak of the bagworm.

CHAPTER 6 CONCLUSION

The outbreak of bagworm in the Malaysia's oil palm plantations has been a blight to the industry, with the exact cause still being investigated. As microbial community affects the health and performance of insects, the microbial community of *M. plana* was investigated. The bacterial commmunities in the larvae of *M. plana* in their early and late instar stages as well as form the non-outbreak and outbreak areas were compared. Generally, the bacteria community of the *M. plana* larvae was dominated by the *Proteobacteria* phylum and *Enterobacteriaceae* family. However, it was found that the bacterial community of the *M. plana* larvae in the non-outbreak area was dominated by *Actinobacteria* phylum while *Proteobacteria* phylum dominated in the outbreak area. In the family level, it was the *Microbacteriacea* which dominated in the non-outbreak area but the *Actinobacteriacea* dominated in the outbreak area. Although the bacterial communities in the comparisons were not significantly different in terms of diversity, richness and evenness, there were some significant difference in abundane of certain bacteria phyla and families. A significant and clear distinction in the bacterial community structure when comparing non-outbreak area and outbreak area was also recorded. The results of the study showed that the bacterial communities of the *M. plana* larvae from different area were indeed different. This differences seen could be due to the several factors such as the amount of rainfall or moisture in the environment and the soil microbiome in the areas, affecting the bacterial commmunities, which in turn could be the contributing factors behind the outbreak of the bagworm. This study also provided a first insight to the bacterial community of the *M. plana* larvae and bagworm as a whole, paving the way to furtther understanding the factors behind the outbreak of bagworm and for future pest management efforts.

6.1 Future recommendations

As the study showed a difference in the bacterial community of the *M. plana* larvae between the non-outbreak and outbreak area, there are a few future works that can be taken to further understand the factors behind the outbreak of bagworm. In this study, there was different amount of rainfall or moisture in both the sampling areas and it could be a contributing factor to the outbreak of bagworm. In order to test this hypothesis, a study comparing the bacterial community of the *M. plana* larvae in a controlled setting with the different amount of moisutre or humidity can be done. Besides that, this study also hinted that the soil microbiome could be a contributing factor. A study on the soil microbiome and the bacterial communities of the oil palm leaves of the sampling sites could also be useful to indicate whether the bacterial community of the *M. plana* larvae was indeed influenced by these factors. On the other hand, identifying the bacterial community of the oil palm leaves could answer the question of whether the larvae has resident bacteria or what bacteria were obtained from the diet. To further understand the role of the microorganisms in the bagworm, a metatranscriptomic study can be done to examine the upregulated or downregulated genes between bagworms from the non-outbreak area and outbreak area.

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APPENDIX A. 16S rRNA sequence of *E. coli*.

>E.coli
ATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAACGGTAACAGG
AAGCAGCTTGCTGCTTCGCTGACGAGTGGCGGACGGGTGAGTAATGTCTG
GGAAGCTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATAC
CGCATAATGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCAT
CGGATGTGCCCAGATGGGATTAGCTTGTTGGTGGGGTAACGGCTCACCAA
GGCGACGATCCCAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTG
AGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACA
ATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTC
GGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGGAGTAAAGTTAATACCT
TTGCTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAG
CAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCG
TAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTC
AACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGGGG
GGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATA
CCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAA
AGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAA
CGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAAC
GCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAA
TGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGA
TGCAACGCGAAGAACCTTACCTGGTCTTGACATCCACGGAAGTTTTCAGA
GATGAGAATGTGCCTTCGGGAACCGTGAGACAGGTGCTGCATGGCTGTC
GTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACC
CTTATCCTTTGTTGCCAGCGGTCCGGCCGGGAACTCAAAGGAGACTGCCA
GTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTT
ACGACCAGGGCTACACACGTGCTACAATGGCGCATACAAAGAGAAGCGA
CCTCGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTAGTCCGGATTGG
AGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGGATCA
GAATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACA
CCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCTTAACCTTCGGGAGGG
CGCTTACCACTTTGTGATTCATGACTGGGGTG

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