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Gut microbiota biodiversity and activity play critical roles in the physiological performance of *Spodoptera frugiperda* (Lepidoptera: Noctuidae)

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Abstract

The nutrition, physiology, and behaviour of the host are significantly influenced by the microbes that dwell inside insects. Although Lepidoptera (Butterflies and moths) are among the most varied insect species, little is known about their microbial symbionts, especially during metamorphosis. Here, the present work studied the diversity and activity of gut microbiotas throughout the holometabolous life cycle of *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae), a well-known agricultural pest in the world, using ribosomal tag pyrosequencing of DNA and RNA. Although Firmicutes and Proteobacteria predominate, they go through a structural "metamorphosis" alongside their host. In the early instar, Enterococcus, Pantoea, and Citrobacter were numerous and active, whereas Clostridia proliferated in the late instar. It's interesting to note that only enterococci survived transformation. Compared to males, female adults had higher concentrations of Enterococcus, Klebsiella, and Pantoea, whereas Klebsiella predominated in males. The development of new pest-management techniques may benefit from an understanding of the metabolic processes carried out by these microbial symbionts that are connected with herbivores.

Introduction

Insects in the lepodopteran order develop in four stages: eggs, larvae, pupa, and adult. The morphological characters for *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae) in the adult stage, the forewing of the male moth is typically dark and brown with triangular white dots at the tip and close to the middle of the wing.

Females have less pronounced markings on their forewings, which can range from a uniform greyish brown to a faint grey and brown mottling. Both sexes have an iridescent silver-white hind wing with a thin dark border (Sparks, 1979). In *S. frugiperda* is possible to identify males and females by observing the terminal segment, the position of the genital, and the shape of the terminal segment (Odhiambo and Ondasi, 2020).

To understand how the microbial community differs in *S. frugiperda* at different stages of life, it needs to identify the metabolically active populations. Despite the fact that 16S rRNA from the RNA pool

represents protein synthesis potential and can be used as an indicator of active microbes, which directly contribute to the current function of the microbiota and characterise the active moiety of a microbial community from diverse sources (Mattila *et al*., 2012).

The aim of the present work is to study the diversity and activity of gut microbiotas throughout the holometabolous life cycle of *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae), using ribosomal tag pyrosequencing of DNA and RNA.

Materials and methods

1. Insect rearing and sample processing:

Trials were performed in the Insect Molecular Biotechnology Unit, Vegetables Pest Department, Plant Protection Institute, Agricultural Research Center. A stock colony of *S. frugiperda* larvae was collected from maize fields at the farm of Plant Protection Institute, Egypt. The colony was kept in an incubator at a temperature of 25.2 °C and a relative humidity of 65.5%.

The lab colonies were eating lettuce. The colonies were raised with slight alterations (Dahi *et al*., 2020 and Gamil, 2020). Fresh lettuce leaves were used to feed the larvae, which were raised in a plastic container (40*20*15 cm) with muslin fabric and rubber bands to facilitate aeration. Food was changed every two days. The third instar was raised separately from the larvae.

This was done in tiny plastic containers that were covered in a thin muslin cloth and rubber band (70 mm in height by 20 mm in diameter). To aid in the effective pupation, a thin layer of fine sawdust was sprinkled on the bottom of each glass jar. Until pupation, larvae were maintained in an incubator at 25.0°C + 1°C, 65.0°C + 5.5% RH, and 14 L: 10 D photoperiod. Puppies are kept together in the same incubator. Up until moths emerged, pupae were monitored every day.

After the moths emerged, a single male and female were partnered and placed in an incubator with a glass cadge for oviposition that was kept at the aforementioned temperature. The moths were given food in the form of a small piece of absorbent cotton wool that was rubber banded to the glass and then soaked in 10% sucrose solution. Paper scraps were placed inside the cage while oviposition was taking place. Every day, the papers were checked for egg batches.

The eggs were kept at 25°C and 65°5% R.H. till hatching for strain establishment. First, a selected insect washed with sterile water three times. Next, surfaces were rinsed with ethanol 70% for 30 seconds before being was washed with sterile water one more. According to Shao *et al*. (2013), each individual's intestinal tissue was collected, homogenised, and then used to extract nucleic acids.

Following homogenization of the intestinal dissection in 500µL of sterile PBS. 100µL of the homogenized material was transferred into 900µL of PBS, rapidly vortexed, and 100µL of each dilution was disseminated onto Brain-heart infusion agar plates (8130, BD). For 48 hours, all plates were incubated at 37 °C. Colony-forming units (CFUs) were used to measure the total number of bacterial cells in each sample.

2. Nucleic acid extraction and reverse transcription:

The dissected gut insect tissues in (Early- larval, late-larval, female adults and male adults) were added to liquid nitrogen in a porcelain morter, and ground with a pistol to a fine powder. Isolation and purification of genomic DNA by using (Phenol extraction and ethanol precipitation of DNA) while isolation and purification of RNA by using (Quanti Tect RNA isolation kit).

The extracted yield of nucleic acid (DNA and RNA) was checked on the agarose gel and quantified using a NanoDrop 1000

(Thermo Scientific) then purification both f DNA and RNA by using (QuantiTect DNA purification kit and RNA purification kit). The reverse transcription for the extracted RNA to cDNA uses (QuantiTect Reverse Transcription kit).

Frist RNA should treat by a genomic DNA Wipeout buffer at 42 °C for 2min to get rid of extracted DNA. Also 7 µL of the DNase was added to the RNA to the transcript to cDNA in a total reaction volume of 10 µL using random primers. Two negative controls were performed, including 7µL of DNase-treated RNA with all RT reagents except for the reverse transcriptase and 7µL of RT-PCR grade water instead of RNA.

3. Denaturing gradient gel electrophoresis (DGGE) of amplified 16S rRNA genes:

As described by Shao *et al.* (2014) that using for PCR primers 968F/1401R were used to amplify the V6-V8 portion of 16S rRNA genes. Specific primers were designed for each of Archaea- and fungus-specific primers to amplify archaeal 16S and fungus ITS genes (Anderson and Cairney, 2004 and Hershberger *et al*., 1996).

Electrophoresis was done using a 16×16 cm, 1 mm thick gel that contained 8% polyacrylamide with a 20 to 80% denaturant gradient (100% denaturant was 7M urea and 40% (v/v) deionized formamide). The gels were run at 100V for 16h at 60 °C in TAE buffer (40mM Tris–acetate, 1mM EDTA; pH 7.4). After electrophoresis, the gels were stained for 30 min in TAE buffer with SYBR-Gold nucleic acid gel stain (S-11494, Invitrogen) for photographs. Gels were scanned using a GS-800 calibrated densitometer (Bio-Rad).

4. Primer design and Amplification of DNA fragments (PCR).

Primer designed according to the hypervariable V1–V3 segment in the 16S rDNA. We used the universal primers Gray28F 5′-GAGTTTGATCNTGGCTCAG and Gray519R 5′-GTNTTACNGCGGCKGCTG, which target variable regions V1–V3 in the 16S gene.

The PCR reaction was made in a sterile 0.5 ml microfuge tube containing the following reagents in the order given: 100 µl solution of 20 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM Mg Cl_2, 0.001% (w/v) gelatin, 0.2 mM each dNTP, 1.25 µM each of forward and reverse primers, 1µL of template DNA, and 2.5 µ of Ampli Tag DNA Polymerase (Perkin - Elmer cetsus).

Amplification was carried out in the PTC-100 programmable Thermal Controller (PCR). The PCR Program was as follows: 3min at 94°C, followed by 35 cycles of 94°C for 45s, 60°C for 30s, and 72°C for 1min, and a final extension time of 10min at 72 °C. The nucleotide sequence of the PCR products was determined by ABT PRISM (310) version 3.2 DNA sequencer with autocycle sequencing kit (Pharmacia).

Results and discussion

1. *Spodoptera frugiperda* microbiota overview:

Insects that develop into the lepodopteran order go through four stages: eggs, larvae, pupae, and adults (Figure 1). Our study case focuses on dividing larval stages into early-instar and late-instar stages. The feeding culture on nutrient agar plates and PCR amplification did not show any fungus contamination. Of course, we discovered a large number of bacteria persisting in the stages of *S. frugiperda* development. The colony-forming unit (CFU) count in the larval stage was 6.13*117 per sample, and adults were 2.82*107 per sample. As shown according to *S. frugiperda* developmental stages the bacterial communities differ.

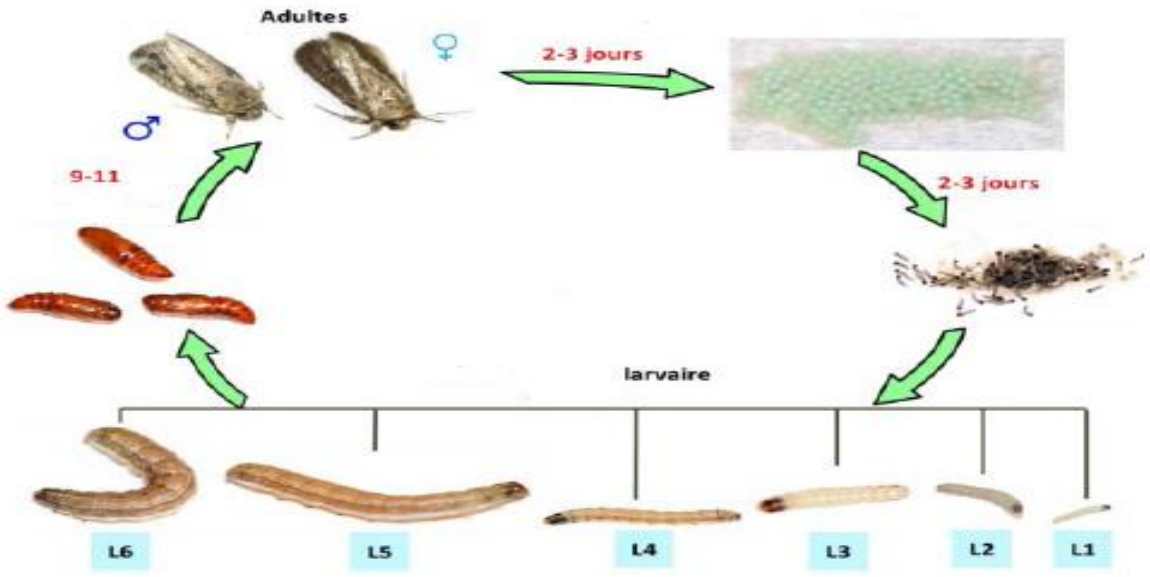

Figure (1): Development cycle of *Spodoptera frugiperda*. Source: Cokola (2018).

The taxonomic analysis of sequences obtained by phylogenetic investigation of communities and comparative functional analysis (NCBI) showed that the common phylum of bacteria in *S. frugiperda* is Firmicutes (52%) in the early-instar and (92%) in the late-instar. Mature adults showed an increase in both Firmicutes and Proteobacteria. In adult female gut dominantes by Proteobacteria (56%) and Firmicutes (42%) while the male gut dominates by Proteobacteria (93%) with Actinobacteria (5%) as shown in (Figure 2).

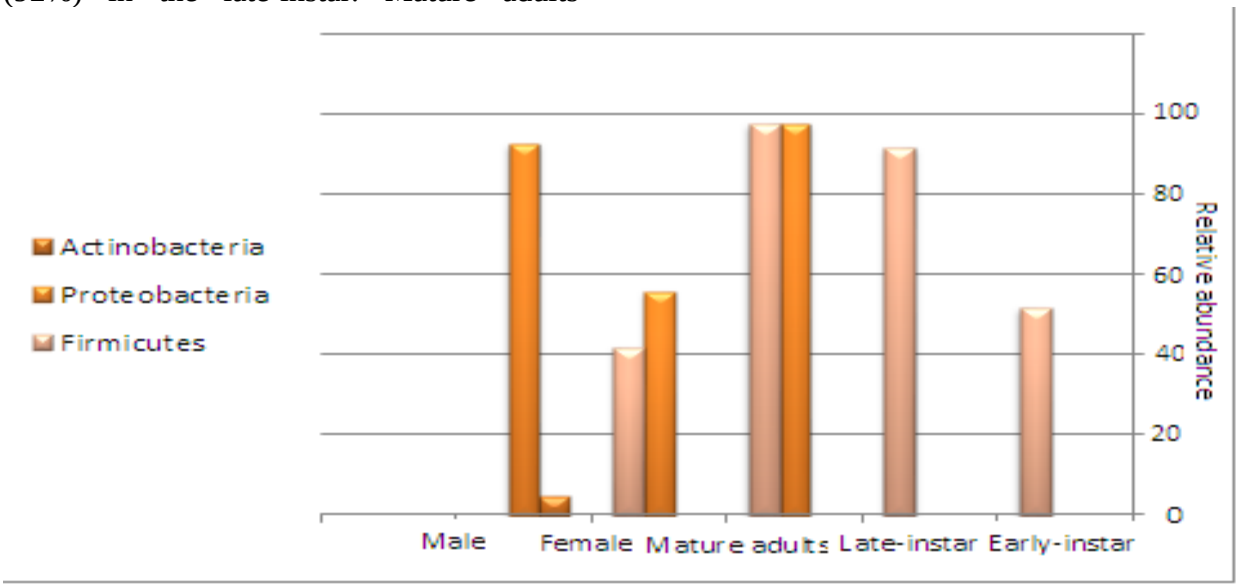

Figure (2): Overview of the microbiota change during host development. Abundance of the 16S rRNA gene at each larva (Early and late-instar) and adult (Male, female) at the phylum level.

By using denaturing gradient gel electrophoresis (DGGE) which is a molecular technique for fingerprint analysis of the microbial community, to amplify 16S rRNA genes to detect the variance of microbiota within an *S. frugiperda* gut sample The DGGE showed that a few variations in larvae were reared in the same conditions as shown in Figure (3a).

On the other hand, the DGGE for adults showed a similar DGGE band in both male and female adults as well as a more varied microbial community in the female adult compared to the male adult. Male DGGE bands are either weak or nonexistent, but female adult DGGE bands have three major bands. The clustering analysis profile of the DGGE is completely different between males and females as shown in Figure (3b). The results of PCR-DGGE showed that samples with the same condition at the same life stage are highly similar in larval gut bacterial structure and membership, while PCR-DGGE in adults showed communities differed between males and females as shown in Figure (3c).

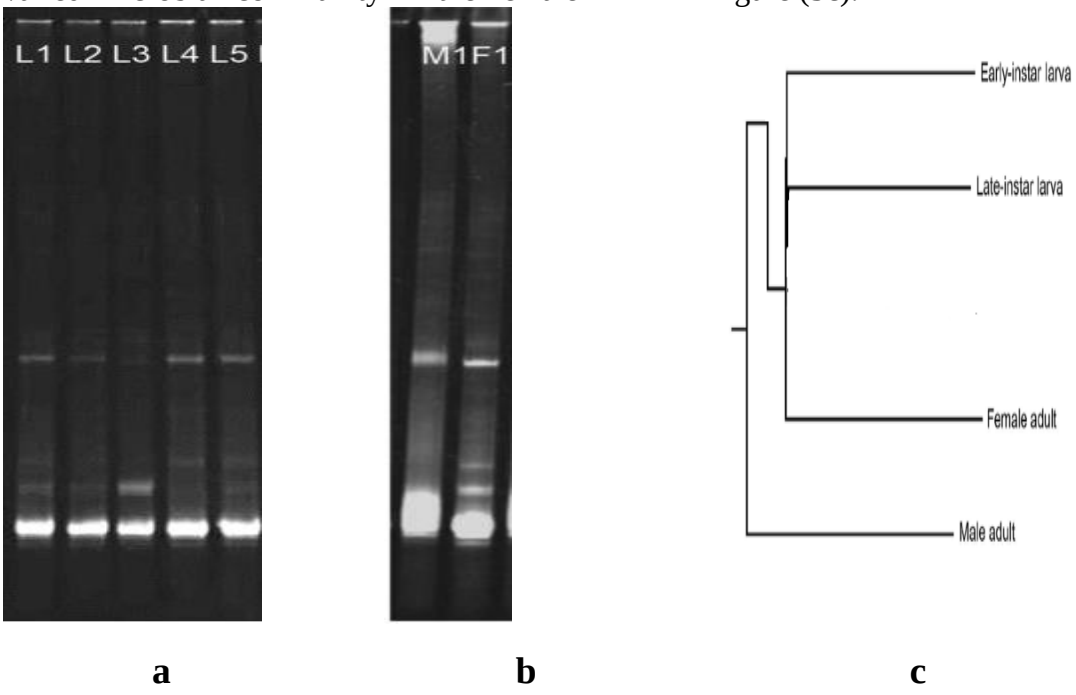

Figure (3a): DGGE profile of the mature larval gut microbiota of different individuals (L=larva).
Figure (3b): DGGE profile of the adult gut microbiota of M=Male adult and F=Female adult.
Figure (3c): Cluster analysis of the DGGE patterns of the male (M) and female (F) samples.

2. Larval microbiota:

Proteobacteria sequences made up about 38.9% of all the sequences in the early 2nd instar gut microbiota, which is a rather low abundance. Pantoea in the larval intestine is decreased by 23% (Figure 4). Another significant Proteobacterium closely related to Citrobacter made up 15.6% of the community. Both DNA and RNA data sets contained detections of these key phylotypes (Figure 4).

Despite having a low abundance in the DNA data set, Clostridium was one of the main members of the RNA-derived fraction, indicating that it has significant metabolic activity in the gut. As the larvae matured, it seemed that Proteobacteria were being replaced by Firmicutes.

With the growth of the host, the gut microbiota continually altered. The community of late-instar larvae had significantly lower species diversity than that of early-instar larvae. The stomach of the

larvae was a haven for the Firmicutes with 62.1% of all sequences, Enterococcus was the microbiota component that was the most stable (Figure 4). Similar to this, it was discovered that a larger percentage (35.4%) of the gut bacterial community belonged to the Clostridium genus.

Proteobacteria, on the other hand, were mostly eliminated from the larval gut microbiota; Pantoea, however, was present in both stages. The RNA-based data collection included a sizable portion of each dominant genus as well. Additionally, functional variations were found in bacterial populations related to various developmental phases.

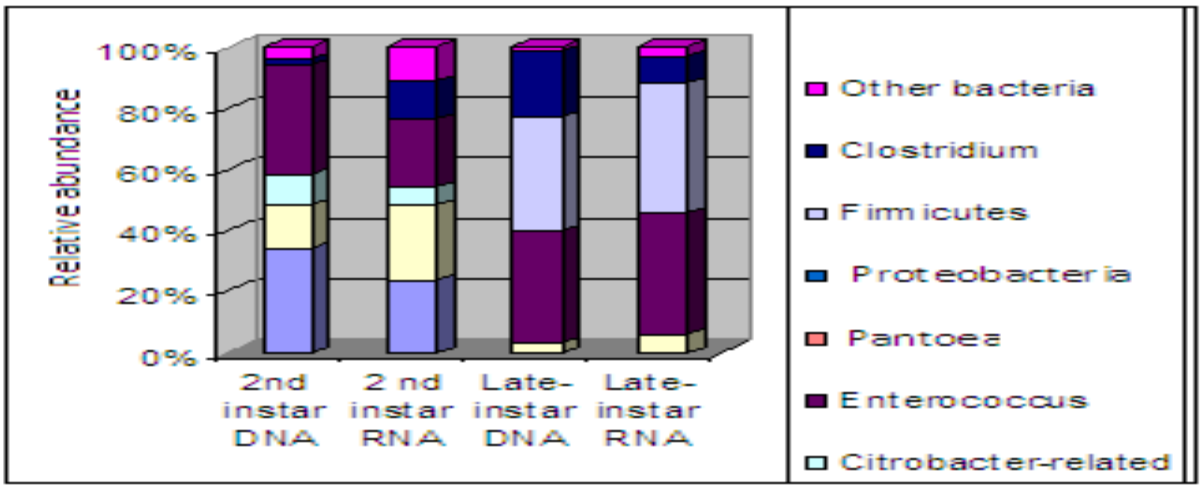

Figure (4): ***Spodoptera frugiperda*** **larval gut flora. Relative abundance of significant taxa (To genera level) in DNA and RNA data sets of early-instar (E-instar) and late-instar larvae (L-instar).**

3. Adult microbiota:

Even nevertheless, enterococci predominated in the bacterial population between larva and adulthood, despite an obvious structural difference between the two. Phylotypes from the Enterobacteriaceae bacterial family were more prevalent in adults than in larvae. Furthermore, the proportional fraction of the most prevalent bacteria that each adult's gut microbiota contained varied significantly between females and males (Figure 5).

Compared to male adults, the bacterial community in female adults was more diverse. Other Firmicutes, including Weissella, Pediococcus, Clostridium, and Lactobacillus spp., were also discovered in female adults in addition to Enterococcus, which accounted for a sizeable fraction (34.3%). Only a minor amount of Citrobacter was found, however, Pantoea made up 29.7% of all sequences and were likewise well-established in the gut flora. Another notable enterobacteria in the DNA data collection (23.8%) but absent from the RNA data set (4.0%) was the Gram-negative bacterium Klebsiella sp.

Male individuals had a much larger percentage of Enterobacteriaceae-related microorganisms than female adults did. Despite Pantoea's significant decline in the male gut flora, Klebsiella sp. was extremely prevalent, accounting for >88% of all sequences in the male sample, with the remainder being made up of Thermomonospora, Serratia, and Citrobacter.

The RNA data set revealed a similar picture, demonstrating that the majority of taxa were active inside the gut. In contrast to the females, Firmicutes, including Enterococcus, were kept at a very low level in the males.

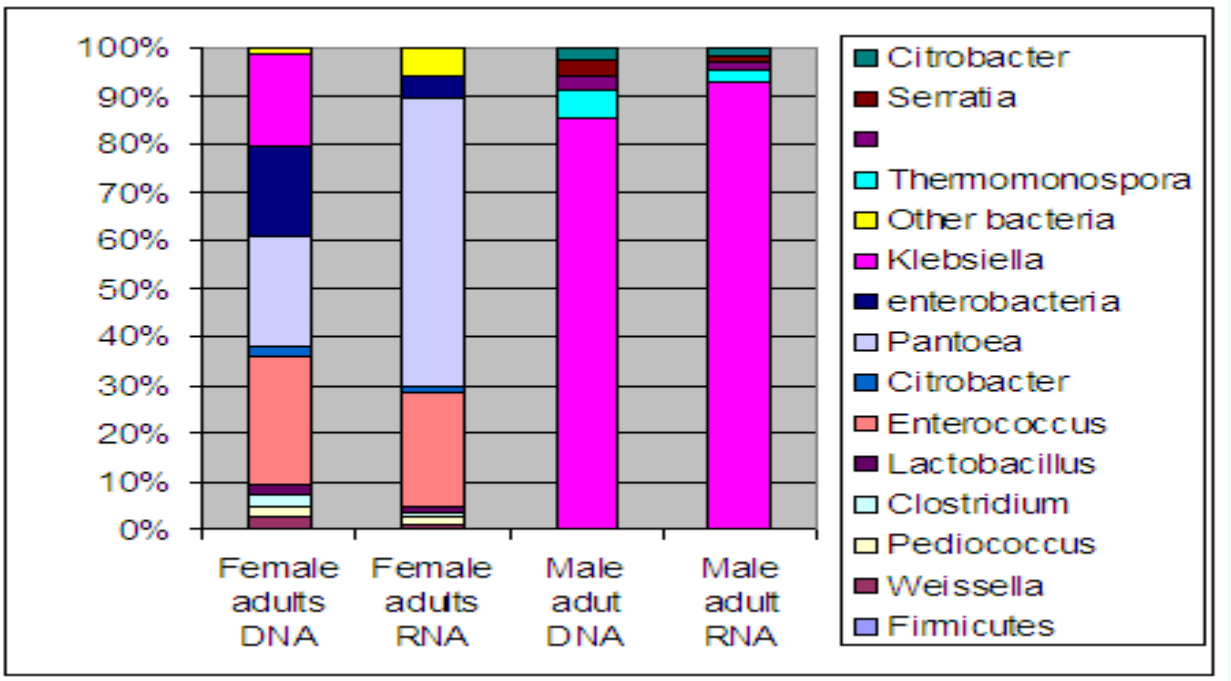

Figure (5): Relative abundance of significant taxa (To genera level) in the DNA and RNA data sets of male and female individuals.

In spite of the different studies on the bacterial community in the insect's gut, there are few studies comparing the metabolic activities of the bacterial community in the larval and adult stages. In this work, we investigated the bacterial community of *S. frugiperda* by using the 16S rRNA gene and their metabolic activity by evaluating the 16S rRNA gene, which gave us a new vision for the metabolic potentials of microbial communities.

The Operational Taxonomic Unit (OTU) showed microbial activities in the host gut were still active. This result agrees with a previous study on wood-feeding huhu beetles' (Prionoplus reticularis, Cerambycidae) gut flora (Reid *et al*., 2011) that showed many bacterial phylotypes are active (Reid *et al*., 2011).

Also, not all active bacteria are able to colonise inside the host. Furthermore, during the development of *S. frugiperda* there is significant reduction in bacterial diversity. The microbiota in wood-feeding termite guts or that of the beetle Odontotaenius disjunctus exhibits higher phylum-level diversity than *S. frugiperda* according to Warnecke *et al.* (2007) and Ceja-Navarro *et al.* (2013).

A few bacterial species belonging to the phyla Firmicutes and Proteobacteria were detected as low-richness species of the microbiota in *S. frugiperda*, which agrees with previous studies in other lepidopterans. For example, the tobacco hornworm, Manduca sexta which is mainly consists of phylotypes belonging to Enterococcus (Brinkmann *et al.*, 2008).

Also, the gypsy moth (*Lymantria dispar* L.) fed on different diets has a similar midgut bacterial community (Mason and Raffa, 2014). The biochemical and physiological conditions of an insect's digestive system, according to Funke *et al.* (2008), play an important role in the type of microbial community.

In lepidopteran larvae, the high PH (>10) could act as a determining factor for the microbial composition. Because Lepidoptera feeds on plant materials containing harmful microbes, it allows the host gut to control and eliminates invading microbes from its environment.

Due to Shao *et al.* (2014) mention that larvae maintain a strongly selective eco-environment for their microbiome, in the young larvae, the most common species are Pantoea and Citrobacter (Proteobacteria), which shift to Enterococcus and Clostridium (Firmicutes) in the mature larvae. Enterococci, in particular, is thought to be the most common bacterial species in Lepidoptera in both wild and laboratory-reared populations (Shao *et al*., 2014).

Enterococcin has been found in the tobacco hornworm (Lepidoptera: Sphingidae), gypsy moth (Lepidoptera: Erebidae), and velvetbean caterpillar (Lepidoptera: Noctuidae), indicating that this bacterium plays a conserved role in phytophagous insects. Pantoea has the ability to degrade and utilise different types of plant materials, which is helpful to herbivores.

In mature larvae, we detect only Clostridium at a dominant level. In late instar, the gut needs anaerobic microorganisms, such as Clostridia, and facultative anaerobic enterococci due to the prevailing anoxic atmosphere (Tang *et al*., 2012). This could be one of the factors driving the shift in the composition of gut microflora from protozoa to firmicutes. Suen *et al*. (2010) stated that there are microbiome genes responsible for the carbohydrate metabolism pathway.

Gammaproteobacteria in the Enterobacteriaceae family, for example, are responsible for the degradation of major structural components of plant materials. In addition, Pantoea spp. secretes special enzymes such as including β-galactosidases (GH2), α-xylosidases (GH31), α-mannosidases (GH47), and α-rhamnosidases (GH78), as well as pectinesterases (CE8) responsible for plant polymer degradation.

Citrobacter amalonaticus also plays an important role in the breakdown of chitin, demonstrating Gammaproteobacteria's metabolic role. This Proteobacteria plays the same function in *S. frugiperda* and in special in the early life stages for the insects. Other research indicates that firmicutes play a role in increasing the ability to harvest energy from the diet.

Fonknechten *et al.* (2010) mentioned that Clostridia species such as C. thermocellum and C. ljungdahlii are capable of degrading cellulose and hemicellulose and metabolising amino acids. Clostridia, which is widespread, is also important in biomass utilisation and nutrition.

The presence of enterococci in gypsy moth gut flora plays a role in preventing colonisation by pathogens (van Frankenhuyzen *et al.*, 2010). In this study, we found that the characteristic gut microbiota found in Spodoptera frugiperda larvae may provide various benefits to the lepidopteran host, ranging from nutrient supplementation to host defense.

Kohl *et al*. (2013) claimed that in Lepidoptera, the shift from the larval stage to the adult stage is a metabolically dynamic and complex process. The microbiota in the gut is undergoing structural changes. During metamorphosis, the microbiota in the gut undergoes a sterilization process and is renewed. It is interesting to find that Enterococcus species are able to survive the metamorphosis and be transmitted to emerging adults (Figures 4 and 5). This enterococcal population had high levels of genes involved in transcription and translation pathways, as well as genes involved in energy and nucleotide metabolism.

In the adult lepidopteran moth, the gut is small and morphologically distinct in contrast to that of the larva. The author found that *S. frugiperda* adults host a bacterial community that differs in both females and males (Figure 5). The female adult gut community contains a significant proportion of Firmicutes, mostly enterococci. While those bacteria still share little in common with male adults. On the other hand,

Klebsiella was found in male adults. According to Chu *et al.* (2013) both Pantoea and Klebsiella belong to the Proteobacteria family Enterobacteriaceae, which is found in Lepodoteria and herbivores, and are potentially beneficial, nonpathogenic microbes.

From previous studies, Arias-Cordero *et al.* (2012) showed that in holometabolous insect groups, adults' guts might have similar or different microbiota as larvae (Wong *et al.*, 2011) or have sexually dimorphic microbiota (Tang *et al.*, 2012). For example, the gut of the adult cockchafer beetle *Melolontha hippocastani* housed the same microbial species that were present in the larval midgut, despite having metamorphosed from larva to beetle (Arias-Cordero *et al.*, 2012).

In contrast, a developmental change in the most abundant gut bacteria was identified in the fruit fly *Drosophila melanogaster* (Wong *et al.*, 2011). In contrast, a developmental change in the most abundant gut bacteria was identified in the fruit fly Drosophila melanogaster (Wong *et al.*, 2011). Also, the bacterial composition of adult black flies *Simulium* spp. differed between males and females, although they were collected from the same habitat (Tang *et al.*, 2012). Similar to this, *Anopheles stephensi* adult males were found to have quite high levels of Klebsiella sp., whereas larvae and pupae were not (Rani *et al*., 2009).

Lately, a close relationship between bacteria and lepodopteran species has been revealed by comparative genomic and metagenomic studies. Thus, it is surprising that in Lepidoptera, the gene encoding the enzyme that detoxifies plant-produced cyanide did not evolve but was transferred from bacteria (Wybouw *et al.*, 2014). Obviously, microbiota and microbial activities play an important role in the host (microbe-host interactions).

Therefore, a better understanding of the relationship of microbial symbionts to the lepidopteran host would lead to new concepts and approaches to controlling insect pests by manipulating their microbiota. For example, Hakim *et al.* (2008) stated that *S. littoralis* is considered an important model to understand complex microbial symbioses due to its simple structure, microbial community, and genetic amenability. This work plays an important role in better understanding the ecological and evolutionary roles of gut symbioses in an important insect group.

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