University of São Paulo
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Modulation of rhizosphere - associated microbiota by insect pests: a holobiont relationship

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Thesis presented to obtain the degree of Doctor in Science. Area: Genetics and Plant Breeding

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I dedicate to my aunt Lucia (in memorian), who even without being understood during her lifetime taught us how to keep the mind alive and the heart warm.

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"See, in all our searching, the only thing we've found that makes the emptiness bearable, is each other." – Carl Sagan

SUMMARY

RESUMO

Modulação da microbiota - associada à rizosfera, por pragas de insetos: uma relação holobionte

Atualmente observamos um crescente número de pesquisas que buscam desvendar as causas, os efeitos e as possíveis utilizações biotecnológicas da modulação de comunidades da microbiota de rizosfera nas interações complexas entre plantas e solo. Sabemos também que o ataque de insetos herbívoros é um fator de considerável prejuízo para a agricultura e que tem relações evolutivas bem estabelecidas em sistemas naturais. O presente trabalho procurou testar algumas hipóteses a cerca da relação direta entre a microbiota de rizosfera e o ataque de insetos praga. Partindo do ponto de que plantas possuem mecanismos de defesa contra insetos, bem conhecidos, foi verificado que a microbiota de rizosfera parece contribuir ativamente para esse sistema, e assim estabelecer relações holobiontes. Tivemos um profundo acesso á comunidades do domínio bactéria e fungi, através da tecnologia de sequenciamento de nova geração para amplicons do gene RNAr 16S, região V3 e região intergênica ITS em amostras de solo, semi-solo e intestino de insetos praga (Ordem: *Lepidoptera*) de comportamento generalista. Nossos resultados, resultaram em três artigos aqui apresentados em capítulos. No primeiro capítulo é discutido o efeito modulador da herbívora da praga agrícola *Spodoptera frugiperda* na microbiota de rizosfera de *Arabidopsis thaliana* em diferentes estágios fisiológicos da planta. Como resultados foi possível perceber que o efeito na modulação da estrutura de comunidades de bactérias é diferente do efeito na modulação de comunidades de fungos após o ataque de insetos herbívoros. Os efeitos são diferentes tanto em abundância relativa quando na diversidade para cada um dos domínios de microrganismos estudados. No segundo capítulo destacamos a diferença na modulação da estrutura de comunidades de bactérias para diferentes famílias de plantas. Utilizamos mudas de *A. thaliana*, *Zea mays* Sh2, *Phaseolus vulgaris*, *Solanum lycopersicum* e *Beta vulgaris*, expostas ao ataque de *Trichoplusia ni* durante uma semana. As análises da microbiota de rizosfera de cada um dos grupos de plantas hospedeiras, sugere que a influência da espécie vegetal deve ser considerada na modulação das comunidades de bactérias da rizosfera após a herbívora. Adicionalmente, determinadas espécies de plantas podem ser menos susceptíveis a modulação da rizosfera pela herbívora. Outro destaque foi o efeito da modulação da microbiota de rizosfera, na perda de biomassa de plantas semeadas em semi-solo transplantado. Com base nos dados fenotípicos das diferentes espécies de plantas avaliadas, sugerimos que a modulação da microbiota de rizosfera após a herbívora, pode estar envolvida na inibição da produção de biomassa vegetal na geração seguinte de plântulas. Por fim, no terceiro capítulo exploramos a modulação na microbiota no intestino de larvas de *Trichoplusia ni* através da carga microbiana obtida na alimentação restrita. Larvas *T. ni* de mesma origem foram divididas em três populações. Cada população foi alimentada de forma específica e restrita com folhas de *A. thaliana* ou *S. lycopersicum* ou dieta artificial calórica. Acessamos a microbiota do intestino das larvas, após três gerações de alimentação restrita e verificamos que a microbiota intestinal em lagartas de comportamento generalista, pode ser alterada devido à obtenção de carga microbiana por via alimentar. Essa modulação pode estar relacionada a degradação de metabólitos que podem ser prejudiciais à homeostase dos insetos. A microbiota intestinal de cada população também pode influenciar diretamente as preferências alimentares de gerações sucessivas. Em resumo, todos os nossos resultados, apresentados em cada um dos capítulos a seguir, são chaves no conhecimento e podem ajudar a clarificar as complexas relações entre plantas, insetos e microrganismos. Contribuindo assim para um maior entendimento desse tipo de sistema holobionte.

Palavras-chave: Interação planta/inseto/microrganismo; rRNA 16S; ITS; NGS

ABSTRACT

Modulation of rhizosphere – associated microbiota by insect pest: a holobiont relationship

Currently, we observe a growing number of researches that seek to unravel the causes, effects and possible biotechnological uses of the rhizosphere microbiota communities modulation in the complex interactions between plants and soil. We also know that the attack of herbivorous insects is a factor of considerable damage to agriculture and that has well established evolutionary relationships in natural systems. The present work tried to test some hypotheses about the direct connection between the rhizosphere microbiota and the insect pest attack. Beginning from the point that plants have well-established defense mechanisms against insects, it was verified that the rhizosphere microbiota seems to contribute actively to this system and thus to establish holobionte relationships. We had broad access to communities of the fungi and bacterial domain, through the new generation sequencing for rRNA 16S gene, region V3, and intergenic region ITS amplicons on soil, semi-soil and, insect gut samples from pest insects with general behavior (Order: *Lepidoptera*). Our results from the data analysis to *Illumina Miseq* sequencing outputs and, additional experiments, resulted in three articles presented here in chapters. In the first chapter, we discuss the modulating effect from the pest insect attack (*Spodoptera frugiperda*), on the *Arabidopsis thaliana* microbiota rhizosphere, for different physiological plant's stages. As a result, it was possible to discuss the differences between the modulation in the structure of bacterial communities and the modulation in the structure of the fungal communities after the attack of herbivorous insects. In the second chapter, we highlight the difference in the modulation of the bacterial community structure for different plant families. We used seedlings of *Arabidopsis thaliana*, *Zea mays* Sh2, *Faseolus vulgaris*, *Solano lycopersicum* and, *Beta vulgaris* exposed to the attack of *Trichoplusia ni* for one week. The rhizosphere microbiota analysis for each host plant groups, suggests that the influence of the plant species should be considered on the bacteria rhizosphere communities modulation after the insect attack. Besides, specific plant species may be less susceptible to rhizosphere modulation by insect attack. Another highlight was the microbiota rhizosphere effect in the biomass loss for plants sown on transplanted semi-soil. Based on the phenotypic data, we suggest the rhizosphere microbiota modulation after the herbivore may be involved in the plant biomass inhibition on the next seedlings generation. Finally, in the third chapter, we explore the *Trichoplusia ni* gut microbiota modulation through the microbial load obtained in the restricted feeding. The *T. ni* larvae from the same original population were divided into three populations. Each population was fed individually and restrictively with leaves of *A. thaliana*, *S. lycopersicum* or artificial caloric diet. We accessed the gut microbiota in *T .ni* after three generations of restricted feeding, and we verified that the gut microbiota in caterpillars of general behavior, could be altered due the obtaining of microbial load through alimentary diet. This modulation may be related to the degradation of metabolites that may be harmful to insect homeostasis. The gut microbiota of each population can also directly influence the food preferences of successive generations. In summary, all our results presented in each one of the chapters are important points that can help to clarify the complex relationships between plants/insects/microorganisms and, contributing to a better understanding of this holobiont system.

Keywords: Interaction plant/insect/microorganism; rRNA 16S; ITS; NGS

1. INTRODUCTION

Plants and insects have coexisted in the most diverse types of relationships more than 400 million years ago (Sugio et al., 2014; Franco et al., 2017). Plants are continually evolving their mechanical or chemical defense processes against insects (Willsey et al., 2017; Hahn et al., 2019), while these invertebrates have to develop mechanisms for overcoming, resistance or increased tolerance in relation to strategies of plant defense (Ryam, 1990; Kessler & Baldwin, 2002; Huang et al., 2016). This coexistence can be compared to a continuous arms race (Mello & Silva-Filho, 2002; Sugio et al., 2014).

As an additional element within this context of interaction, plants and insects have relationships with microorganisms that may have resulted directly or indirectly in the interactions between each one (Cardosa et al., 2013; Babikova et al., 2014). The holobionts relationships between microorganisms, insects and plants are currently undergoing in-depth discussion (Hassani et al., 2018). These relationships are of mutual, bi- or tri-directed effect among their components. The holobiont relations have a high capacity for modulation and a direct impact within the evolutionary process and the plasticity of the system. The system as a whole evolves together, and all components have a participation in the action of cause or effect in the face the temporal changes of the system itself (Rosenberg et al., 2006).

Microorganisms can modulate the plant's primary and secondary metabolism (Reichling, 2010; Badri et al., 2013; Mussilova et al., 2016) and plant defense systems (Pieterse et al., 1996; 2012) against insects for the benefit of plants (Kang et al., 2015) or insects' benefit (Antoniou et al., 2017). Microorganisms can also alter insect biology, including behavior (Colman et al., 2012) and may influence plant-insect interactions (Franco et al., 2019). This set of microorganisms (bacteria, fungi, and viruses) that inhabit a particular environment is called microbiota (Schlaeppi & Bulgarelli, 2015). For understand these relationships more profoundly, our work was restricted to access to communities of fungi and bacteria. We focus on two main points which are the influence by the insect pest attack on the microbiota in the region of the soil highly influenced by the root, and the effect of the microbial load of the plants on the herbivorous insects' gut microbiota that act as an agricultural pest.

We maintained our focus based on the holobionts relationships between plants, herbivorous insects and microorganisms by accessing the soil microbiota in contact with the root, and the *Lepidoptera* insect pest gut microbiota. Concerning, the region where the soil and roots of the plants come into contact, that is, the portion of the soil directly influenced by the root system of the plants is called rhizosphere (Vandenkoornhuyse et al., 2015). The community of microorganisms in the rhizosphere is highly differentiated in quantity and community composition found in free soil (Marschner, 1994; Kent & Triplett, 2002). The soil residue

adhered to the root of a plant represents the portion of higher activity of the rhizosphere and is called a rhizoplane (Montiel & Aguilar, 2003).

In different plant species, the rhizosphere microbiota presents different structures, and the microorganisms can be distributed in varying amounts according to the genome and development of the plant (Chaparro et al., 2013). Several studies indicate that these differences in the rhizosphere microbiota for different host plants are directly related to the type and quantity of exudates released by each plant (Haichar et al., 2008, Chaparro et al., 2013; Badri et al., 2013). The root exudates may drive the differentiation of the soil biome in the rhizosphere and have host genotype-dependent factors (Badri & Vivanco, 2009; Sugio et al., 2014). In order to obtain an overview of these relationships, we have studied the rhizosphere microbiota of *A. thaliana* ecotype Col-0, and of different plant species such as Sweet Corn (*Zea mays* Sh2), Bean (*Phaseolus vulgaris*), Tomato (*Solano lycopersicum*), Red Beet (*Beta vulgaris*) after insect attack.

Insects of the order *Lepidoptera* present a variety of species with herbivorous characteristics that can cause enormous economic losses in a considerable number of cultivated plants (Lukhtanov, 2019). Currently, many studies have focused on associations between different insect's species and the bacterial diversity correlations (Colman et al., 2012; Jones et al., 2013), but we still require information from some important insects' herbivorous groups in a general context (e.g., *Lepidoptera*). The general overview emerging from this work, is that herbivorous insects harbor microbial communities of limited diversity, dominated by a few taxa (Colman et al., 2012, Jones et al., 2013).

Several studies analyzing the microbial communities of insects from different orders pointed out that bacterial species diversity is low because that both evolutionary history and insect feeding habits which contributed for its gut microbial diversity are restricted (Colman et al., 2012; Jones et al., 2013). However, it is still unknown for insect pests of the order Lepidoptera (e.g., *Spodoptera frungiperda* and *Trichoplusia ni*), whether the microbial load of different plants may influence insect gut microorganism community' structures. In this study, we used insect pests of the order *Lepidoptera* with generalist behavior so that we could test the same herbivorous model in plants of different species.

Current knowledge about some of the interactions between insects/plants/microorganisms, the specific roles of these microbial communities in the rhizosphere of plants and herbivorous insects remain mostly uncertain. Recently, the new generation of sequencing methods and molecular tools for detection of fragments or entire genomes of microorganisms that cannot be cultivated, are advancing our understanding of the influence of the microbiota on plant-insect interactions at the molecular level (Smalla et al., 1996; Kniff, 2014). Our studies about the rhizosphere microbiota of plants and insect gut allowed an advance in our current knowledge on plant/herbivory interactions that are influenced by the associated microorganisms.

The diversity, structure and abundance of bacterial and fungal communities in the host plant (*Arabidopsis thaliana*) rhizosphere and pest insect gut were characterized. In the first chapter, we aim to understand whether there is a significant modulation on rhizosphere microbiota after the attack of the generalist *Spodoptera frugiperda* (*Lepidoptera*) insect pest. In this study sought to verify possible differences in both bacterial and fungal communities after the insect attack. In addition, we seek to understand whether different physiological plant developmental stages (pre-flowering, flowering, and senescence), modulate the rhizosphere-associated microorganism composition by herbivorous insects.

In the second chapter, we compared the rhizosphere microbiota of different plant species after the *Trichoplusia ni* insect attack. It has been accepted that several natural systems can present convergence effects of their components when modulators by some factor inserted in the system. Thus, we tested the hypothesis that the rhizosphere microbiota of different host plants follows a convergence pattern for the communities of bacteria after the insect attack. Second, we checked whether the modulation of the rhizosphere microbiota by the insect attack might reflect some differences in the next plant's generation sown in the same soil that was used in the first experiment.

In the third chapter, we sought to understand the microbial load effect from different plant diets on the modulation of *T. ni* gut microbiota. We have also tested whether there is a difference in the gut microbiota of generalist insects if the population is restricted to one type of diet, for successive generations, and whether this effect is long-lasting. We hypothesized that the gut microbiome modulation could be related to the food preference behavior of the different insects' populations.

This complex network of interactions can be better understood through advances in new generations of sequencing, molecular techniques of genome extraction and amplification for microorganisms that cannot be grown. The increases in proper statistical methodologies also open new interesting perspectives in this field. In the present work, we address some of the open questions of the complex relationships among plants, insects, and microorganisms. The outcomes of this study may provide a scientific basis for the technological advancement of agricultural systems and food security.

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2. HERBIVORE INSECTS MODULATE RHIZOSPHERE - ASSOCIATED MICROBIOTA DIVERSITY IN DIFFERENT DEVELOPMENTAL STAGES OF *Arabidopsis thaliana*

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ABSTRACT

Plants have developed defense mechanisms over time to recognize insect pest attack and launch specific responses against the mechanical damage done by these consumers. Currently, the biological role of interactions between host plants, insects and microorganisms in defense responses has been discussed. Thus, the objective of this work was to test whether the *Spodoptera frugiperda* attack can modify the community structure and the abundance of microorganisms in different developmental stages of *Arabidopsis thaliana* rhizosphere microbiota. Our present study sought to understand: (1) a differentiation in the structure of fungal and bacterial communities of *Arabidopsis thaliana* rhizosphere after *Spodoptera frugiperda* attack, (2) alterations in the frequencies of microbiota communities in the rhizosphere after insect attack and (3) the role of plant developmental stages on the rhizosphere microbiota after the pest insect attack. These issues were addressed by methodologies that allow the extraction of DNA from cultivable and non-cultivable microorganisms, and molecular techniques such as *TRFLP* and *qPCR*. Our results demonstrate that there is a change in the structure of fungal and bacterial communities after the herbivorous insect attack. However, there is not a significant change in the abundance of the number of amplicon copies for bacterial and fungi. In addition, plant developmental stages appear to have a discriminating effect on the modulation of the rhizosphere microbiota after herbivorous insect attack. The initial discussion of these effects on the modulation of the rhizosphere microbiota in a model plant opens new perspectives of strategies aimed at increasing the resistance of cultivated plants to pest insects and consequently, improving food productivity.

Keywords: Plant-insect-microorganism interactions; Rhizosphere; Microbiota; 16S rRNA; ITS; *Spodoptera frugiperda*

2.1 INTRODUCTION

Plants have different features in the recognition and resistance to pest insect attack (Franco et al., 2017; War et al., 2012). Plants developed a wide range of defense systems to recognize insect pest attack and to launch specific responses against mechanical damage (War et al., 2012). The elicitors contained in the saliva of the chewing insects allow the plants to differentiate between a general mechanical injury and insect feeding action (War et al., 2011). In response, plants release volatile organic compounds called “VOCs”, that may attract natural predators from pest insects and thus are beneficial to plants (Arimura et al., 2009; Hare, 2011, War et al., 2011), or may repel insect pests (Dudareva et al., 2006; Arimura et al., 2009).

The action that insects perform when feeding on only part of the plant tissue may be susceptible to induce the systemic production of VOCs (Dudareva et al., 2006; Arimura et al., 2009), even in undamaged parts of the plant tissue (Johnson & Dowd, 2004). The VOCs, when released, can act on neighboring plants, triggering the production of similar defense compounds (Karban, 2011). On the other hand, the production of these compounds requires a high metabolic cost of the host plant (Agrawal et al., 2011, Duffey & Stout, 1996), which may not be strategic when plants are not under attack by insect pests (Karban, 2011).

Consumption of a small area of plant tissue by herbivore insects can induce systemic production of these chemicals in whole plant parts (Johnson & Dowd, 2004), which can act in

different parts of the plant systemically (Bruin & Dick, 2001; Preston et al., 2001). The production of these chemicals requires a high metabolic cost to the host plant (Agrawal et al., 2011, Duffey & Stout, 1996), so many of these compounds are not produced in large quantities until the insects have begun to start feeding (Herms & Mattson, 1992).

The involvement of the root system on a specific response to herbivore insects is still not fully understood, although it has long been established the function of the roots on nutrient and water uptake. Plant roots have been shown to communicate with the roots of neighboring plants (Bruin et al., 1995; Bruin & Dick, 2001; Preston et al., 2001). It is known that organisms in the rhizosphere, which is the region where the soil and roots have a strong interaction, may play an essential role in this communication system (Abrahim et al., 2000, Akiyama 2005).

Secondary metabolites and volatile organic compounds secreted by the roots have been shown to play an important role in plant defense to pathogens or symbiosis. This association takes place in the portion of soil strongly influenced by the roots, known as rhizosphere (Anaya et al., 2011; Pelayo-Benavides, 1997; Bakker et al., 2013).

The rhizosphere microbiota has been the target of studies directed to the understanding of the pathogenic or mutualistic interactions that result in effects in plant fitness (Nihorimbere at al., 2011). In addition to known beneficial and pathogenic fungi and bacteria, the rhizosphere microbiota harbors several broad ranges of the less-characterized group of microorganisms that may have a direct relationship on nutrient uptake, plant development stages and indirect protection against other pathogens (Chaparro et al., 2013).

It has been shown, in *Arabidopsis thaliana*, that roots can secrete over a hundred proteins constitutively in the different stages of plant development (De-La-Peña et al. 2010; Chaparro et al., 2013). It is interesting to note that proteins related to plant defense systems, such as chitinases, glucanases, myosinases, among others, have increased secretion during flowering, which demands the metabolic restriction of plant resistance for the maintenance of homeostasis (Chaparro et al. 2013).

Proteomic studies have also been shown to be positively correlated with enzyme activity assays for defense proteins and *in situ* analysis of genes specifically expressed on *Arabidopsis* roots (Badri et al., 2009). The sum of these findings shows a clear correlation between the defense proteins secreted by the roots and the physiological stage of the plant (Chaparro et al., 2013). Thus, changes in exudation according to the plant developmental stage modulate the composition of the rhizosphere microbiota community, such that the microbes most beneficial to the balanced homeostasis of the plant appear to be recruited (Chaparro et al., 2013). Thus, rhizosphere microbiota communities are affected by plant age and genotype (Micallef et al., 2009).

Studies on the biological functions of the root and the functional capacity of the rhizosphere microbiota are still unclear. Our understanding of the structure established between fungal and bacterial communities under different biotic stress conditions of the plant remains limited, specially related to plant development and the defense mechanisms triggered by insect attack.

Although many reports show changes in leaf and root protein profile in response to injury (Kendziorski et al., 2005; De-la-Peña et al., 2010) and fungal infection (Lundberg et al., 2012), a small number of studies have addressed the biological functions of the rhizosphere microbiota after insect pest attack, limiting to bacterial communities (Berendsen et al., 2012; Bakker et al., 2013, Badri et al., 2013, Hubbard et al., 2018).

The present work seeks to elucidate how the rhizosphere microbiota of *Arabidopsis thaliana* undergo changes after the attack of the caterpillar *Spodoptera frugiperda*, a generalist pest, which causes significant damage in a variety of crop species (Páre et al., 1998). *Spodoptera frugiperda* larvae can damage plant tissues extensively, appearing the symptom of "*scraping*" (Páre et al., 1998). These caterpillars can be found feeding on new plants, causing damage such as destruction or weakening of the plant, cutting off stalks at ground level, defoliation of developed plants and damage to flowers. Although there is a significant amount of information about on plant genes related to insect defense (Milligan et al., 1998), little is known whether the same genetic defense systemic effect may control the rhizosphere microbiota and how different plant developmental stages modulate this response.

The understanding of this tripartite relationship (host plant, pest insect, rhizosphere microorganisms) may help us to reveal new strategies to promote an increasing resistance of cultivated plants and consequently increase food productivity.

2.2 MATERIAL AND METHODS

2.2.1 *Arabidopsis thaliana* COL-0 growth conditions

Arabidopsis thaliana COL-0 WT (Columbia) seeds originated from self-fertilization multiplications were used to maintain the seed bank of the Laboratory of Molecular Biology of Plants of the Department of Genetics ESALQ/USP. Seed bank seeds were purchased from *Lehle* (Round Rock, TX). The individual ecotype COL-0 WT (Columbia) plants were sown and separated into three large experimental groups according to the physiological stage of the plant: pre-flowering (15 days after sowing), flowering (25 days after sowing) and post-flowering stage (35 days after sowing).

These groups were subdivided into (1) subgroup "inst": test group with herbivory attack for 48h (n=10), (2) subgroup "mec": positive control with mechanical injury (n=10) (wound on sheet run with sterile scissors), (3) Subgroup "crt": negative control (without mechanical injury or insect attack) (n=10). All groups were maintained under the same experimental conditions, in pots of 400ml. The *Arabidopsis thaliana* seeds were sterilized in a mixture of sodium hypochlorite (2.5%) and washed three times with sterile water. The sterilized seeds were seeded directly into a non - sterile mixture of vermiculite by *Plantmix-Trimix*® and Peat moss by *Basaplant Florestal*® (1:1).

The plants were grown in a growth chamber (*Fitotron*®), under controlled environmental conditions: temperature of 25 °C ± 1 °C, photoperiod of 16 h light and light intensity of about 35 m/s^2. The experiments were performed using a completely randomized design (CRD).

2.2.2 The herbivory promoted by *Spodoptera frugiperda* (J. E. Smith)

Third instar caterpillars of *Spodoptera frugiperda* (*Lepidoptera: Noctuidae*) were used in the laboratory under controlled conditions. The larvae of *S. frugiperda* were supplied by the Laboratory of Insect Biology, Department of Entomology and Acarology, Luiz de Queiroz College of Agriculture, University of São Paulo, Piracicaba, SP. The photoperiod used to create the larvae was from 14 h from clear to 10 h from dark at 25 ± 1 ° C and 60 ± 10% relative humidity. Five larvae of *S. frugiperda* were used per plant. The larvae were placed on the leaves of *Arabidopsis* for two days (48h) in the "ins" subgroup for each plant stage tested. After the experimental process, the larvae were discarded adequate.

2.2.3 Collection of rhizosphere samples

The *A. thaliana* rhizosphere samples were collected at the end of two days (48h) in the control group (crt), mechanical injury group (mec) and the group that had the insect attack(inst).

The experiment started (time t0) according to the day when the plants reached each one of the physiological stages: pre-flowering (15 days after sowing), flowering (25 days after sowing) and post-flowering stage (35 days after sowing).

Samples of A. thaliana rhizosphere were collected with sterile tweezers. The samples collected correspond to the soil adhered in A. thaliana roots. Only the soil within a radius of +/- 2 mm around the roots was collected. The soil samples collected were packed in sterile 15 mL Falcon® tubes, frozen in liquid nitrogen and subsequent -80 freezer until the total DNA extraction.

2.2.4 Total DNA extraction from *Arabidopsis thaliana* rhizosphere samples

Ten replicates were used for each experimental group for the extraction of total DNA from the *A. thaliana* rhizosphere. Each sample was composed of 400mg rhizosphere soil. The total DNA was extracted using the *MoBio Laboratories* kit (*DNA* PowerSoil®) according to the manufacturer's instructions. The DNA concentration was measured by a Nanodrop® spectrophotometer (*Thermo Fisher Scientific*). The extracted DNA was stored at -20°C until the sample amplification.

2.2.5 Amplification by *PCR* and *T-RFLP* technique

We performed T-RFLP experiments to verify spatial modulations on the structure of fungi and bacterial communities. We assessed the range of diversity detectable by T-RFLP analysis in cultivation-independent approaches by 16S rRNA gene and ITS region amplicons.

T-RFLP was performed to verify differences in the bacterial and fungal community structures in the different experimental groups studied. The total DNA extracted from *A. thaliana* rhizosphere samples was amplified by PCR (polymerase chain reaction). The primers 8FM-6FAM (AGAGTTTGATCMTGGCTCAG) and 926r (CCGTCAATTCCTTTRAGTTT) were used to amplify the genomic DNA of the bacterial domain, targeting the conserved sequence of the 16S rRNA gene; and the primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) were used to the amplification of genomic DNA to fungal from the ITS (Internal Transcribed Spacer) sequence.

For bacterial analysis, the amplification reaction was performed as follows: 95°C for 4 minutes, 95°C for 30 seconds; 57°C for 30 seconds, 72°C for 45 seconds, 72°C for 10 minutes, and the second, third and fourth steps were repeated 30 times and finally 10°C for tempo time. The PCR reaction was performed in a total volume of 50 µl containing: 33.1 ultrapure H_2O (milli-Q), 5 µl Buffer, 6 µl MgCl, 4 µl dNTP, 0.1 µl to each primer (10 µM), 0.5 µl BSA (10%), 0.2 µl Taq Polymerase and finally 10 µg from DNA template.

The reaction for ITS amplicon amplification from total DNA extracted was defined under the following conditions: 95°C for 10 minutes, 94°C for 20 seconds; 55°C for 30 seconds, 72°C for 40 seconds, 72°C for 7 minutes, and the second, third and fourth steps were repeated 35 times and finally 10°C for infinite time. The PCR reaction was performed in a total volume of 50 µl containing: 32.6 ultrapure H_2O (Milli-Q), 5 µl Buffer, 5 µl MgCl, 4 µl dNTP, 0.2 µl to each primer (10 µM), 0.5 µl BSA (10%), 0.5 µl Taq Polymerase and 10 µg DNA extracted from each sample.

After amplification, the amplicons followed the restriction step. Restriction enzymes, *HhaI* (*Thermo Scientific*® 2,000 U) and *BsuRI (HAEIII)* (*Thermo Scientific*® 3,000 U), were used to cut the 16S rRNA and ITS PCR product respectively. The PCR products after the restriction step followed to the purification and precipitation steps as optimized by Moeseneder et al. (1999) for the TRFLP technique. Finally, samples with restricted amplicons for the bacterial domain and fungal amplicons were analyzed in a sequencer ABI PRISM® 3500 Genetic Analyzer - Applied Biosystems®. The peak areas of the electropherogram were statistically analyzed through the "Principal Coordinate Analysis" (PCoA), based on the dissimilarity of the Bray-Curtis index and, the analysis of similarities (ANOSIM) using the *PAST* free software.

2.2.6 Quantitative *PCR* (*qPCR*) analyses

The abundance of copies of the 16S rRNA gene (g $soil^{-1}$) was determined using quantitative PCR (qPCR). The total DNA samples extracted as reported above were amplified by the universal primers ITS1 (CTTGGTCATTTAGAGGAAGTAA) and 5.8s (CGCTGCGTTCTTCATCG) for fungi, P1 (CCTACG GGAGGCAGCAG) and P2 / Eub518R (ATTACCGCGGCTGCTGG) to the bacterial domain. Quantitative PCR was performed with the *Step One TM Real*-time PCR systems - *Applied Biosystems* and the Maxima® SYBR Green / ROX qPCR Master Mix reagent (2X) was used 3µL of the extracted total DNA sample. All reactions were performed in triplicates.

The reaction for amplification and quantification of 16S rRNA gene molecules was performed under the following conditions: 95 °C for 3 minutes, 94 °C for 30 seconds; 55°C for 30 seconds, 72°C for 30 seconds, and these last three steps were repeated 35 times and finally 72°C for 10 seconds, 94°C for 10 seconds, + 5°C for 10 seconds, to obtain the Melt curve. The PCR reaction was performed in a total volume of 25µl containing: 12.5µl SYBR Green/ROX, 0.5µl from each of the primer (10µM), 10.5µl Milli-Q© water to make up the volume, and 1µl of DNA (sample obtained from each dilution).

Likewise, following the specificity for the reaction for gene amplification with the universal fungi primers the reaction was carried out under the following conditions: 50 °C for 2 minutes, 95 °C for 2 minutes; 95°C for 15 seconds, 60°C for 1 minute, and these last two steps were repeated 44 times and finally 50 °C for 10 seconds, 95 °C for 10 seconds, + 5 °C for 10 seconds, to obtain the melting curve. The PCR reaction will be performed in a total volume of 25 µl containing: 12.5 µl of SYBR Green / ROX, 0.2 µl of each primer (10 µM), 11.1 µl of Milli-Q© water to make up the volume and 1µl of DNA (sample obtained from each dilution).

Amplification specificity was confirmed by melting curve analyses obtained from serial dilutions (10^{-2} to 10^{-8} gene copies μL^{-1}). Consistency in *Ct* values and consequently in quantification values validated our approach. Amplification reaction efficiency was obtained with R^2 values higher than 0.98 for all calibration curves.

Standard curves were generated with serial dilutions of the DNA from the extracted samples as described above. The amplification efficiency for all runs and also, the absolute quantification of DNA in the number of molecules, were provided by the Applied Biosystems *StepOneTM* Systems program.

The one-way ANOVA and the Tukey's test to means comparison test ($p < 0.05$) were performed using the free software *PAST* to verify the differences in the mean of molecules number obtained per gram of soil in each sample.

2.3 RESULTS AND DISCUSSION

2.3.1 *Spodoptera frugiperda* attack on *Arabidopsis thaliana* leaves

We performed experiments with *A. thaliana* seedlings at different developmental stages. The attack of *S. frugiperda* was considerable after 48 hours on *A. thaliana* leaves for all plant developmental stages (Figure 1).

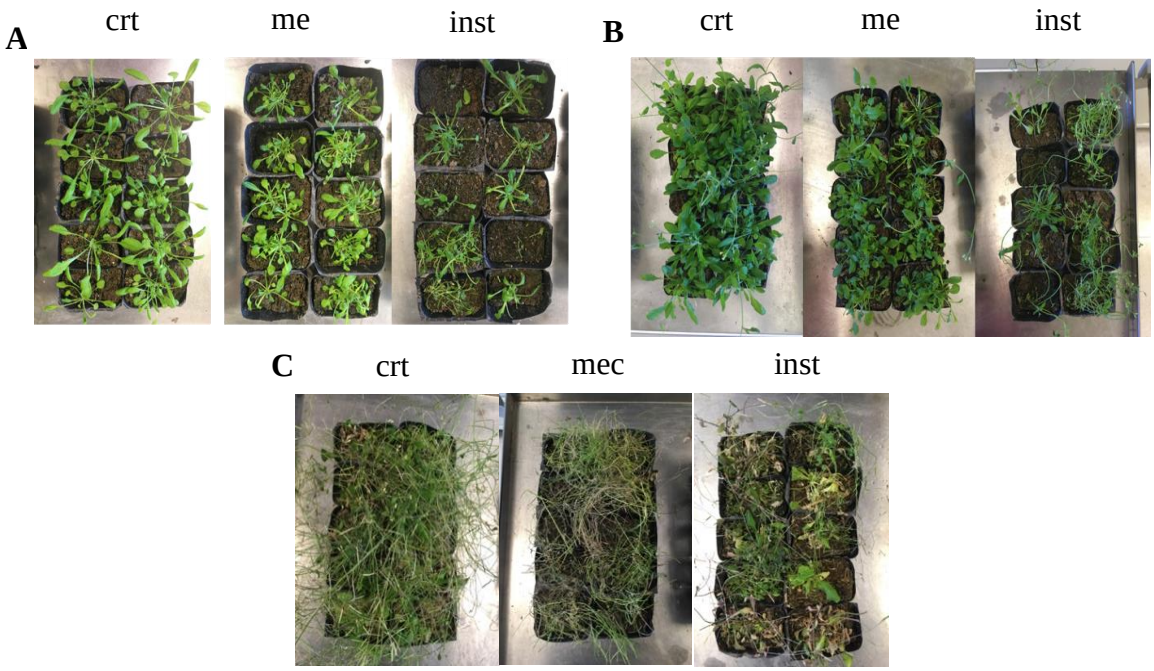

FIGURE 1: *Arabidopsis thaliana* seedlings in different experimental subgroups ctr (Control), mec (Mechanical Injury), inst (48h after insect attack). In figure (A) Pre-flowering plant stage; (B) the Flowering plant stage; (C) the Post-flowering plant stage.

2.3.2 Microbiota communities structures modulation, and microbial communities abundancy on *Arabidopsis thaliana* rhizosphere after *Spodoptera frugiperda* attack

The bacterial communities detected by the TRFLP technique revealed a modulation in the spatial structure. Most of the experimental treatments, control (crt), mechanical injury (mec) and insect attack (inst) are grouped in different quadrants in the PCoA (figure 2). Additionally, the grouping of the three experimental groups also seems to have a modulation in its temporal structure, which can be related to different stages of the Arabidopsis life cycle. As shown in figure 2a, 2b and 2c, the samples from the insect attack group seem to present a higher similarity according to the stage of pre-flowering, flowering stage, ending at the stage of post-flowering of the plant. In the post-flowering plant stage, rhizosphere microbiota samples from insect attack groups appear to be structurally more similar than in the previous plant life stage.

Figure 2 shows that for the bacterial domain in all plant stages the group attacked by insects shows a different rhizosphere microbiome pattern in comparison to mechanical injury and control plant groups. We considered that the changes in microbiota modulation are a result of plant defense mechanisms after insect damage. A large number of reports show changes in leaf- and root-protein profiles in response to mechanical injury (Kendziorski et al., 2005; De-la-Peña et al., 2010) and fungal infection (Lundberg et al., 2012). More recently, few studies have suggested a microbial influence on plant tissue chemistry, which in turn is related to inhibition of insect feeding (Badri et al., 2013, Zhou et al., 2018).

However, the direct influence of insects on the whole rhizosphere microbiota is in large part unknown. There are few indications that a change in the rhizosphere community structure is possible due to herbivory in the plant aerial part since it has been shown that herbivores above ground can reduce the insect attack on the ground (Van Dam & Heil, 2011). This observation is supported by our studies which suggest a relationship between components in the soil and the components in the plant aerial parts.

The control and mechanical injury groups are similar to each other when compared to the insect attack group. It is interesting to note that the analysis of PCoA for the bacterial domain, in the stage of the flowering plant (figure 2b), a greater distance between the control and mechanical injury group when compared with the PCoA analyzes for the same groups in the other stages of life of the plant (figure 2a, 2c).

This effect may be due to a more pronounced effect of specific defense proteins in the flowering phase of the plant (Dela-Penã et al., 2010).

PCoA studies from fragments detected for the ITS showed modulation in the structure of fungal communities present in the rhizosphere microbiota of *A. thaliana* when attacked by insects (figure 3). In the developmental stages of pre-flowering and flowering, a lower similarity between the group attacked by insects, control and mechanical injury groups was observed. This difference in the structure of rhizosphere communities attacked by insects can be stimulated by the different proteins that are released as exudates in each phase of the plant (Chaparro et al., 2013, Dela-Penã et al., 2010).

The presence of fungi in the rhizosphere of plants is also strongly influenced by the plant developmental stage. This observation can be explained by differences in its metabolome and number of debris cells in the rhizosphere (Fesel & Zuccaro, 2016). Root exudates and cell debris enrich the soil substrate, making it a suitable habitat for fast-growing organisms. Species-specific and even genotype-specific microbial communities are assembled in response to plant exudates (Broeckling et al., 2008; Sloan & Lebeis, 2015). Moreover, rhizosphere microbiome may be modulated by plant health (Panke-Buisseet et al., 2015). Beneficial and detrimental effects on plant health have been observed, indicating a fungal function along a parasitic to a mutualistic life strategy spectrum (Junker et al., 2012; Fesel & Zuccaro, 2016).

Summarizing, the first part of the study, showed here, supports the initial discussion about the influences from insect attack on the rhizosphere modulation in *A. thaliana*. There is a modulation on the fungal and bacterial communities after the action of the insect disturbing on the plant leaves. However, the structure of the communities is not equivalent in all phases of the plant.

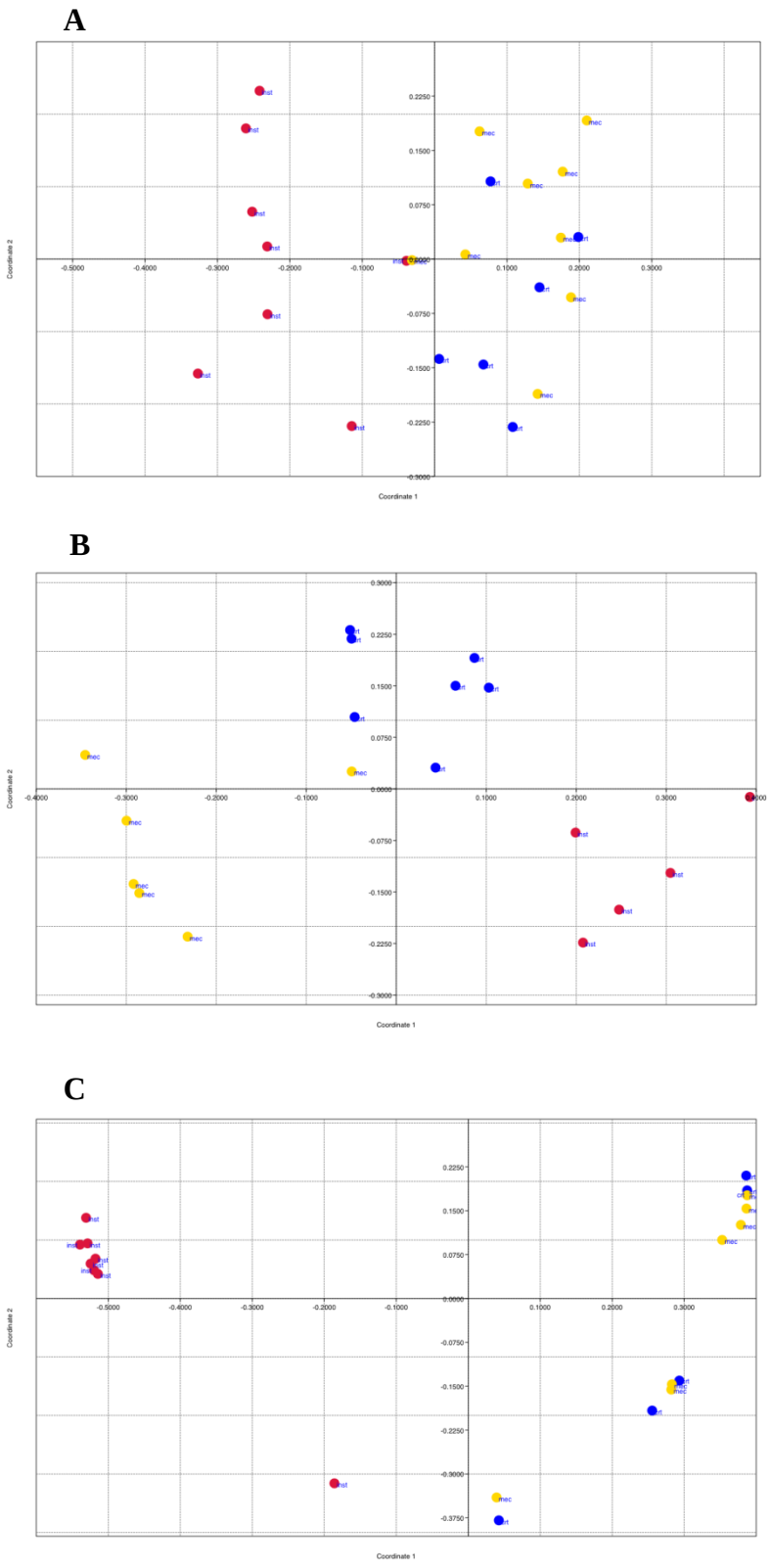

FIGURE 2: PCoA based in Bray-Curtis index dissimilarity to fragments generated by T-RFLP. Structure of bacterial domain communities tagged by primers that amplify to the conserved 16S rRNA gene. The samples are from *Arabidopsis thaliana* rhizosphere to (A) pre-flowering plant stage (17 days); (B) flowering plant stage (28 days); (C) post flowering plant stage (35 days). The red dots correspond to the subgroup insect attack after 48h (inst); the dots in yellow correspond to the subgroup mechanical injury (mec) and the dots in blue correspond to the control subgroup (crt). ANOSIM ($p<0.05$).

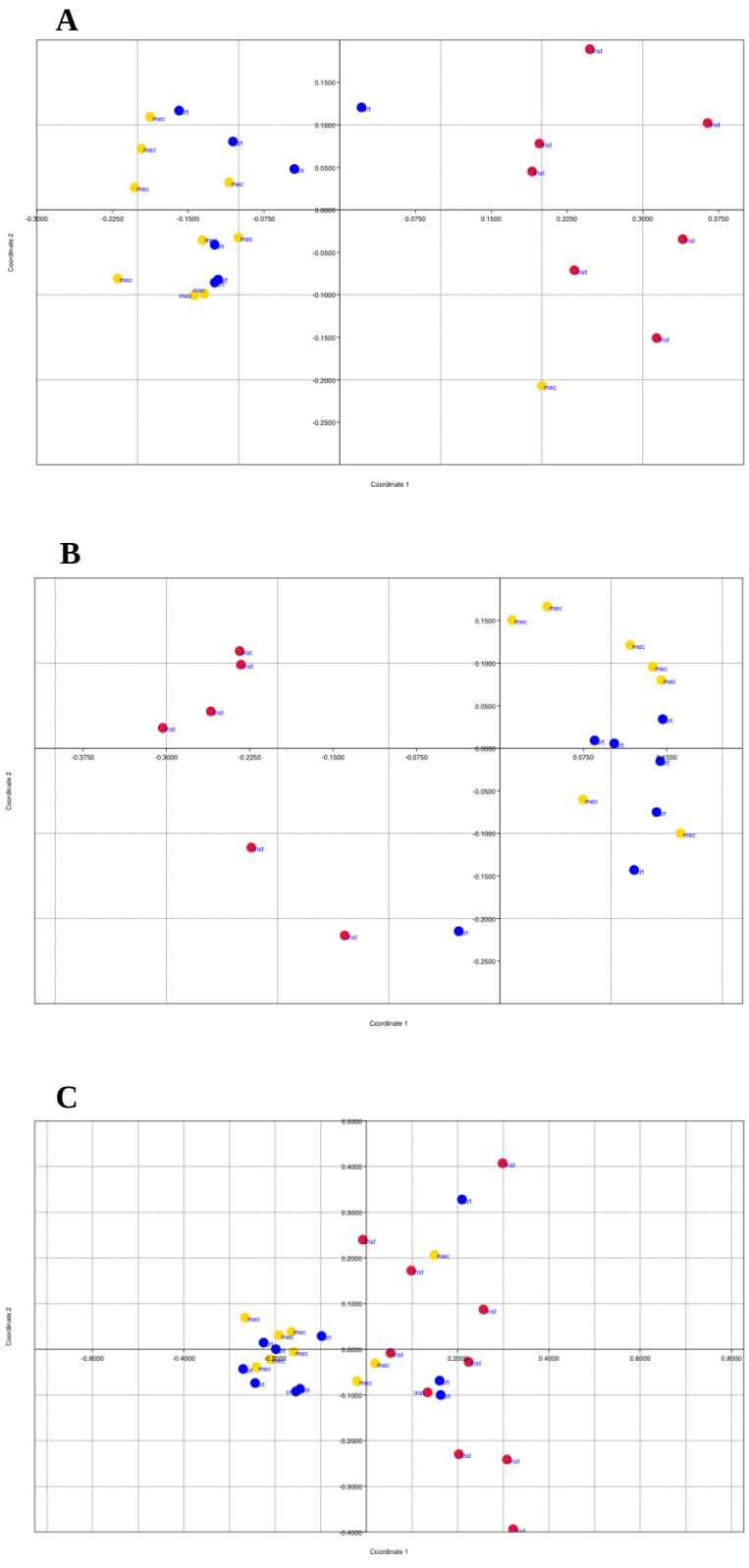

FIGURE 3: PCoA based in Bray-Curtis index dissimilarity to fragments generated by T-RFLP. Structure of fungal kindon communities, tagged by primers that amplify to the conserved ITS region from 45S rRNA gene. The samples are from *Arabidopsis thaliana* rhizosphere to (A) pre-flowering plant stage (17 days); (B) flowering plant stage (28 days); (C) post flowering plant stage (35 days). The red dots correspond to the subgroup insect attack after 48h (inst); the dots in yellow correspond to the subgroup mechanical injury (mec) and the dots in blue correspond to the control subgroup (crt). ANOSIM (P<0,05).

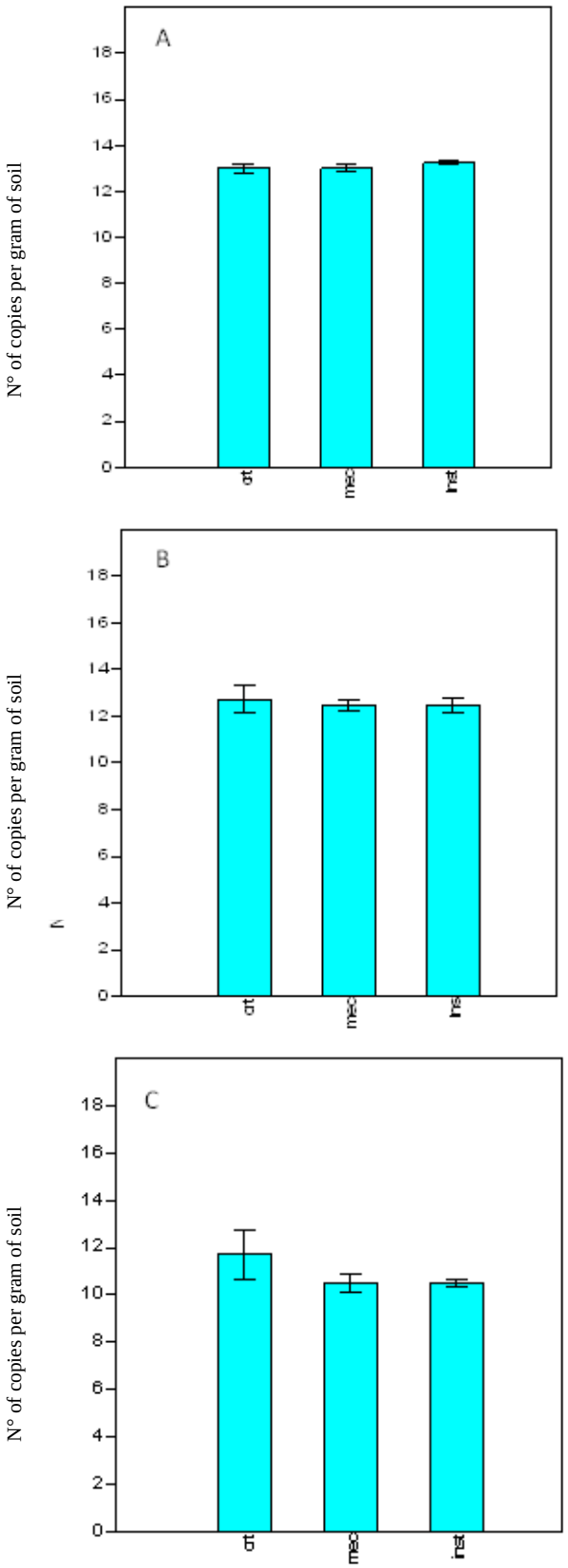

FIGURE 4: Abundance (n=10) of the16S rRNA gene. (A) Pre-Flowering plant stage; (B) Flowering plant stage; (C) Post Flowering plant stage. The bar on the chart is the mean standard error with 95% interval.

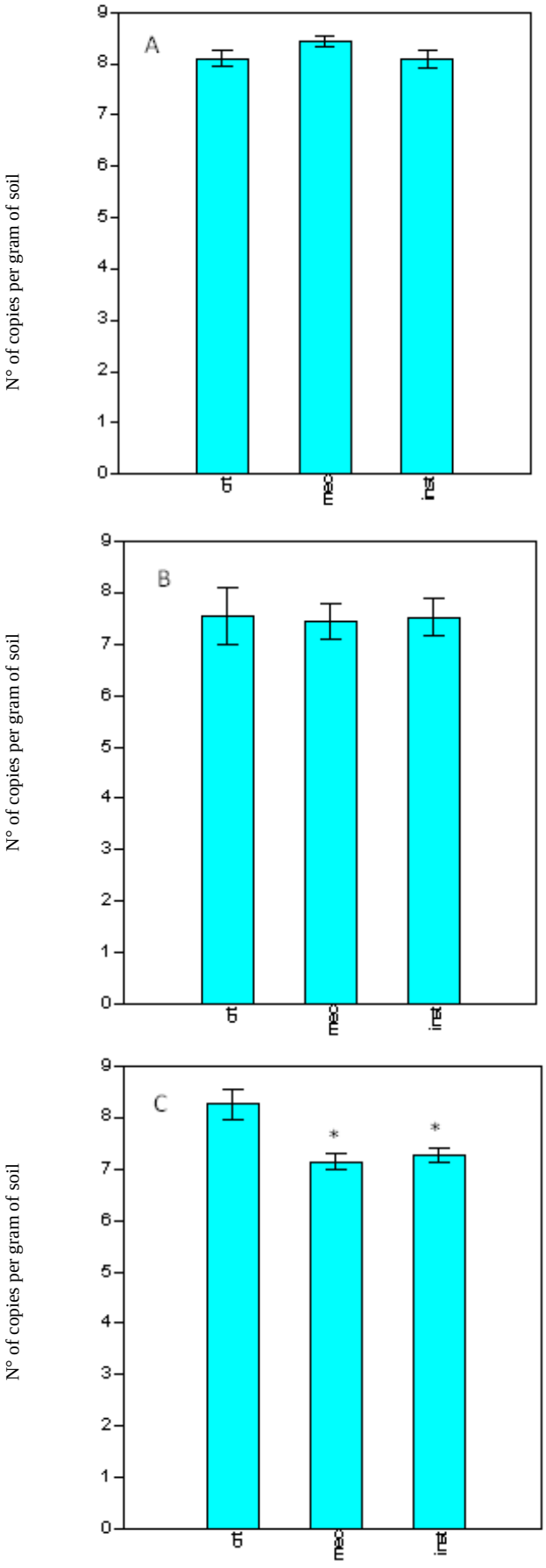

FIGURE 5: Abundance (n=10) of the Intergenic ITS region among the 18S and 5.8S rRNA gene. (A) Pre-Flowering plant stage; (B) Flowering plant stage; (C) Post Flowering plant stage. The bar on the chart is the mean standard error with 95% interval. (*) significant to $p < 0.05$.

2.4 CONCLUSION

Spodoptera frugiperda damage on distinct *Arabidopsis thaliana* developmental stages promotes a distinct pattern of microbiota on the bacterial and fungal communities in the rhizosphere.

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CONFLICT OF INTEREST

We have no declaration of conflict of interest.

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3 THE HERBIVORE INSECT MODULATES RHIZOSPHERE MICROBIOTA IN A HOST-PLANT DEPENDENT MANNER AND AFFECTS SUBSEQUENT PLANT PERFORMANCES

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ABSTRACT

The rhizosphere is a region where the soil and plant's roots come into contact. It is known that the rhizosphere system structures may be affected by abiotic and biotic factors. In this context, our investigation sought to understand the complex relationship between different host plants, insect pests and the rhizosphere microbiota. In the present study, the rhizosphere bacterial composition on different host plants was challenged by *the Trichoplusia ni* attack. The rhizosphere microbiota was evaluated not only in distinct plant populations, but also on subsequent plant generations. We have shown that the *T. ni* attack differently alters the rhizosphere microbiota structure depending on the host plant species. In addition, we also observed that genus-specific bacteria were recruited in the rhizosphere microbiota after insect attack. Finally, the rhizosphere microbiota modulation by insect attack may have adverse effects on the plant's biomass in the subsequent generation.

Keywords: 16S rRNA sequencing; Holobionts; Insect herbivorous; Soil memory; Interaction plant-insect-microorganisms

3.1 INTRODUCTION

It is known that mechanisms in plants against insect pest are directly related to specific genes of host plants that activate pathways of systemic resistance (War et al., 2012), local (Franco et al., 2019) or production of toxic chemicals such as terpenoids, alkaloids, anthocyanins, phenols and quinones that kill or delay the development of herbivores (War et al., 2012). In the other hand, indirect defenses against insects are mediated by the release of a blend of volatiles that attracts explicitly natural enemies of the herbivores and by providing nutrition to enhance the effectiveness of the natural enemies. In this second context, the relation between host plants, insects and microorganism have been studied in the recent science moment (War et al., 2012; Medeiros et al., 2012; Franco et al., 2014, 2019).

In the last years, several studies attempted to understand the complex interaction between microorganisms, host plants and insects (Tesla et al., 2017; Kang et al., 2018; Medeiros et al., 2012; Franco et al., 2014; 2019). It has been demonstrated that insects-host plants and microorganism interactions can critically affect the behavior, physiology and fitness of each player involved (Badri et al., 2013; Franco et al., 2017; 2019; Kang et al., 2018). The authors suggest that the evolution and ecology in host plants, insects and microorganisms, may only be understood in a holobiont context (Hassani et al., 2018; Haag, 2018). In summary, a holobiont system can be understood as a complex system between a host plant and its surrounding organisms.

The characteristic feature within this system is that they may behave such as an integrated system and co-evolve together. As an example, a novel mechanism has been studied in sugarcane. In infested areas the sugarcane borer *Diatraea saccharalis* has been found to occur associated with opportunistic fungi, such as *Fusarium verticillioides* and *Colletotrichum falcatum*, leading to the rotting disease in sugarcane. A class of pathogenesis-related (pr4) proteins named Sugarwins are upregulated after insect damage. Interestingly, Sugarwins have been shown not to affect insect mortality and development, and instead, it promoted fungal cell death (Medeiros et al., 2012; Franco et al., 2014; 2019).

An increasing number of studies have shown that host plants, microorganisms and herbivore insects have close interactions (Badri et al., 2013; Hubbard et al., 2018; Franco et al., 2017; 2019). In addition, this process might be mediated by primary and secondary metabolites released by host plants, modulating the rhizosphere microbiota after insect attack (Badri et al., 2013 and Hubbard et al., 2019).

The microbiota rhizosphere relies heavily upon chemical exudates that create communication highways of signaling molecules produced and secreted by microbes and plants (Sugio et al., 2014). Mediated by this intense exudate change, the plant microbiota serves as an extension of the plant genome and have potential impacts on microbial functions (Rout & Southworth, 2013; Mendes et al., 2013). Several benefits of rhizosphere microbiota on plant health have been described, which includes disease suppression (Mendes at al., 2011; Ritpitakphong et al., 2016), host-plant systemic resistance induction (Zamioudis et al., 2015), increase in nutrient acquisition (Van der Heijden et al., 2016) and adaptation to environmental variations (Haney et al., 2015). These complex interactions between host plants and rhizosphere microbial communities are bidirectional, and the host plant also provides novel metabolic capabilities to its microbial associates, leading to the microbial adaptation on rhizosphere environmental (Thrall et al., 2007).

High-throughput 16S rRNA gene sequencing and access to a set of microorganisms that cannot be cultivated provide a new approach to explore the holobiont system interactions, in such a way that allows us to study in more details the complex association between host plants-insects-rhizosphere microbiota (Knief, 2014, Kuske et al., 2002, Smalla et al., 1993). However, it is still largely unknown how host plants attacked by herbivore insects interact with rhizosphere microorganisms. Depicting this scenario may allow us to more accurately predict the adaptation of plants to the rhizosphere microbiota modulated by the biotic stress. In humans, this holobionts system also appears to be established between the phenotypic responses to a stressor agent and particular microorganisms (Sze and Scholss, 2016). As an example, it is possible to predict gene expression in humans, such as those encoding carbohydrate-active enzymes and proteases, from bacterial species assemblages (Muegge et al., 2011).

Furthermore, we know that for a variety of cause and effect relationships there are convergent responses of these complex systems. However, it is not yet known whether the rhizosphere microbiota

to different host plants in this holobiont context follows a convergence or divergence pattern when altered by the insect attack. According to the literature, there are several examples of systems convergence in nature. In *Myrmecophages*, for example, the diet adaptation appears to be a major driving factor of convergence in gut microbiota composition over evolutionary timescales (Delsuc et al., 2014). This process also applies to the aquatic environment. Different populations *of the Fundulus heteroclitus* fish have a conserved response to pollution. The authors suggest that the interacting organisms independently evolved mechanisms for adaptation to the environmental (Fisher and Oleksiak., 2007).

In *Escherichia coli*, it has been shown that populations submitted to different environments have the same growth phenotype at the endpoint and the evolution is convergent to the system (Fong et al., 2005). In addition to these concepts, it has been proposed that bacterial communities on leaves have particular microbes termed "hub microbes," due to their central position in the microbial network, and these "hub microbes" are important in shaping microbial structure on plant hosts (Agler et al., 2016). Another compelling case happens between ants, beetles and termites that share symbiotic fungus by diet behavior, which shows gut bacterial microbiota convergence (Aylward et al., 2014).

In the current study, we aimed to test whether the modulation of plant rhizosphere microbiota after insect attack also follows a unique structure to different plant species. In addition, we checked whether this response applies to bacterial-isolated groups or hubs. Finally, we analyzed whether soil microbial communities' modulation after insect attack in host plants can interfere with the subsequent plant generation.

Our results demonstrate that the modulation of the rhizosphere microbiota by herbivorous insect attack depends on the host plant species. Besides, some bacterial genera contribute to the rhizosphere microbiota modulation after insect attack. Moreover, the microbiota on semi-soil, modulated by insect attack, may influence the biomass of next-generation seedlings negatively.

3.2 MATERIAL AND METHODS

3.2.1 Plant material

In order to obtain the rhizosphere of different plants with and without insect attack, we sowed the plants in the same condition in a plant growth chamber under a photoperiod of 16 h light and 8 h dark at 25 +/- 2 °C.

The Sweet Corn Super Sweet Hybrid (*Zea mays* - Sh2) seeds; Beans *Phaseolus vulgaris* L. Seychelles) seeds; Tomato (*Solanum lycopersicum* Rutgers) seeds and Red Beet (*Beta vulgaris* L. Burpee bred) seeds, were purchased from W. Atlee Burpee & Co. (Warminster, PA). The *Arabidopsis thaliana* ecotype Col-0 seeds were purchased from Lehle (Round Rock, TX).

All the seeds were surface sterilized in 2% sodium hypochlorite for 2 min, followed by three washes with sterile distilled water. The surface-sterilized seeds were placed directly on semi-soil.

3.2.2 Semi-Soil and plant growth conditions

In the first experiment, we sowed the five different plant species in semi-soil. This semi-soil was composed of ¼ cover crop soil that was collected at the Ag Research Development & Education Center (ARDEC) by Colorado State University at 4616 NE Frontage Road, Fort Collins, CO 80524 (DMS 40° 38' 59.172" N and 104° 59' 44.34" W), where only experiments without agrochemicals were performed. Additionally, ¼ no sterile horticultural vermiculite (#2 grosses 3,5 cub. ft.) by *Thermorock*®, ¼ Peat moss by *Promix Bx*®, ¼ sand no sterile by *QUIKRETE*® Play Sand (Atlanta, GA) were used. Seeds of Sweet Corn, Arabidopsis, Beans, Tomato and Red Beet were previously sterilized in sodium hypochlorite solution and *Milli-Q*® water at 2% (V / V) and subsequently rinsed four times with sterile distilled water. The seeds were seeded directly into the semi-soil contained in 1-liter plastic pots.

More than one (1) seed was placed for each pot to ensure the germination of at least one seedling per pot. The pots with the seeds were incubated in growth chambers with a photoperiod 16 h light / 8 h night at 25 °C +/- 1 °C for seven days. After seven days the excess of plants per pot was thinned. Remaining only one plant per pot for all species studied. We used a completely randomized design with six replicates per treatment and were maintained one plant per pot. The seedlings of plants grew for four weeks in growth chambers until they were used in different treatment. Plants were watered every two days in a sufficient volume (+/- 50ml per pot or less to *A. thaliana*) of sterilized water to keep the semi-soil on the pots moist enough for each plant species studied.

3.2.3 Herbivory experiment using *Trichoplusia ni* (Hübner)

In the fifth week each of the different plant species were divided into three experimental groups: (1) Test group: Plants with herbivore attack (n = 6); (2) Positive Control Group: Plants with induced mechanical injury by sterile scissors (n = 6); (3) Negative Control Group: Plants without mechanical injury nor herbivorous attack (n = 6). The original population of *Trichoplusia ni* larvae was used in the third instar stage and were obtained from Frontier Agricultural Sciences (Newark, DE). Four *T. ni* larvae were kept in plant leaves of the test group to each one five different species of plants studied.

The herbivory was maintained for one week with intervals of two from day zero to day seven of experimentation. After a week of experimentation, part of the rhizosphere soil of the plants was removed about 2mm from the root. Most rhizosphere soil and total soil per pot were maintained in the

plant growth chamber for use in re-sowing the next day. The dry biomass, fresh biomass, root height, and root biomass data were collected on all repetitions and experimental groups. The biomass data were analyzed by ANOVA (F test), and the means that have differences were compared by *PostHoc T-Student* (*Bonferroni*) test, at 5% probability level.

3.2.4 DNA rhizosphere extraction and *Illumina Miseq* Sequence

Six replicates were used for each experimental group for the extraction of total DNA from different plants families to compare the bacterial communities in the rhizosphere. Each sample was composed of 50 mg of soil rhizosphere. The total DNA was extracted using the *MoBio* kit (*PowerPlant*® DNA Isolation Kit) according to the manufacturer's instructions. The DNA concentration was measured by a Nanodrop® spectrophotometer (*Thermo Fisher Scientific*). The extracted DNA was stored at -20 °C until the samples amplification to *Illumina Miseq*.

3.2.5 *PCR* amplification for *Illumina Miseq* bacterial 16S rRNA gene

The 16S rRNA gene region V3 was targeted using the forward primer F5′–TCGTCGGCAGCGTCAGATGTGTATA AGAGACAGCCTACGGGAGGCAGCAG-3′ and reverse primer R5′-GTC TCGTGGGCTCGGAGATGTGTATAAGAGACAGATTACCGCGGCTGC TGG-3′. They amplified to 230 base-pairs (bp) V3 region (Klindworth et al., 2013).

The Polymerase chain reaction (PCR) was performed in 25 μl reaction volume. The reaction was formed by 1 μl of DNA from each sample (5 ng/ μl), 5 μl of primer forward and the same aliquot from reverse primer (1 μM total), 12.5 μl of 2× KAPA HiFi HotStart ReadyMix. The PCR was done followed the conditions: (1) initial denaturation at 95 °C for 3 min, (2) followed by 25 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and ended with (3) a final extension step at 72 °C for 5 min. Two microliters of amplified product were analyzed on bioanalyzer. PCR clean-up was done with AMPure XP beads to 16S rRNA gene V3 amplicon. The amplicons at the 16S rRNA gene to specific region V3 were quantified in the mix reaction for equimolar concentrations with Illumina sequence adapter and index primers (Nextera XT Index kit).

3.2.6 Bacterial 16S rRNA gene sequence analysis

Bacterial community analyzes were generated using *myPhyloDB* (Manter et al. 2016). The OTUs are assigned to ecological guides using the phyla and genera levels. For diversity, microbial community composition (relative OTUs) was analyzed using principal coordinates analysis (*PCoA*) based on the Bray-Curtis index distance dissimilarity. A complementary non-parametric multivariate

statistical test, *perMANOVA*, and non-parametric univariate ANOVA analyses were used to test the differences in microbial bacterial communities with the Bray-Curtis distance with 999 permutations in myPhyloDB.

The linear discriminant effect size was used to identify bacterial rate that was significantly associated with each treatment using an alpha value of 0.05 to Tukey's HSD post-hoc test. The OTUs database generated in *myPhyloDB* by phyla and genera levels were then used to verify the groups shared between each experimental group studied. The shared and unique OTUs among treatments were counted, and their distributions were shown in the *Venn* diagram constructions which were done with the script described for the plugin to '*jQuery*' JavaScript library (Bardou et al., 2014).

The means in the distance between dissimilarity vectors in the *PCoA* data matrices were analyzed for the control and insect groups. The means data were tested by ANOVA (F test).

3.2.7 Experiment to test rhizosphere microbiota modulation effect on the subsequently plant generations

Using the semi-soil and the part of the resident rhizosphere that was stored for a day in the plant growth chamber, were sowed new seeds of the respective species used, originating from the same batch of seeds previously used. The seeds were sterilized and seeded according to the protocol previously described. The plants were kept in the growth chamber in the same conditions as the first sowing, without the addition of fertilizers. After four weeks the plants were collected and the dry biomass, fresh biomass, root height and root biomass for all replicates were measured. The biomass data from the second experiment were analyzed by ANOVA (F test) and the means compared by the *PostHoc T-Student* (*Bonferroni*) test, at 5% probability level. The free software *Past* and *Sisvar* were used for the statistical analysis and software *Origin 8.5* for the elaboration of the graphs.

3.3 RESULTS

3.3.1 Herbivory effect in plants biomass

In order to verify whether the herbivory attack was significant to different plants used in this study, we observed that *T.ni* feeds on the leaves and stems of the plants in an unspecific way (figure 1). Each plant presented significant damage (p<0,05) due to the presence of the herbivore *T. ni*. The biomass consumption by *T. ni* was observed in different percentages in each plant genotypes. In Red Beet the consumption by *T. ni* larvae was around 86%, followed by Tomato (79%). Thus, the Red Beet and Tomato plants were the most consumed plants by *T. ni*. The insect pest consumed Corn and Beans in the percentages around 50% and 46.80% respectively (figure 1). The *A. thaliana* biomass

was also significantly consumed (p<0,05) in the rate of 57% compared with *A. thaliana* control group plants.

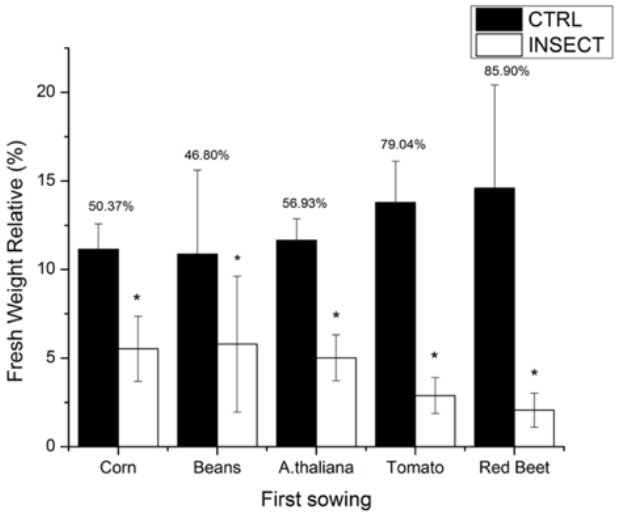

FIGURE 1. Relative difference between the Control group (CTRL) and insect group (INSECT) for the variable's fresh biomass in the first sowing; The numbers above the bars correspond to the difference in percentage between the two groups for each cultivated plant. (*) ANOVA significant for p<0.05 followed by the Post-Hoc Test T-Student (Bonferroni).

3.3.2 Rhizosphere microbiota *Illumina Miseq* sequence

The filtering reads by basal quality control and singleton OTUs clearance on sequencing generated by Illumina Miseq, allowed a total of 14851quality bacteria sequences. We removed from the analysis samples with fewer than 3000 reads. The average read length of bacteria for the 16S rRNA subunit was 601 bp. The coverage of each sample, which reflects the captured diversity, was higher than 94.05% for all samples. Therefore, the sequencing depth was adequate to assess the diversity of bacterial communities in the rhizosphere of Corn, Beans, *A. thaliana*, Tomato and Red Beet (Supplementary chart 1).

3.3.3 Rhizosphere bacterial communities' structure before and after insect attack between different plants species

In our study, we sought to understand the structure of bacterial communities in the rhizosphere of different plants after the attack of herbivorous insects. We have observed through the analysis of *PCoAs* (figure 2) that the rhizosphere has been modified either after the insect attack (figure 2C) either after the mechanical injury (figure 2A). In addition, the microbiota structures to each plant species were clustered. This observation suggests that plant species strongly influence the rhizosphere structure even after the herbivorous insect attack. On the other hand, the community structures could be separated plant species, although Corn and Red Beet are grouped closely (figure 2A).

It is interesting to note that the mechanical injury (figure 2B) and insect attack (figure 2C) treatments shifted the community structure in comparison with the control group.

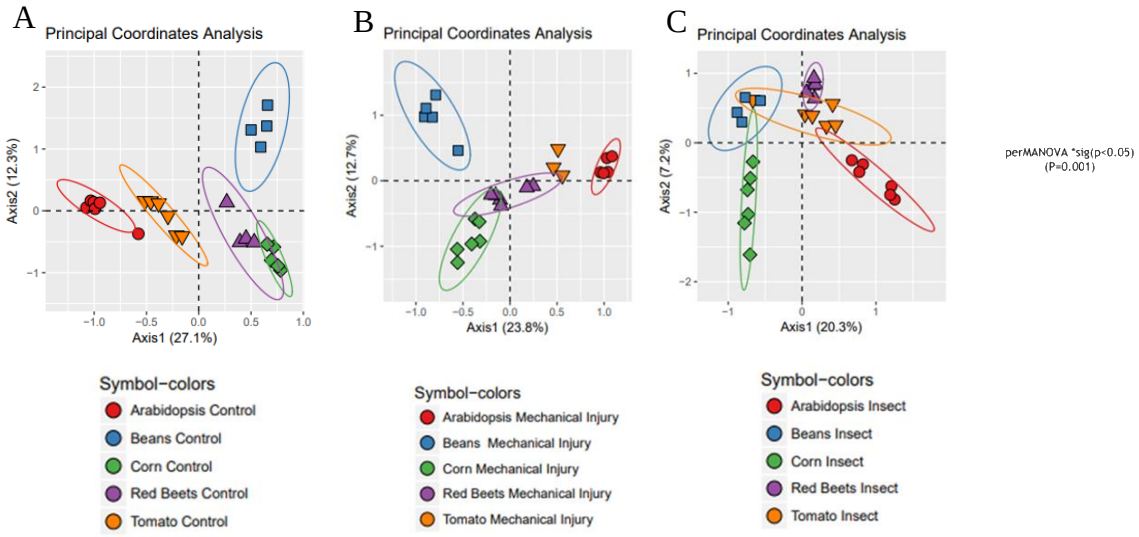

FIGURE 2. Principal coordinate analysis (*PCoA*) of *pairwise* dissimilarities (Bray–Curtis index) by OTUs for bacterial communities in *Arabidopsis*, Tomato, Red Beet, Corn and Bean rhizosphere of (A) Control group; (B) Mechanical injury group; (C) Insect attack group.

According to the *PCoA* clustering analysis, the bacterial communities structure on different plant species is shown to be closer in herbivorous insect attack samples (figure 2C) than in the samples from the control group (figure 2A). *The PCoAs* analysis suggests that insect treatment results in a convergence in rhizosphere microbiota clustering to different plant species.

TABLE 1: Distances between each crop plant. Comparison in pair to insect and control rhizosphere microbiota by pairwise dissimilarities matrix (Bray–Curtis index) to OTUs for bacterial communities. Ns (no significative to test F p<0,05).

Crop Plant Pair	Vector distances		
	Control	Insect	Difference (%)
Beans/Tomato	0.67805738	0.59692804	8.112934
Arabidopsis/Red Beet	0.67862325	0.5987591	7.986415
Corn/Beans	0.6316512	0.57635113	5.530007
Tomato/Red Beet	0.6025	0.56754293	3.495707
Beans/Arabidopsis	0.73626238	0.71272929	2.353309
Corn/Tomato	0.6595883	0.64383011	1.575819
Corn/Arabidopsis	0.72841808	0.71386261	1.455547
Beans/Red Beet	0.6108145	0.60102795	0.978655
Arabidopsis/Tomato	0.59668442	0.61032864	-1.364422
Corn/Red Beet	0.5288917	0.61546497	-8.657327
Total Means	0.64514912	0.62368248	2.146664^{Ns}

Ns(p=0.41909418)

Dissimilarity distance analysis among the plant species (in pairs) to control and insect attack groups are shown above (table 1). It was demonstrated no significant difference (test F) between the

distance vectors means in control and insect group (table 1). To each plant species, the rhizosphere microbiota seems to establish a convergence or divergence concerning other plant genotypes according to the specific pair comparison.

An example is to Beans and Tomato that had a convergence of 8,1% to the rhizosphere communities, after the insect attack (table 1). The whole list of communities convergence in the rhizosphere samples per plant pair is showed in table 1.

In contrast, the analysis among the plant pairs Arabidopsis/Tomato, Corn/Red Beet after the *T. ni* attack showed a divergence in the microbiota communities as indicated in table 1. There are not significantly different from the total means by the F test ($p > 0.05$) (table 1).

Moreover, it was demonstrated no significant difference (test F) between the distance means in control and insect group. To each plant species, the rhizosphere microbiota established a convergence or divergence concerning other plant genotypes according to the specific pair comparison.

3.3.4 The difference on structure and abundance in microbiota rhizosphere by bacterial phylum level before and after insect attack

The understanding of the rhizosphere bacterial phyla presented for each plant species has great importance in building the whole scenario of rhizosphere microbiota modulation after the insect's attack.

In this context, grouping analyzes were performed comparing the control and insect groups for each of the different rhizospheres of the studied plants (figure 3). It is interesting to observe that almost all the studied plant species presented significant differences in the structure of their bacterial communities before and after the *T. ni* attack (figure 3A, 3B, 3C, 3E). For Tomato, a significant difference ($p = 0.32$) was not observed between the community structure presented in the rhizosphere samples that had an herbivorous insect attack and the control group sample (figure 3D).

Some plant's species presented greater prominence in the differentiation of rhizosphere microbiota after the attack of *T.ni*. The *PCoAs* for Bean (figure 3B), *A. thaliana* (figure 3C) and Red Beet (figure 3e) show the structurally differentiated clusters for the communities in the control group and the group in which insects attacked the plants.

Statistical analyses on the phylum level showed that for most cases there are significant differences ($p<0.05$) between the rhizosphere microbiota for the same plant species when compared before and after herbivory (figure 3F, 3G, 3H, 3J). The phyla *Actinobacteria*, *Verrumicrobia*, *Bacteroides*, *Firmicutes*, and *Acidobacteria*, presented significative differences in the rhizosphere microbiota among control samples and insect attack samples (figure 3). However, it was not observed

for Tomato significant difference (p>0.05) in the relative abundance among the phyla present on the control and insect attack group (figure 3I).

We emphasize here, the phylum Verrucomicrobia, which was found more abundantly in the Corn rhizosphere samples after insect attack (supplementary table 1). Indeed, on the Bean's rhizosphere, the *Verrucomicrobia* phylum (supplementary table 2) was found less abundantly on the insect attack group samples (p<0.05).

Some contrast was observed among *A. thaliana* and Red Beet rhizosphere after insect attack. It was verified a reduced abundance for the Bacteroides phylum in the *A. thaliana* rhizosphere (supplementary table 3) after insect modulation. However, the same phylum is present with a higher relative abundance in the Red Beet rhizosphere modulated by the insect, when the samples are compared with the control samples (p <0.05) (supplementary table 5).

At the same time, some phylum were only found in the rhizosphere of one of the plants studied, like the Nitrospira phylum in Corn (supplementary table 1) and, Armatimonadetes, Cyanobacteria, Actinobacteria in Beans (supplementary table 2). Such as the Chlorobi, Proteobacteria and WPS-2 phylum in Red Beet rhizosphere (supplementary table 5)

The whole data about the phylum found on the rhizospheres from different plant's families studied, with their respective relative abundances for the control group and the insect attack samples are in the supplementary tables from 1 to 5.

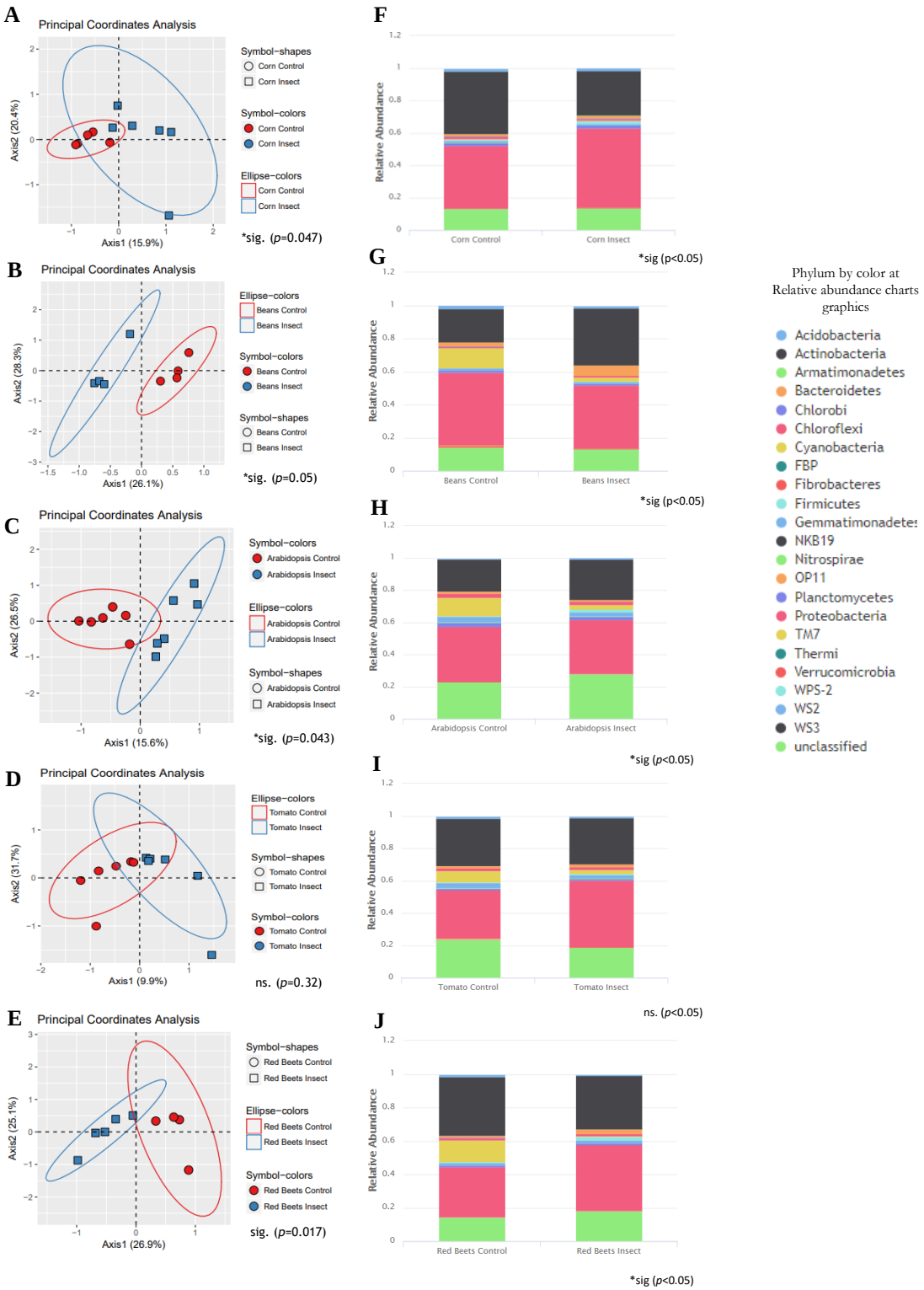

FIGURE 3: (A, B, C, D, E). Principal coordinates analysis (*PCoA*) of pairwise dissimilarities (Bray–Curtis index for bacterial rhizosphere communities in taxonomic composition by OTU level presents in **(A)** Corn, **(B)** Beans, **(C)** *A. thaliana*, **(D)** Tomato and **(E)** Red Beet. The *PCoA* is a comparison between the rhizosphere in control groups and rhizosphere after the insect attack. **perMANOVA* (p</=0,05) and *ns perMANOVA* (p>0,05). **(E, F, G, H, I).** Taxonomic composition of OTUs to bacterial by phylum level in **(F)** Corn, **(G)** Beans, **(H)** *A. thaliana*, **(I)** Tomato and **(J)** Red Beet. The size of each segment in the charts is proportional to the increasing relative abundance of OTUs assigned to the indicated the specific phylum in the legend. *ANOVA & Tukey's HSD post-hoc test (p<0,05) and ns ANOVA & Tukey's HSD post-hoc test (p>0.05).

3.3.5 Microbiota analyzes about genus bacterial influence on the rhizosphere after insect attack

We searched understanding if there is a relationship among the bacterial genera level present in the plant rhizosphere with and without insect attack through the OTUs analyses.

The Venn diagram (figure 4) demonstrate that the representation of the number of bacterial genera overlapped in the rhizosphere is higher when herbivorous insects attack the plants (figure 4B).

We observed that the number of OTUs, which match for all plants studied on the control samples, are fifty-eight (58) OTUs. (figure 4A). For the insect attack group, the samples are sixty (60) OTUs.

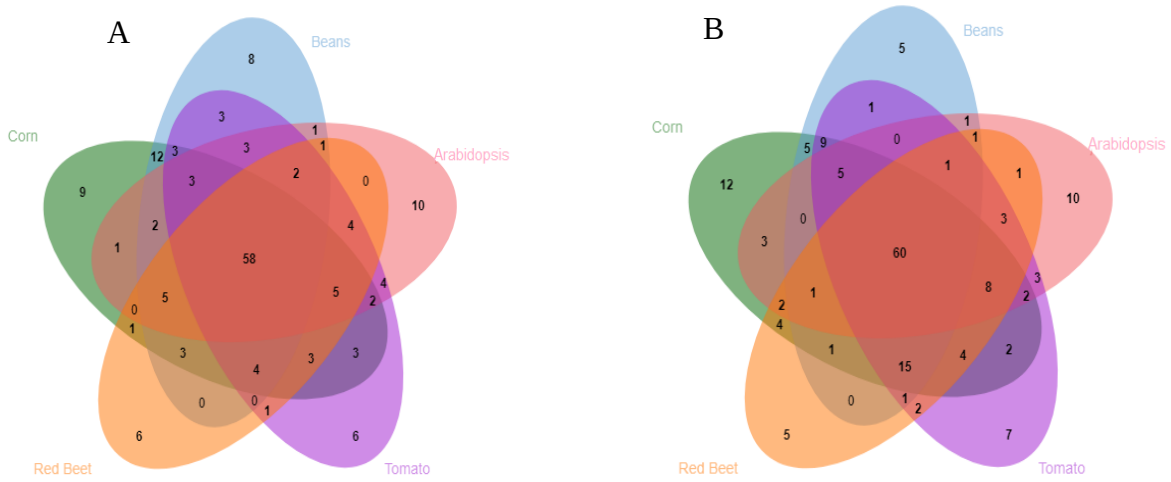

FIGURE 4. (A). Venn diagram showing the distribution of common OTUs assigned to the bacteria genera among different plants families from the Control group. (B) Venn diagram showing the distribution of common OTUs assigned to the genera bacteria among different plants families from the Insect attack group.

Exploring the OTUs at the genus level, which are shared among the rhizosphere of all plant species studied after the insect attack, we found forty-nine (49) OTUs shared, which are present both in the control group and in the group of samples attacked by insects (Figure 5).

However, some genera are shared exclusively among the samples from the control group, and others are exclusively shared among the rhizosphere samples with insect attack (Figure 5).

Interesting observe that there are more OTUs, at the genus level, that are shared exclusively among the rhizospheres sample after the plants have been attacked by insects (Figure 5 and Table 2).

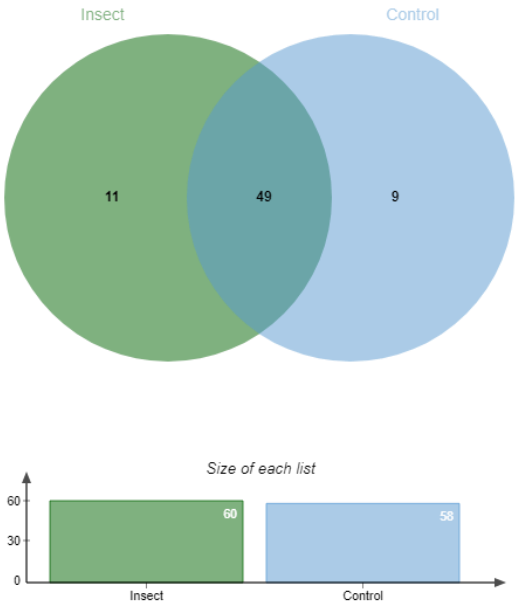

FIGURE 5. Venn diagram showing the distribution of common OTUs assigned to the indicated genus among different species plants for control and insect attack group. The names from the genera in the Venn diagrams that are specific for each experimental group and shared between the groups are shown in supplementary table 6.

Table 2 shows the relative abundances for each of the genera that were exclusively found in the rhizosphere of the studied plants when there is the attack of *T.ni*. For most genera, there is no significant difference in abundance for each of the rhizospheres of the studied plants.

Only the genera *Achromobacter* and *Burkholderia* presented significant differences ($p<0.05$) when compared among all the samples of the different plants studied after the insect attack. The genera *Azospirillum, Arthrobacter, Hydrogenophaga*, presented with an abundance above 10% for most of the rhizosphere of the studied plants after the attack of *T. ni* (Table 2).

The relative abundances for all genera present in the rhizosphere samples of plants after insect attack are found in supplementary material 1. There are genera presented in supplemental material 1 that may also be present in the control samples.

TABLE 2: Relative abundance by bacterial genus level to all the species plants studied just to rhizosphere samples from *insect attack* group. *sig ($p<0.05$).

Genus (exclusives for insects' group) #11	Corn	Beans	*A. thaliana*	Tomato	Red Beet
Azospirillum	5.981089	0.069046672	0.01539711	0.055317997	0.057418
**Achromobacter*	0.578311	0.034821665	0.011447008	0.146993503	0.234646
Arthrobacter	0.352697	0.258378083	0.172953682	0.261005038	1.568947
Hydrogenophaga	0.219714	0.178434102	0.112818218	0.062577345	0.134871
**Burkholderia*	0.101112	0.054544551	0.004253956	0.010789578	0.016945
Geodermatophilus	0.087333	0.054801445	0.036258066	0.026363476	0.022481
Nonomuraea	0.082654	0.028018099	0.036153098	0.052815345	0.093556
Virgibacillus	0.068538	0.031879806	0.097034385	0.007060461	0.060442
Kibdelosporangium	0.0307	0.053865023	0.04624105	0.055063865	0.059487
Agrococcus	0.021601	0.024802771	0.035180765	0.018966013	0.102771
Terracoccus	0.014154	0.050318218	0.075223195	0.051351321	0.034089

*sig ($p<0.05$) ANOVA and LSmeans & Tukey's HSD post-hoc test.

3.3.6 Influence from rhizosphere microbiota modulation by insect attack on the next generation plant seedlings

The composition of the rhizosphere microbiota was verified for different plant species concerning the richness and abundance of the bacterial groups after the herbivorous insect attack. We then sought to understand if the effect of modulation of the rhizosphere microbiota by herbivorous insect attack could be maintained on the next generation of seedlings planted in the semi-soil following the experimental groups used in the first sowing. Additionally, if the microbiota modulation after insect attack has some influence in the next generation of seedlings.

Figure 6 shows the data of a second sowing cycle, using the stored semi-soil where insects attacked the plants of the previous cycle or were harvested without any passing through the herbivory, thus belonging to the control group.

According to what is shown in figure 6, the plants that grew in the semi-soil in which there was insect attack previously obtained a decrease in the fresh biomass concerning the plants sown in the soil where only plants without the attack of *T. ni* were prior harvested.

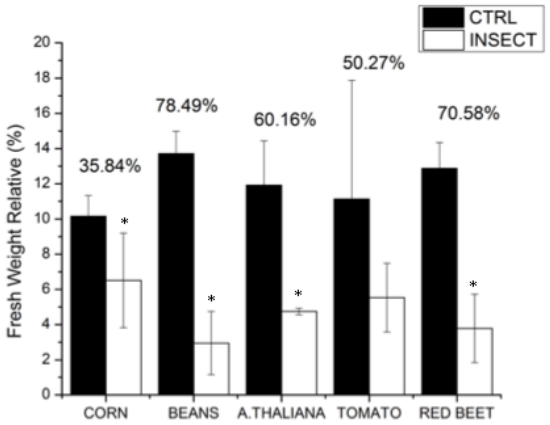

FIGURE 6. Relative Difference between the control group (CTRL) and insect group (INSECT) for the variables Fresh Weight in the second sowing; The numbers above the bars correspond to the difference in percentage between the two groups for each cultivated plant. (*) ANOVA significant for $p<0.05$ followed by the Post-Hoc Test T-Student (Bonferroni).

The most significant reduction in biomass was for bean (78.49% less), and Red Beet (70.58% less) and these plants families have a significant difference by test *T-Bonferroni* ($p < 0.05$) between control groups and insect group.

Still, the Tomatoes grown in the semi-soil that in the previous cycle had plants attacked by insects have not presented a significantly different in fresh biomass of the Tomatoes sown in the semi-soil with control plants in the last sowing cycle.

3.4 DISCUSSION

In recent years the triad relationship between microorganisms, plants, and insects has been approached in different ways (Badri et al., 2013b; Franco et al., 2019). In the present study, we sought to understand how the herbivore insect attack acts on the rhizosphere microbiota in different plant species and how the modulation of this microbiota can influence the next generations of plants phenotypically. At first, we tried to establish a unique source of herbivory for different types of plants sown in the same kind of semi-soil. We have verified that the use of generalist insects such as T.ni can guarantee a significant herbivory effect for the different genotypes of plants used.

Depending on the plant genome, there was the consumption in different proportions of the aerial plant biomass by the larvae of *T. ni*. However, the biomass consumption for all plants studied was significant (Figure 1). This fact may be related to the different types of metabolites, and concentration levels present in the leaves of each of the families of plants used (Oliver et al., 2000; Tognetti et al., 1990). Based on a significant insect herbivory effect in different plant species (Figure 1), we then accessed the rhizosphere microbiota from each of the vegetables used in the study and compared the intact rhizosphere and the rhizosphere microbiota after insect attack.

The plant species has a significant effect on the rhizosphere microbiota (Haichar et al., 2008; Chaparro et al., 2013; Badri et al., 2013b). That is, rhizosphere microorganisms can be distributed in different amounts according to the plant genome and development stage the host (Chaparro et al., 2013, 2014). Some studies point out that these differences in the rhizosphere microbiota in different host plants are directly related to the type and amount of exudates released by each plant (Haichar et al., 2008, Chaparro et al., 2013). Thus, root exudates may drive the differentiation of the soil biome in the rhizosphere, and by host genotype-dependent factors (Badri & Vivanco, 2009).

In this context, the effect of insects on the rhizosphere microbiota is still unknown. Some studies that began to clarify this panorama relate that different structures of rhizosphere microbiota can alter the tolerance of the plant to herbivory. For example, in a recent article (Hubbard et al., 2018) it was found that differences in the rhizosphere microbiota may contribute to different plant responses against insect attack and that these responses are due to exudation and the glycosylate production for plants. In previous work, it was seen that plants are grown in the presence of an intact rhizosphere microbiota that reduces herbivory compared to those grown with a microbiota influenced by herbivory (Badri et al., 2013). In both papers, it is speculated that host metabolome responses to the microbiota are a mechanism to allow plant differences concerning to herbivory resistance (Badri et al., 2013, Hubbard et al., 2018).

In our study, we tried to test the hypothesis that once the attack of herbivorous insects may modulate the structure of the rhizosphere microbiota in the different families of plants used, these bacterial communities would be closer, even for different plant genomes. In other words, the bacterial community of rhizosphere microbiota would converge after the insect attack in the host plant. The

literature has several examples of systems convergence in nature (Delsuc et al., 2004; Fisher and Oleksiak 2007; Fong et al., 2005).

The effect of the convergence on the bacterial community structure in different rhizosphere microbiota was no significant after herbivory attack (p>0.05). The absence of a significant effect of the convergence means that the communities of microorganisms are not on average closer to each other (Figure 2 and Table 1). Thus, it is clear that this convergence or divergence effect between the bacterial community structure of the rhizosphere microbiota in the different plant species depends on the genome of the plant (table 1).

That is, the effect of herbivory on the rhizosphere microbiota is high to the point of modulating the structure of the communities. However, the type of community structure that will be established through the modulation triggered by herbivory seems to depend mainly on the genome of the host plant.

We have noticed that some plant species after herbivorous insect attack converge in the community structure of bacteria present, for example, *A. thaliana* / Red Beet (7.9%), Corn / Beans (5.5%), Tomato / Red Beet 3.49%), Beans / *A. thaliana* (2.3%), Corn / Tomato (1.5%) and Corn / *A. thaliana* (1.4%) (Table 1). The effect of the plant species again shows that herbivory generates different responses when we compare rhizosphere from different genomes. Therefore, the convergence effect cannot be considered a similar behavior for all rhizospheres after the insect attack in the compared host plants.

We have shown the effect of herbivory on the structure of most genomes of host plants (Figure 3). The genotype of the plant is the one that can direct how this modulation will establish itself in the selection of the communities of the rhizosphere microbiota. This supposition corroborates with the knowledge that the family of different host plants presents differences in the release of exudates and can select communities of microorganisms that present differences in diversity and abundance (Chaparro et al., 2013, 2014; Haichar et al., 2008; Badri & Vivanco, 2009).

Additionally, it has been discussed that bacterial communities in the plant rhizosphere, present specific microbes called "hub microbes." Due to their central position in this microbial network microorganisms are disproportionately influential in modeling the microbial structure in plant hosts (Agler et al., 2016). In the present work, we suggest that some communities of microorganisms, recruited by different plant genotypes, have a more significant influence on the modulation of the rhizosphere microbiota after *the T .ni* attack.

Recent research has demonstrated the influence of rhizosphere microbiota on herbivory (Badri et al., 2013; Hubbard et al., 2018). However, our target is reversely, and we present the influence of herbivory on the modulation of the rhizosphere microbiota. We suggest that insect effect in the host plant can act directly by modulating the rhizosphere microbiota even on different plant species (genomes). However, the host plant species seems to have a more significant influence on the control of this modulation.

In a recent publication (Hubbard et al., 2018) suggests the possibility of predicting the effect of herbivory on the host plant according to the plant rhizosphere microbiota. We believe that there is a possibility of phenotypic prediction through the genomic analysis of the plant's rhizosphere microbiota, as are suggested in the prediction of immune system responses through the genome study of the intestinal microbiota in humans (Sze & Schloss, 2016). It should be noted that in plants with insect attack, the genome of the plant should be considered because, since each host plant belonging to different species may be different in the modulation of the rhizosphere microbiota as is demonstrated in our study (Figure 1, Table 1).

In a more restricted context, the phenotypic prediction through the genome of the host microbiota may be accurate when comparing the genomes of the nearest hosts, that is when we compare the same species of plants in different situations (Badri et al., 2013; Hubbard et al., 2018). However, among the more distant genomes such as different host families used in this study, the effect of the genotype may not be convergent for the same modulation and the phenotypic predictions should be made more cautiously.

Observing the differences in the modulation of the rhizosphere microbiota according to the genome of each of the species used in the study, we sought to understand how the rhizosphere modulation of the rhizosphere is established with the insect attack in each of the different species studied (*A. thaliana, Zea mays Sh2, Faseolus vulgaris, Solano lycopersicum, and Beta vulgaris*).

From the analysis of *PCoA*, it was verified that the genome of each of the different host species might have a strong influence on the structure of rhizosphere microbiota after the attack of herbivorous insects (Figure 3). Most host plant species showed significant differences between the structure of the rhizosphere microbiota in the comparison between the intact plant groups and the group of plants after insect attacked (p= 0.047), Bean (p= 0.05), *A. thaliana* (p= 0.043) and Red Beet (p= 0.017) (Figure 3A, B, C, E), when the rhizosphere of plants attacked and not attacked were compared (p<0.05) (figure 3F, 3G, 3H, 3J).

Also, in Corn (supplementary table 1) and Red Beet (supplementary table 5), the *Acidobacteria* phylum reduced the relative abundance ($p<0.05$) significantly in plants under insect attack in comparison to the control group. This *Acidobactia* phylum possesses acidophilic bacteria, with few possible groups to be cultivated and still little known in a useful way. However, it is known about them is that *Acidobacterial* has species that are heterotrophic and, some members seem to be more versatile in carbohydrate utilization, the ability to use nitrite as Nitrogen sources and have an excellent response to macro and micronutrients and soil acidity (Kielac et al., 2016). The Firmicutes phylum increased significantly on the rhizosphere after insect attack ($p<0.05$) in the Corn (supplementary table 1), in *A. thaliana* (supplementary table 3) and Red Beet (supplementary table 5) and this phylum are able of producing several short-chain fatty acids (SCFAs) like butyrate (Marciano et al., 2017).

Besides, the Bacteroides phylum was found in a lower abundance in the *A. thaliana* (supplementary table 3) rhizosphere after the *T. ni* attack. Bacteroides are a phylum able to use the host-derived glycans as energy sources (Sonnenburg et al., 2005) and some Bacteroides fragilis strains contribute to the maturation of the immune system in humans (Mazmanian et al., 2005).

It seems complicated to compare, but we know that human studies on the host and microbiota interactions are more explored. In this context, some studies with humans indicate that obese adult population has a significantly higher level of Firmicutes and lower level of Bacteroidetes compared to healthy weight, and lean adults (Koliana et al., 2017). The differences in the levels of Firmicutes and Bacteroidetes are correlated to more upper infections and deficiency in the immune system (Koliana et al., 2017). Our study also verified this relation in *A. thaliana*, for example, and, this would be in agreement with the modulation direction of insect-attacked plant rhizosphere microbiota in defense system responses in plants.

The *Verrucomicrobia* phylum (supplementary table 1) was found more abundantly in the rhizosphere of the samples attacked by insects in Corn, and the oppositional occurred to the bean (supplementary table 2) ($p < 0.05$). We have known that the bean is a predominantly heterotrophic group. It has carbohydrate-degrading metabolism, with biogeochemical roles (Cardman et al., 2014). In Beans, the phyla *Armatimonadetes* and *Cyanobacteria* also decreased in abundance after the attack of T.ni, contrary to the phylum Actinobacteria, which has a significant increase in the abundance in the rhizosphere of Beans plants attacked by insects (supplementary table 2 and table 2).

The Actinobacteria is involved in the production of extracellular enzymes and the formation of a wide variety of secondary metabolites (Schrempf, 2001). However, it remains unclear whether these groups of bacteria are recruited only due to the release of secondary and primary metabolites in the plant's rhizosphere (Badri & Vivanco 2019; Chaparro et al., 2013) or also has a strong influence of host plant defense pathways (Hubbard et al. al, 2018).

When we compare the rhizosphere microbiota of plants attacked by insects, we have the presence of 60 genera that are shared between the rhizosphere of the different plants (Figure 4B). When we eliminate the groups that overlap in the rhizosphere of plants not attacked by herbivorous insects, we observe the specific bacterial genera that are shared by plant's host rhizosphere after insect attack (Figure 5 and Table 2). For most of the genera, there is no significant difference in abundance for each of the rhizospheres in the plant species studied after the *T .ni* attack ($p>0.05$) (table 2).

Thus, among the eleven bacterial genera present in the rhizosphere microbiota of all the different species of plants studied, there is no difference in relative abundance between different plants rhizosphere studied. Here we focused on the genera of bacteria that showed a higher relative abundance. The genera *Azospirillum, Arthrobacter, Hydrogenophaga*, present an abundance above 10% for most of the rhizosphere of the studied plants after the attack of *T. ni* (Table 2). It is essential to understand that the last genera mentioned are present exclusively in the plant's rhizosphere microbiota after herbivorous insect's attack.

We know that the genus *Azospirillum* have free-living species, are nitrogen-fixing and are widely recognized as plant growth promoters (PGPRs) (Cecagno et al., 2015). The bacteria from genus *Azospirillum* have many mechanisms to promote plant growth including nitrogen fixation and phytohormone production (Bashan et al., 2004). *Azospirillum* can secrete phytohormones such as auxins, gibberellins, cytokinins, and nitric oxide signals from plant growth promotion (Fibach-Paldi et al., 2012; Kochar et al., 2012).

The *Arthrobacter* genus can be found in ordinary soil but are also related to soils under extreme stress conditions such as deep underground areas, arctic glaciers, chemical contaminated sites and environments with high radiation rates (Fong et al., 2001). *Arthrobacter* strains can survive long periods under highly stressing conditions that may be nutrient depletion, abrupt changes in temperature, ionizing radiation, reactive oxygen species and toxic chemicals that may accumulate in the soil, such as *Endothal* herbicides (Boylen, 1973, Zevenhuizen, 1966, Jensen, 1964). *Arthrobacter* may also act as a nitrogen fixer (Smyk & Ettlinger, 1963) and are also considered PGPRs (Bianco & Roberto, 2011).

Hydrogenophaga is a hydrogen-oxidizing bacteria genus that grows autotrophically using hydrogen as an electron donor and oxygen as the terminal electron acceptor (Sato et al., 2006). We have some applications of hydrogenase as a source of reducing power for bioremediation, and regeneration of NAD(P)H for biological conversion processes (Mertens et al., 2003; Ihara et al., 2006). These microorganisms can be used to remove or reduce concentrations of contaminating metabolites, or that can cause harm in environments where they are found, both in the transformation of nitrite into nitrate and in the degradation of other substances.

It is also interesting to note that there are genera that are not unique to the rhizosphere of plants attacked by insects. However, on the other hand, they are the ones that have higher relative abundances in the more significant part of the rhizosphere after the insect's attack (Supplementary Material 1). For example, Streptomyces are higher than 10% and *Cellvibrio* higher than 1% for most rhizospheres of plants attacked by insects. At the same time, the differences in relative abundance were not significant ($p > 0.05$) between the rhizospheres of the different plant species studied to the genera cited above.

The genus Streptomyces is not only related to the production of antibiotics by its strains. They can be great competitors in natural environments (Chater, 2016). Some studies have shown that strains of Streptomyces when grown in agar culture with other bacteria, they have caused lysis in the different groups of microorganisms and can be interpreted attacking prey in potential (Kumbhar et al., 2014). This predatory action establishes perspectives on the benefits of Streptomyces for plants and holobiont relationships with insects (Chater, 2016). Thus, some authors were reporting that the inoculation of the pine rhizosphere with *Streptomyces sp* has mycorrhizal development and plant defense mechanisms stimulation, leading to an increase in resistance to powdery mildew infection (Kurth et al., 2014).

Streptomyces are also carried by ants that have a mutualism with black yeasts in host plants (Hanshewet et al., 2015).

In this relation, the plants secrete sugary exudates for the ants and yeasts, while the ants remove the predators and generally clean the host. Streptomyces produce inhibitors of other unwanted fungi that can attack ants, for example (Hanshewet et al., 2015). Streptomyces may have associations with various arthropods (Kaltenpoth et al., 2015 Hanshaw et al., 2015) that feed on plant biomass. They are highly enriched in relationships with leaf-cutting ants and beewolf wasps (Seipke et al., 2012) with the ability to completely degrade cellulose, compared to random soil isolates, suggesting the valuable relationship of the genus Streptomyces with insects (Book et al., 2016).

These observations open a new perspective because the increase of the genus Streptomyces in the rhizosphere of different plants may not only reveal us the mutualistic strategies between plants, insects, and microorganisms in the vegetal defense against opportunistic fungi and plant pathogens. This result may be related to possible microorganisms' recruitments aimed at the defense of the host against the herbivory too. However, it may also prove to be an evolutionary strategy already established among bacteria of the genus Streptomyces, plants and insects. Mechanisms such as co-evolutionary relationships between microorganisms, plants, and insects have been widely demonstrated among the most different groups of insect plants and microorganisms (Franco et al., 2019).

The genus *Cellvibrio* is often reported as degrading cellulose and chitin (Blackall, 1986, DeBoy et al., 2008). We do not discard the fact of the herbivory through *T. ni* to release in the soil the rest of the vegetable matter surplus of the feeding of the insects that can guarantee a more considerable amount of organic vegetal resources in the semi-soil where the experiments were installed. What justifies the fact that in the rhizosphere of *A. thaliana* attacked by insects the relative ambiguity of the genus *Cellvibrio* is not so superior concerning the rhizosphere of the other plant families since the biomass of *A. thaliana* is much lower than the biomass of the other plants used (Supplementary material 1).

With all this information about bacterial genera recruited in the microbiota rhizosphere after insect attack, it is possible to notice that the microorganisms recruited by the rhizosphere of plants attacked appear to be associated with degradation of organic material. Farther, the genus *Cellvibrio*, it is also associated in the auxiliary greater stability on the plant's physiology, by acting against possible opportunistic fungi such as the genus *Azospirillum*, and in other mechanisms like the degradation of substances that can establish a homeostasis imbalance to the system. Also, the genera *Hydrogenophaga* and *Arthrobacter* can even settle in environments in extreme conditions, such as the interrupted rhizosphere by direct herbivory effects on plants.

It is still unclear, but there are indications of the recruitment of several groups of bacteria that are strong candidates to operate in symbiotic relationships between plants and insects such as the

genus Streptomyces. Nitrogen fixers and PGPRs, as the strains of the genus *Azospirillum* and *Streptomyces* also appear to be recruited.

Finally, we have been discussing the adverse effects on the next generation of seedlings sown in the soil with the microbiota interference by the insect attack and possible restructuring of the rhizosphere microbiota (Figure 6). This effect was related to the non-significant modulation of the rhizosphere microbiota in Tomato after insect attack. It was not observed for Tomato plants, attacked for insects, a significant difference between the control rhizosphere. Then, the significative differences observe to fresh-biomass in most of the plant species sowing on the semi-soil with insect attack modulation was not observe to seedlings Tomatoes in the next generation.

To the loss of biomass of the second-generation plants in semi-soil that underwent the herbivorous effect, the modulation will also depend on the genotype of the plant (Figure 6). The primary and secondary metabolites present in the exudates of each of the different families studied are released into the soil by the attacked plants and remain residual in the soil. The metabolites appear to be herbivory-induced compounds in host plants (Maag et al., 2015). These compounds may accumulate in the soil at the root exudation (Okazaki & Saito, 2011). Moreover, they can be understood by plants as elicitors of the plant defense system. We assume that the plant to maintain its homeostasis would displace the energy expenditure in the early activation of the defense system against herbivory, even without the presence of the insect.

The effect would be antagonistic to the energy expenditure and the biomass production by the plants through a process of soil memory by remaining metabolites (Lapsansky et al., 2016). The drastic effect on the biomass decrease to new plants when compared to seedlings planted in semi-soil without rhizosphere microbiota interruption can demonstrate that the phenotypic effects on the host plants by the root metabolome remnant (Hartmann et al., 2008). The cited effect may be more intense in short-term soil memory than the modulation of the microbiota by the insect attack.

In the present work, we assume that more studies about the metabolome effect on host plants attacked by insects and, the forces of this soil exudation need to be better understood. However, our results presented in this work can be considered as an initial step for further studies on this exciting topic.

3.5 CONCLUSION

The effect on the structure modulation of rhizosphere microbiota by the insect attack significantly depends on the host plant.

There are specific groups of bacteria recruited in the rhizosphere microbiota after insect attack, and those groups may have relations with defenses mechanisms to help plants.

The modulation of the rhizosphere microbiota by the insect attack has negative influences on the plant biomass to the next plant generations sown in the same semi-soil.

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CONFLICT OF INTEREST

We have no declaration of conflict of interest.

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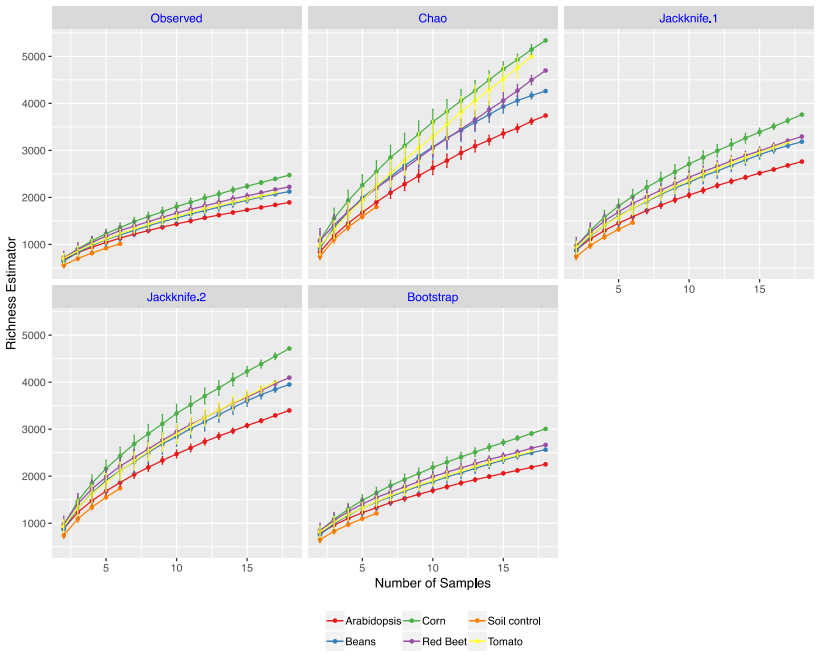
SUPPLEMENTARY MATERIAL

Supplementary chart 1: The Good's coverage by sample (captured diversity).



Supplementary table 1: Bacterial relative abundance in Corn to rhizosphere for control group (Control) and insect attack group (Insect). The amount of bacterial to relative abundance in percentage was determined by comparing the numbers of OTUs by phylum. Asterisks indicate statistically significant differences in the phyla by pairs of values (*p<0,05).

Phylum (Corn)	Control	Insect
**Acidobacteria*	40.54514718	1.62312163
Proteobacteria	38.61994829	48.55901397
Chloroflexi	1.625676213	1.163097764
Planctomycetes	1.48785468	1.744215725
Bacteroidetes	1.396479714	1.467294263
Gemmatimonadetes	1.058134447	0.84162026
**Firmicutes*	0.688139751	1.633270353
**Verrucomicrobia*	0.434188544	0.885607266
**Nitrospirae*	0.430144524	0.176942456
Chlorobi	0.226286131	0.254789844
Cyanobacteria	0.146207902	0.543053346
TM7	0.064326439	0.01509878
NKB19	0.049363564	0.030153363
WS2	0.034380801	0.017126315
Fibrobacteres	0.023508353	0
Armatimonadetes	0.014558473	0.058688014
Actinobacteria	0	27.57360448
FBP	0	0.007049412
unclassified	13.155655	13.40625276

*sig ($p<0.05$) ANOVA and LSmeans & Tukey's HSD post-hoc test

Supplementary table 2: Bacterial relative abundance in Beans to rhizosphere for control group (Control) and insect attack group (Insect). The amount of bacterial to relative abundance in percentage was determined by comparing the numbers of OTUs by phylum. Asterisks indicate statistically significant differences in the phyla by pairs of values (*p<0,05).

Phylum (Beans)	Control	Insect
Proteobacteria	40.86572	37.98241
**Actinobacteria*	27.22295	34.39742
**Cyanobacteria*	7.159581	2.339913
Bacteroidetes	4.225893	6.165838
Acidobacteria	1.871271	1.669923
Planctomycetes	1.229792	1.09588
Gemmatimonadetes	1.195692	1.109197
Chloroflexi	0.936485	0.908081
**Verrucomicrobia*	0.895643	0.614616
Firmicutes	0.201289	0.254815
Chlorobi	0.159581	0.136022
TM7	0.123392	0.049813
Nitrospirae	0.117032	0.097753
**Armatimonadetes*	0.09191	0.04552
WS2	0.015161	0.021206
Thermi	0.005759	0
OP11	0.004939	0
WPS-2	0.001997	0.003994
unclassified	13.67591	13.10761

*sig (p<0.05) ANOVA and LSmeans & Tukey's HSD post-hoc test

Supplementary table 3: Bacterial relative abundance in *A. thaliana* to rhizosphere for control group (Control) and insect attack group (Insect). The amount of bacterial to relative abundance in percentage was determined by comparing the numbers of OTUs by phylum. Asterisks indicate statistically significant differences in the phyla by pairs of values (*p<0,05).

Phylum (A. thaliana)	Control	Insect
Proteobacteria	34.06247	33.52404
Actinobacteria	19.57879	25.15943
Cyanobacteria	11.5816	2.855714
Gemmatimonadetes	3.54297	2.619227
Planctomycetes	2.553904	2.072914
Chloroflexi	2.266668	1.898165
**Bacteroidetes*	1.441793	1.060323
Acidobacteria	1.013165	0.77227
**Firmicutes*	0.323041	1.664197
Verrucomicrobia	0.312682	0.265403
Nitrospirae	0.21904	0.176594
Chlorobi	0.068737	0.019275
NKB19	0.055765	0.038319
Fibrobacteres	0.026861	0
TM7	0.0099	0.008414
Thermi	0.009331	0.01032
Armatimonadetes	0.004784	0
unclassified	22.92849	27.85539

*sig (p<0.05) ANOVA and LSmeans & Tukey's HSD post-hoc test

Supplementary table 4: Bacterial relative abundance in Tomato to rhizosphere for control group (Control) and insect attack group (Insect). The amount of bacterial to relative abundance in percentage was determined by comparing the numbers of OTUs by phylum. Asterisks indicate statistically significant differences in the phyla by pairs of values (*p<0,05).

Phylum (Tomato)	Control	Insect
Proteobacteria	29.9480023	40.7639
Actinobacteria	29.22680213	28.4478
Cyanobacteria	7.048594537	2.5655
Gemmatimonadetes	3.153788783	2.13785
Bacteroidetes	1.596288562	1.805395
Acidobacteria	1.472244321	1.187533
Chloroflexi	1.449333731	1.642541
Planctomycetes	1.191488774	1.14806
Verrucomicrobia	0.626425351	0.413871
Firmicutes	0.483288031	0.740547
Nitrospirae	0.218327367	0.22704
TM7	0.038976178	0.003177
FBP	0.007684743	0
Armatimonadetes	0.006165473	0
NKB19	0.003657297	0.00269
Chlorobi	0	0.012359
OP11	0	0.004287
WS2	0	0.003348
unclassified	23.52893242	18.8941

ns (p>0.05) ANOVA and LSmeans & Tukey's HSD post-hoc test

Supplementary table 5: Bacterial relative abundance in Red Beet to rhizosphere for control group (Control) and insect attack group (Insect). The amount of bacterial to relative abundance in percentage was determined by comparing the numbers of OTUs by phylum. Asterisks indicate statistically significant differences in the phyla by pairs of values (*p<0,05).

Phylum (Red Beet)	Control	Insect
Actinobacteria	35.36894	32.16462
**Proteobacteria*	29.86109	39.14406
Cyanobacteria	12.83892	0.147189
Chloroflexi	1.547948	1.455469
**Acidobacteria*	1.407708	0.777771
Gemmatimonadetes	1.169642	1.619869
**Bacteroidetes*	1.102344	2.674185
Planctomycetes	1.061522	0.983579
**Firmicutes*	0.653043	2.399669
Verrucomicrobia	0.328709	0.326319
Nitrospirae	0.190591	0.111575
**Chlorobi*	0.143811	0.008221
WS2	0.023129	0
Armatimonadetes	0.015621	0
Fibrobacteres	0.014038	0
TM7	0.013789	0.019478
WS3	0.00334	0
NKB19	0	0.0108
**WPS-2*	0	0.002433
unclassified	14.25582	18.15477

*sig (p<0.05) ANOVA and LSmeans & Tukey's HSD post-hoc test

Supplementary table 6: Bacterial genus that are shared to all plant's species in control group (58) and insect attack group (60) by the OTUs richness match. The OTUs distribution is showed at the Venn Diagram (Figure 5).

Genus shared to all plant's species present in control groups and insect attack group (49)	Genus shared in all plant's species exclusive in insect groups (11)	Genus shared in all plant's species exclusive in control groups (9)
Nannocystis	*Arthrobacter*	*Sorangium*
Ramlibacter	*Azospirillum*	*Lamia*
Nocardia	*Nonomuraea*	*Actinocatenispora*
Novosphingobium	*Hydrogenophaga*	*Fimbriimonas*
Sphingomonas	*Geodermatophilus*	*Bradyrhizobium*
Mycoplana	*Burkholderia*	*Steroidobacter*
Pedomicrobium	*Terracoccus*	*Plesiocystis*
Streptomyces	*Achromobacter*	*Hyphomicrobium*
Salinibacterium	*Agrococcus*	*Bosea*
Pseudomonas	*Virgibacillus*	
Sphingopyxis	*Kibdelosporangium*	
Cellvibrio		
Dyella		
Blastococcus		
Phycicoccus		
Kaistobacter		
Mycobacterium		
Opitutus		
Rhodoplanes		
Mesorhizobium		
unclassified		
Microbacterium		
Kribbella		
Lysobacter		
Nocardioides		
Limnohabitans		
Methylibium		
Rhodocytophaga		
Pseudoxanthomonas		
Rubrobacter		
Nitrospira		
Agrobacterium		
Shinella		
Rhodanobacter		
Asticcacaulis		
Phenylobacterium		
Fluviicola		
Planctomyces		
Phaeospirillum		
Nitrobacter		
Balneimonas		
Devosia		
Skermanella		
Arenimonas		
Agromyces		
Bacillus		
Amycolatopsis		
Luteimonas		
Dokdonella		

*sig ($p<0.05$) ANOVA and LSmeans & Tukey's HSD post-hoc test

Supplementary table 7: Relative abundance by genus to all Crop Plants after herbivorous insect attack. The genus above may be presents in the control group. *sig (p<0.05).

Genus presents in insects' groups	Corn	Beans	*A. thaliana*	Tomato	Red Beet
Streptomyces	16.10247061	21.43809666	12.4017723	16.50480642	16.77079024
Cellvibrio	6.517524087	2.175939737	0.024523778	1.433975294	1.853593211
Azospirillum	5.981089234	0.069046672	0.01539711	0.055317997	0.057418457
**Skermanella*	2.915815876	0.991862238	0.602094935	1.095758199	1.189180589
Rhodoplanes	2.056102493	2.13508519	1.944648855	1.506193096	1.053248475
**Devosia*	1.857161009	1.130925152	1.427660656	0.94725095	2.376942456
**Agrobacterium*	1.542964952	1.036429329	0.40918081	0.395849907	1.788305489
Pseudomonas	1.246033324	0.377519226	4.58858172	3.468625696	0.898289578
**Kaistobacter*	1.161534297	0.915514784	0.425749138	0.545213471	0.773382392
Sphingopyxis	1.115912004	1.134969173	1.695118448	0.801981128	0.960739857
**Bacillus*	0.820466499	0.193035667	0.530959958	0.474288429	1.318191461
**Balneimonas*	0.729360028	0.767642867	0.267783744	0.449471847	0.62456245
Mycobacterium	0.663075665	0.861790639	0.381320163	0.622243216	0
**Achromobacter*	0.578311456	0.034821665	0.011447008	0.146993503	0.234645982
Methylibium	0.521816715	0.429295943	0.245392469	0.255043976	0.192243437
Pseudoxanthomonas	0.451698267	0.667213604	0.205687041	0.2987658	0.392787059
**Sphingobium*	0.404341245	0.017485415	0	0.354813047	0
**Kribbella*	0.401518165	0.538318748	0.267286529	0.322985724	0
Nannocystis	0.393717405	0.16495956	0.094830063	0.202101565	0.310289048
Phenylobacterium	0.386568549	0.54675484	0.301616503	0.137098913	0.317720764
**Nitrobacter*	0.384927738	0.679262795	0.186964112	0.409628304	0.404057279
**Microbacterium*	0.38113785	0.136551976	0.17977106	0.144894148	1.083678069
Arthrobacter	0.352697118	0.258378083	0.172953682	0.261005038	1.568947229
**Planctomyces*	0.351006585	0.26286131	0.940533015	0.315566163	0.250450809
**Mesorhizobium*	0.34067555	0.419078162	0.934124459	0.24393397	0.712311058
Myxococcus	0.327256254	0	0	0	0
**Dokdonella*	0.31364912	0.443748343	0.118801379	0.243569345	0.181801909
Opitutus	0.309500133	0.137090626	0.121917263	0.154623	0.140904269
Nocardia	0.303196544	0.381621254	0.191798153	0.419169318	0.086396181
**Lysobacter*	0.295390259	0.317646181	0.274998895	0.557395253	1.160129939
Phycicoccus	0.28453991	0.23088206	0.129508088	0.074433174	0.231112437
**Paracoccus*	0.281827323	0.210264187	0	0.128149032	0.324058605
Sphingomonas	0.281109122	0.386336516	0.106138955	0.312477902	0.320597985
Brevundimonas	0.277363431	0.110771347	0	0.320764828	0.296618934
Flavobacterium	0.276589985	5.414222023	0	0.56806329	0.562795015
Rhodanobacter	0.26733625	0.474998343	0.135822726	0.261159728	0.211164147
**Paenibacillus*	0.254353399	0	0.018623486	0	0.204216388
Shinella	0.236127685	0.182560992	0.483575312	0.356161054	0.723289578
**Plesiocystis*	0.228382171	0.062226531	0.260380757	0.082416247	0
Hydrogenophaga	0.219714046	0.178434102	0.112818218	0.062577345	0.134871387
**Limnohabitans*	0.216819146	0.352608724	0.068118536	0.186848095	0.173183506
Rubrobacter	0.216205914	0.167023005	0.260585168	0.339089985	0.229262795
Mycoplana	0.210719968	0.132856006	0.340686599	0.185903385	0.296201273
**Steroidobacter*	0.197582427	0.028415871	0.075317113	0.055152258	0
**Luteolibacter*	0.188776187	0.147979647	0	0.03424158	0.044643331
Nitrospira	0.176942456	0.092855012	0.1318671	0.169207991	0.088113233
**Sorangium*	0.163230354	0	0.028667241	0.052157916	0
**Bosea*	0.158744365	0.424970167	0	0.100912667	0.134400689
Rhodococcus	0.156324582	0.028051246	0	0.035865818	0.106012994
**Amycolatopsis*	0.154656148	0.332728388	0.105708035	0.284015071	0.1039048
Rhodobacter	0.154291523	0	0	0	0.02167197
**Thermomonas*	0.132038363	0.329380469	0	0.018761602	0.025815434
Salinibacterium	0.131701361	0.205896977	0.020258773	0.174158048	0.037437019
**Rubellimicrobium*	0.128176655	0	0	0.007408512	0.03656192
**Phaeospirillum*	0.127392159	0.058729448	0.354674931	0.194599134	0.103805357
Hyphomicrobium	0.125475117	0.022225537	0	0.017032396	0.046075312
**Novosphingobium*	0.121237735	0.322734354	0.029020817	0.191610316	0.522679661
**Agromyces*	0.117138469	0.048768563	0.602730266	0.358205162	0.238332007
Planomicrobium	0.103000972	0	0.012695571	0	0
Clostridium	0.101879475	0	0.005695881	0.003723592	0.01097852
Arenimonas	0.101862901	0.18070472	0.575234244	0.126397728	0.112437019
**Burkholderia*	0.101111553	0.054544551	0.004253956	0.010789578	0.016945107

Stenotrophomonas	0.095542738	0.088380072	0	0.1134425	0.073793423
Delftia	0.095531689	0.163285601	0	0.073295103	0.018794749
Chelativorans	0.089808185	0.033205715	0	0	0
**Bradyrhizobium*	0.089581676	0.208035004	0	0.087117696	0.055509149
Dyella	0.089399364	0.093136767	0.009623884	0.04848957	0.023879608
**Acidovorax*	0.087537567	0.116621917	0	0.014656811	0
Geodermatophilus	0.087333157	0.054801445	0.036258066	0.026363476	0.022480774
**Iamia*	0.086045921	0.180257226	0.055594228	0.031562141	0
Nonomuraea	0.082653805	0.028018099	0.036153098	0.052815345	0.093556086
**Nocardioides*	0.082377574	0.299787855	0.030799744	0.139446875	0.063338637
Adhaeribacter	0.074753602	0.010267502	0	0.022534916	0.007067091
Luteimonas	0.06951074	0.024123243	0.087355255	0.090664501	0.209002917
Virgibacillus	0.068538407	0.031879806	0.097034385	0.007060461	0.060441527
**Dyadobacter*	0.064621453	0.080764386	0	0.0173473	0.002519226
Rhodoferax	0.06373199	0.016126359	0	0.018822372	0
**Fimbriimonas*	0.058688014	0.045520088	0	0	0
Ochrobactrum	0.054666092	0	0	0.023347034	0.012881199
Fluviicola	0.051240829	0.047873575	0.002088305	0.050434235	0.024913816
Ramlibacter	0.051008795	0.031912954	0.032888049	0.076190003	0.038046937
Rhodocytophaga	0.04985415	0.020758751	0.003778839	0.00556329	0.008830549
Actinomadura	0.049097277	0	0.035479095	0.027871696	0.038524264
Blastococcus	0.045434456	0.043257757	0.133021745	0.159490188	0.048329356
Actinoallomurus	0.044191417	0.19429528	0.148324936	0.059384116	0
Sinorhizobium	0.043395872	0	0	0	0.036058075
Erwinia	0.042114161	0	0	0	0.058777513
**Asticcacaulis*	0.042103111	0.249204455	0.009651507	0.118469902	0.287894458
Aminobacter	0.042009193	0	0.054312517	0	0.059142137
Pirellula	0.034749845	0.011966322	0.040976089	0.020639972	0
Pedobacter	0.034434942	0.002162888	0	0.025302749	0
**Arthrospira*	0.033617299	0.533620061	0	0.046699593	0
Rathayibacter	0.033578626	0.115412026	0	0.031330107	0.054793158
Kibdelosporangium	0.030700301	0.053865023	0.04624105	0.055063865	0.059486874
Coprococcus	0.030512464	0.012372381	0	0	0.01344471
Sphingobacterium	0.029352294	0	0.006298064	0.36474631	0.017475471
Prosthecobacter	0.026717051	0	0	0.020507381	0
Rubrivivax	0.024910501	0	0	0	0
Sedimentibacter	0.024518253	0	0	0	0
Caulobacter	0.023954742	0	0	0	0
Kaistia	0.023037656	0	0	0.007833908	0
Candidatus_Entotheonella	0.022932688	0	0.010778529	0	0
Magnetospirillum	0.022628834	0	0.017916335	0.007635022	0.008651551
Agrococcus	0.021601255	0.024802771	0.035180765	0.018966013	0.102771148
Marinibacillus	0.021402369	0	0	0	0
Variovorax	0.018778176	0	0	0	0
Candidatus_Xiphinematobacter	0.016778264	0	0	0	0
Microbispora	0.016446787	0.018769889	0	0.039644657	0
Janthinobacterium	0.015032485	1.396529435	0	0.078819721	0
Terracoccus	0.014154071	0.050318218	0.075223195	0.051351321	0.034089101
A17	0.013043622	0	0.018369354	0.018535092	0.007464863
**Cellulomonas*	0.012899982	0.087601101	0	0	0
**Janibacter*	0.012259127	0	0.0632182	0.143082074	0.487410501
**Actinoplanes*	0.011938699	0.290183307	0	0.050130381	0
Pseudonocardia	0.011806108	0	0.028059533	0.010342084	0.008552108
Roseomonas	0.011176302	0.04252022	0	0.006474852	0
Gemmatimonas	0.010629364	0	0	0	0
**Actinocatenispora*	0.010618315	0.138118205	0.040340758	0	0.06097852
Edaphobacter	0.010369707	0	0	0	0
**Pedomicrobium*	0.009668081	0.014096062	0.057207416	0.172964731	0.10230708
Terribacillus	0.008933307	0	0	0	0
Gemmata	0.008452665	0.011775723	0	0	0
Flavihumibacter	0.007839433	0	0.015618094	0	0
**Bdellovibrio*	0.006226244	0.114831941	0	0.004756696	0.002041899
Exiguobacterium	0.005883718	0	0.052588836	0.02868934	0.017667727
Actinocorallia	0.005878193	0	0	0	0
Alicyclobacillus	0.005176567	0	0.006563246	0.010076903	0
Methylophaga	0.004182136	0	0	0	0.032398568
DA101	0.004027446	0	0	0	0

Pelosinus	0.003800937	0	0	0	0
Telmatospirillum	0.000917087	0	0	0	0
Rhizobium	0	0.35768861	0	0.148258641	0
**Serratia*	0	0.161470764	0	0.350238663	0.991288783
**Azohydromonas*	0	0.123690666	0	0	0
**Pontibacter*	0	0.047674688	0.028219747	0.041003713	0.212642535
Aeromicrobium	0	0.025101101	0.112597233	0	0
Ardenscatena	0	0.020394126	0.005126845	0	0.025384513
Methylotenera	0	0.018977062	0	0	0
Edaphobacter	0	0.013623707	0	0	0
Flavisolibacter	0	0.008726134	0	0	0
Thermogemmatispora	0	0.003969438	0	0	0
Pelomonas	0	0.001731968	0	0	0
**Yonghaparkia*	0	0	0.756933395	0.021590206	0
**Promicromonospora*	0	0	0.272673031	0.014916468	0.021267568
**Cellulosimicrobium*	0	0	0.167594802	0.07615133	0.480648369
Cryocola	0	0	0.135076903	0.028611995	0
Prauseria	0	0	0.112950809	0	0
Xylanimicrobium	0	0	0.062058031	0	0.702618669
Actinotalea	0	0	0.036037081	0	0
**Parvibaculum*	0	0	0.034650402	0.066665562	0.020611244
Pilimelia	0	0	0.014446875	0	0
Inquilinus	0	0	0.011408336	0	0
Catellatospora	0	0	0.011336516	0	0
Sporichthya	0	0	0.009480244	0	0
B-42	0	0	0.007016264	0	0
**Nitrosovibrio*	0	0	0.006966543	0.033114558	0
Nostoc	0	0	0.006270441	0	0
Truepera	0	0	0.003154557	0	0
Candidatus_Amoebophilus	0	0	0.00173473	0	0
Denitrobacter	0	0	0	6.07015712	0
Chitinophaga	0	0	0	0.01359056	0
Candidatus_Solibacter	0	0	0	0.011590648	0
Solibacillus	0	0	0	0.009076947	0.038782816
Sediminibacterium	0	0	0	0.007375365	0.002108194
HTCC	0	0	0	0.005911341	0
**Chryseobacterium*	0	0	0	0.004712499	0.033267038
Roseococcus	0	0	0	0.004635154	0
Acinetobacter	0	0	0	0.003994299	0
Candidatus_Koribacter	0	0	0	0.0028949	0
Terribacillus	0	0	0	0.002193273	0.049801114
Klebsiella	0	0	0	0	0.392117475
Niabella	0	0	0	0	0.036237072
Glycomyces	0	0	0	0	0.034559798
Patulibacter	0	0	0	0	0.024045346
Methylocaldum	0	0	0	0	0.009718907
Cryocola	0	0	0	0	0
unclassified	42.40409264	45.39237768	64.09202908	53.12450831	52.61339167

*sig ($p<0.05$) ANOVA and LSmeans & Tukey's HSD post-hoc test

4 GUT MICROBIOME IN *Trichoplusia ni* MAY INFLUENCE THE FEED PREFERENCE IN SUBSEQUENT INSECT GENERATIONS

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ABSTRACT

Insects are the most abundant animals on the planet, and the microbiota inside their intestine provides benefits and pathological interactions. Insects are known plant pests, and some of them such as *Trichoplusia ni* feed widely on a variety of crops of economic importance. In this study, *T. ni* was fed for four generations on distinct diets: (1) *Arabidopsis thaliana* Col-0 leaves; (2) *Solanum lycopersicum* Rutgers leaves; and (3) highly artificial caloric diet. Subsequently, the insects were given a choice to feed on either one of the three diets. In general, all populations preferred the diet that they were fed for four generations. The microbial composition of the insects was evaluated to determine if the diet influenced the structure of the microbial communities. The phyla Proteobacteria, Firmicutes and Actinobacteria were the most abundant regardless of the diet. The population fed with *A. thaliana* presented a prominence of the genus *Shinella*, *Terribacillus*, and *Propionibacterium* that have been shown to degrade glucosinolates; which are insect deterrent compounds produced by *A. thaliana*. Interestingly, these genera were also present in the leaves of *A. thaliana* in considerable proportion. The population fed on *S. lycopersicum* was enriched in *Agrobacterium* and *Rhizobium*. These microbial members can degrade alkaloids; which are produced by *S. Lycopers*icon. These two genera were also abundant in the leaves of the vegetable. Finally, the population fed a hypercaloric diet presented the genus Pseudomonas in high abundance, which is widely known to be a pathogen for a variety of invertebrate. This investigation provides information on the relationships between generalist insects, the types of diet, their intestinal bacterial communities, and how these communities may affect the feeding preference of the insects.

Keywords: Insect; Bacterial diversity; Feeding preference; Diet restriction

4.1 INTRODUCTION

High throughput sequencing analyses have revealed a comprehensive panorama of the diversity of bacterial communities living in the intestine of a variety of insects (1, 2, 3). It is often seen that the gut of these animals is predominantly dominated by Proteobacteria followed by Firmicutes (1, 3). *Gammonaproteobacteria* (Proteobacteria) is capable of increasing the tolerance of insects to high temperatures and specificity to certain host plants (4). Proteobacteria living in the gut of Hymenoptera insects provided resistance to pathogenic parasites and fungi (5). Some Firmicutes such as Spiroplasma can manipulate host reproduction, inducing male-killing, feminization, and parthenogenesis (6, 7).

It has been found that the insects' diet, environmental habitat, and stage of development strongly regulate the colonization and assembly of bacterial communities within the gut (1, 3, 8). For instance, when young larvae of *Spodoptera littoralis* were fed with toxic beans containing cyanogenic glycosides the larval mortality increased dramatically. Accordingly, the intestinal microbiota of the poisoned *S. littoralis* larvae was composed of 25% of *Enterococcus mundii* and 50% of *Pantoea agglomerans*. However, the intoxicated larvae of *S. littoralis* showed a recovery capacity after four days of ingesting a diet rich in barley; and presented *Clostridia* and *Enterococcus casseliflavus* as the dominant groups in their intestinal microbiota (9). In termites, it has been reported a correlation

between bacterial communities and the recalcitrance of the plant diet (10). As such, the secondary metabolites present in the plant diet modulated the microbial community (10). Besides, other studies have shown that changes in the termite diet affected the functional genes expressed in the termite gut (11, 12).

There is limited information about the gut microbiome composition of insects that are pest crops such as Lepidoptera. Additionally, the insect microbiota gut has been the subject of studies that seek to understand the relationship between the resistance of herbivores to possible plant compounds or pathogenic microorganisms. As an example, *Bacillus thuringiensis* has been shown the greater or lesser effect as an insect pathogen depending on the intestinal microbiota associated with the insect host (13).

The same type of protective microbiome effect can be seen against *Baculoviral*. The insect's natural microbiota can play a modulating and protective role against baculoviral infections by stimulating the basal level of insect immunity in *Spodoptera exiguous* larvae (14). Some endogenous intestinal bacteria in insects detoxify secondary metabolites in plants that may be harmful to the host and can protect them from pathogen colonization (8,13). The endogenous microbiome is also involved in the insect's nutrition, maintenance of fitness (8), homeostasis against plant defense system (15) and as targets for novel strategies to control pests' insects.

Trichoplusia ni (*Lepidoptera*; Noctuidae) larvae are generalist herbivores and considered to be critical agricultural pests, which feed on more than 100 species of plants, including tomato, potato, corn, cotton, and many other cultivated plants (19). Food and nutrient uptake pass rapidly through the intestines of the larvae, usually within a few hours, and most of the resident bacterial strains present in the viscera of these insects are unknown (19). However, it is known that *Trichoplusia ni* larvae can detect and respond to microbes present in the food, using epithelial tissue of the midgut as a sensing organ (45). Generalists insects, like *Trichoplusia ni*, have genetic adaptations that allow the expression of digestive proteins, which metabolize plant compounds that could be potentially toxic to the insect (20; 21).

In this study, we sought to understand how different diets may change the gut microbiome of *T. ni* and whether these microbial changes might contribute to the generalist behavior of the insect. Thus, we fed *T. ni* with three distinct diets for several generations and then explored the composition of their gut microbiome.

4.2 MATERIAL AND METHODS

4.2.1 Insect growth conditions

An initial colony of *Trichoplusia ni* larvae in the third instar was obtained from Frontier Agricultural Sciences (Newark, DE), a company that maintains and sells uniform insect colonies fed

on an artificial diet without the addition of antibiotics. The larvae were then divided into three groups; each population was composed of twenty individuals and was kept in sterile Magenta® boxes. Each Magenta box® had on average five larvae. The Magenta® boxes were kept on shelves and covered with Nylon® screens that prevented the insects from moving from the Magenta® boxes to the outside.

The larvae were fed, every day with tomatoes leaves, *Arabidopsis thaliana* leaves or artificial diet cubes (4g per Magenta® box by day) and kept at room temperature (26 °C), 75% humidity and cycles of 16h light, 8h dark. When the larvae reached the pupae stage, they were transferred to 1/2 Gal and ¾ " plastic bottles until they reached the moth stage. The moths were fed daily through filter paper soaked with 100µl (per plastic bottles) of a 9: 1 solution of Milli-Q® water and honey. The moths on the plastic bottles were kept under free crossing. The females then laid their eggs on clean surface filter papers kept inside the bottle and, such was used to close the central hole bottle. The eggs deposited in filter papers at each cycle were transferred without sterilization to sterile Magenta® boxes. The eggs were incubated in Magenta® boxes until the moment of hatching. After the emergence of *T.ni* larvae in the first instar in the Magenta® boxes, *Arabidopsis* leaves, tomato leaves or artificial diet cubes were daily delivered in the boxes. The larvae in each box were fed respectively according to the population from which they originated.

4.2.2 Plant growth conditions

4.2.2.1 *Arabidopsis thaliana* Col-0

Arabidopsis thaliana ecotype Col-0 seeds were purchased from Lehle Seeds (Round Rock, TX). The seeds were surface sterilized in 2% sodium hypochlorite for 2 min, followed by three washes with sterile distilled water. The surface-sterilized seeds were placed directly on the fibrous peat moss Promix Bx® substrate mixed with vermiculite (1:1) for germination. The plants were grown in plastic trays and incubated in a growth chamber. A photoperiod of 16 h light and 8 h dark at 25 +/- 2 ° C was maintained for growing conditions. After growing *A. thaliana* for 15 days, leaves and stems were used as food for the insect populations.

4.2.2.2 *Solanum lycopersicum* Rutgers

The seeds were surface sterilized in 2% sodium hypochlorite for 2 min, followed by three washes with sterile distilled water. Surface-sterilized seeds were placed directly on the fibrous peat moss Promix Bx® substrate mixed with vermiculite (1:1) for germination and grown in plastic trays. A photoperiod of 16 h light and 8 h dark at 28 +/- 2 °C was kept for growing conditions. Rutgers tomatoes were grown for 30 days before excision of leaves and stem as food for the insect populations.

4.2.3 Food preference trial

After four complete life cycles of *Trichoplusia ni* fed on a particular diet, the populations were separated according to diet, and the insects were exposed to a food preference test with a choice between the three different food sources as described by Raffa, K.F., Havill, N.P. & Nordheim, E.V. (2002). In the choice trial, twelve replicates were used for each population, and each replicate was composed of fourth instar larvae. The larvae were devoid of food for 24h before initiating the choice study. The larvae were arranged in a Petri® dish (140 mm diameter) and lined with filter paper to maintain humidity. Three Empendorff® tubes of 2ml were placed in the Petri dishes; each tube was filled separately with pieces of tomato leaves, fragments of *A. thaliana* leaves or parts of the artificial diet.

All three tubes containing food were weighed before the start of the experiment and after 24 hours to determine how many milligrams each larva had consumed from each diet after 24h. The difference in weight of specific diets at 0h and 24h was considered the measurement of choice in this study. It should be noted that some larvae fed on more than one diet during the 24h period. Each set of plates, with all treatments were arranged randomly. The trays were kept in growth chambers (*Percival Scientific*®) regulated at 25^{o}C +/- 1^{o}C, 70 +/- 10% relative humidity, and 16 light hours.

After 24h the trays were removed from the growth chambers and weighed. *Trichoplusia ni* larvae were also weighed before being placed in the Petri dishes and 24h later to verify if there was weight gain among the three different populations tested. The experiment was repeated twice. The data of all the tests were subjected to analysis of variance (F test) and the means compared to the Tukey, at the 5% probability level. The free software *Past* and *Sisvar* were used for the statistical analyses and software *Origin 8.5* for the elaboration of the charts.

4.2.4 DNA extraction

4.2.4.1 Gut insect DNA extraction

Samples were obtained from fourth instar larvae, after three complete cycles of each of the populations. Each gut was removed with sterile tweezers, and the gut tissue was then homogenized by shaking in a sterile tube containing glass beads (0.5 mm diameter) and 0.5 ml of buffer PBS pH 7.5 for 15 minutes using a Vortex. Intestinal DNA of 20 larvae per replicate was extracted using the Qiagen DNA kit (Power Soil®) according to the manufacturer's instructions. A total of three replicates per treatment were obtained. The DNA concentration was measured by a Nanodrop® spectrophotometer (*Thermo Fisher Scientific*). The extracted DNA was stored at -20 °C for further analysis. The same population used for the food preference trial was used for DNA extraction. Due to a large number of individuals needed per replicate, the third generation was chosen for DNA extraction.

4.2.4.2 Plant and Artificial Diet DNA extraction

Three replicates were used for each experimental group in the extraction of total DNA from the plants and artificial diet to compare the bacterial communities in plants and those found in the gut of *Trichoplusia ni*. Each sample was composed of 50 mg of plant leaves or artificial diet according to the experimental group to which it belonged. The total DNA was extracted using the MoBio kit (PowerPlant® DNA Isolation Kit) according to the manufacturer's instructions. The DNA concentration was measured by a Nanodrop® spectrophotometer (*Thermo Fisher Scientific*). The extracted DNA was stored at -20°C for further analysis.

4.2.5 Sequence *Illumina Miseq* of bacterial 16S rRNA genes

4.2.5.1 The *PCR* amplification for *Illumina Miseq*

The sequencing of all gut samples was performed according to standard protocols devised by Illumina. The V3 region of the 16S rRNA gene was targeted to characterize and estimate bacterial communities present in the gut sample. Briefly, primer set of forwarding primer F5′–TCGTCGGCAGCGTCAGATGTGTATA AGAGACAGCCTACGGGAGGCAGCAG-3′ and reverse primer R5′-GTC TCGTGGGCTCGGAGATGTGTATAAGAGACAGATTACCGCGGCTGC TGG-3′ was used to amplify the 230 base-pairs (bp) V3 region of the 16S rRNA gene (Klindworth et al., 2013).

Polymerase chain reaction (PCR) was performed in 25 µl reaction volume containing 1 µl of template DNA (5 ng/ µl), 5 µl of each primer (1 µM) and 12.5 µl of 2× KAPA HiFi HotStart ReadyMix. The PCR was done at following conditions; initial denaturation at 95 °C for 3 min, followed by 25 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and ended with a final extension step at 72 °C for 5 min. Two microliters of the amplified product of 230 bp were analyzed on the Bioanalyzer. PCR clean-up was done by AMPure XP beads to purify the 16S rRNA gene V3 amplicon away from free primers and primer dimer species.

4.2.5.2 *Miseq* library preparation

The Miseq library was prepared by standard library construction protocol as detailed in the "16S metagenomics sequencing library preparation procedures" available at https://support.illumina.com/downloads/16s_metagenomic_sequencing_library_preparation.htm. The amplicons from V3 region rRNA16S were quantified in each reaction mixture and, were utilized equimolar concentrations of the Illumina sequence adapters and index primers (Nextera XT Index kit) in PCR emulsion to generate amplicon libraries. The steps were finalized by a PCR clean up.

Then, the prepared library was validated by running an aliquot through the *Bioanalyzer* chip and, denatured by alkaline (0.1 N NaOH) such as heat treatment. PhiX (5%) was used to serve as an internal V3 library control for low diversity libraries in Miseq sequencing. MiSeq libraries were quantified and then subjected to 150-nucleotide paired-end multiplex sequencing (MiSeq v3 reagent kits) on GAIIx sequencer at Genotypic Technology Pvt. Ltd.

4.2.5.3 Bacterial 16S rRNA gene sequence analysis

Bacterial community alpha diversity index (Shannon) was generated using *myPhyloDB* (23). Bacterial OTUs were assigned to ecological guilds using the bacterial guide at the phyla and family levels. For beta diversity, microbial community composition (relative OTU or ecological guild abundance data) was analyzed using principal coordinates analysis (PCoA) based on the Bray-Curtis distance dissimilarity. A complementary non-parametric multivariate statistical test, including analysis of similarities (perMANOVA) and non-parametric univariate ANCOVA analyses, were used to test the differences in microbial communities with the Bray-Curtis distance and 999 permutations by *myPhyloDB*. Linear discriminant effect size (LEfSe) analysis was used to identify bacterial taxa that were significantly associated with each treatment using a threshold of with an alpha value of 0.05 for the LSmeans & Tukey's HSD post-hoc test.

The tables of OTUs generated as output in *myPhyloDB* were then used to verify at the family level the OTUs shared between each experimental group studied. The shared and unique OTUs among treatments were counted, and their distributions were shown in a Venn diagram with the *'jvenn'* that is a plugin for the '*jQuery' Javascript* library (24). The tables with the shared and unique OTUs among treatments at the family level were prepared with a tool that may be found at URL: http://bioinformatics.psb.ugent.be/webtools/Venn/.

4.3 RESULTS

4.3.1 Food Preference

The experiment of food preference was carried out for three different populations of insects, originally from a parental population fed with artificial diet, as described in the methodology. The larvae that were used in the food preference test came from the different populations (*Arabidopsis*, *S. lycopersicum*, artificial diet), maintained for four complete cycles in a restricted diet. At the end of the food preference test, no significant differences were found for insect weight among the three different populations (Fig. 1).

The difference of weights in the fed consumption of each diet gave a clear representation of preference to a particular diet according to a specific population. The population fed with *Arabidopsis*

preferred the artificial diet (51.7%) and *Arabidopsis* (39.4%) (Fig 2A). In contrast, the insects fed on *S. lycopersicum* preferred *S. lycopersicum* (70.6%) (Fig 2B), and the population fed on artificial diet preferred the artificial diet as well (Fig 2C). It should be noted that the rate of feeding was higher in the artificial diet population compared to the other two groups.

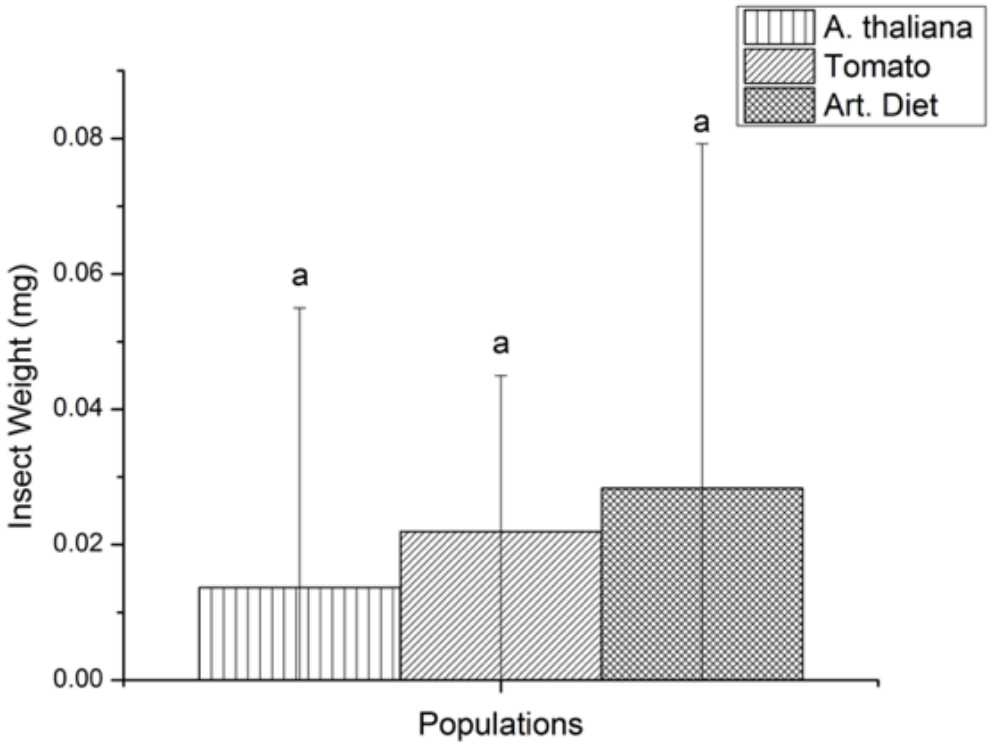

Figure 1. The difference in the weight of the insects after 24h of preferential feeding with the possibility of choice. The different populations are represented in the x-axis. ANOVA (test F). Each letter at the top of the bars represents the mean difference by the Tukey test with a significance $p < 0.05$.

4.3.2 *Illumina* sequencing data

After filtering reads by basal quality control and removing singleton OTUs, *Illumina Miseq* sequencing generated 52,890 quality bacteria sequences in total. Samples with fewer than 3000 reads were excluded from the analysis. The average read length of bacteria for the 16S rRNA subunit was 601 bp. The Good's coverage of each sample, which reflects the captured diversity, was higher than 98.01% for all samples. Rarefaction curves of OTUs at 97% sequence similarity of all samples tended to approach the saturation plateau. Therefore, the sequencing depth was adequate to assess the diversity of bacterial communities of *Trichoplusia ni* gut, *A. thaliana*, and *S. lycopersicum* leaves, and the artificial diet.

4.3.3 Microbiome composition of the diets

The microbiome composition was found to be distinct among the three diets based on PCoA analysis. Samples of the leaves of *A. thaliana*, leaves of *S. lycopersicum* and the artificial diet formed distinct and significantly different clusters (p = 0.004) (Fig 3C).

Most OTUs shared at the phylum level among the different diets were Cyanobacteria, Proteobacteria, and Firmicutes (Figure 3A). For *A. thaliana* leaves, 93% of the relative abundance of OTUs were identified as the phylum Cyanobacteria, followed by the phylum Proteobacteria (7%), and on *S. lycopersicum* leaves the phylum Cyanobacteria (87%) and Proteobacteria (12%) were also highlighted. Within the composition of the artificial diet, we found the same phyla present in plant samples: Cyanobacteria (55%) and Proteobacteria (44%). Besides these, we found a small percentage of the Firmicutes phylum (0.3%) in the artificial diet. The relative abundance, for the mentioned phyla to each diet, presented significant differences ($p < 0.05$) the *p-value* and the whole date are showing in table 1.

The different percentages between the relative abundance for the genera level in each of the diet treatments presented significant differences through the univariate test as well ($p<0.05$). The genus with significant difference in relative abundance between the different diets were Agrobacterium (p=2.7e-05), Azospirillum (p=0.0229), Delfitia (p=0.00092), Propionibacterium (p=0.027), Pseudomonas (p=6.98e-07), Sphingobium (p=0.00565), Streptomyces (p=0.0116), unclassified order Streptophyta (p=6.29e-07), family Moraxellaceae (p= 4.19e-07), family Enterobacteriaceae (p=0.028) and family Caulobacteraceae (p=3.42e-05).

The supplementary table 1 shows in detail the percentage for each genus target by OTUs and percentages of other groups that present a relative abundance lower than 0.01% for each diet.

4.3.4 Gut Microbiome composition of populations

A significant difference was found with a confidence interval of 0.95 in the grouping between the OTUs communities present in the intestine of each population (p=0.041) by PCoA. These clustering patterns suggest that there are consistent differences in bacterial community composition attributed to feeding the larvae three different diets (Figure 2d).

For the microbiome at the *T. ni* gut to different populations, were detected the phyla Proteobacteria, Actinobacteria, Firmicutes, Cyanobacteria, Acidobacteria, Bacteroides and Chloroflexi (Fig 3B). The percentages of the remaining clusters for each population and rates of unclassified OTUs that do not appear on the bar chart (Figure 3B) are presented in table 2. No significant differences were detected by ANCOVA test between each phylum to different populations.

However, the different percentages between the relative abundance for the mentioned genus level, in each of the diets presented significant differences (ANCOVA & Tukey's HSD post-hoc test $p<0.05$). The genus with significant difference in relative abundance between the different diets were

Achromobacter (p=0.0392), Enterococcus (p=0.0418), Mesorhizobium (p=7.86e-06), Microbacterium (p=0.0177), Shinella (p=0.000231), Veillonella (p=0.0367), unclassified family Nocardioidaceae (p=0.0158), Comamonadaceae (p=0.00597), Streptomycetaceae (p=0.03), Alcaligenaceae (P= 0.000218) and Planococcaceae (p= 0.0212). The supplementary table 2 shows in detail the percentage for each genus target by OTUs and percentages of other groups that present a relative abundance for each different population of *Thichoplusia ni* by genus.

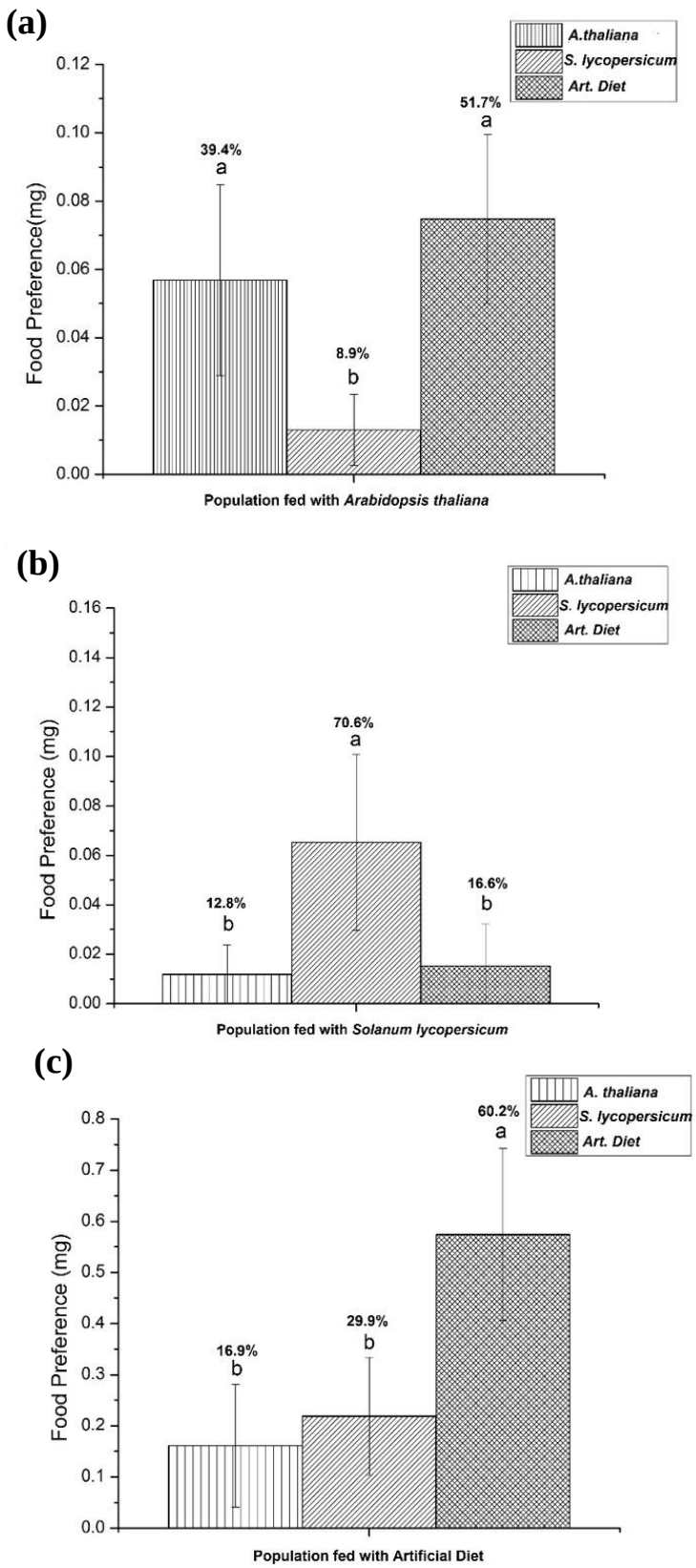

Figure 2. Results of the experiment feeding preference for populations of *Trichoplusia ni* fed during three cycles with different diets. The different populations are represented in the x-axis. ANOVA (test F). Each letter at the top of the bars represents the mean difference by the *Tukey post-hoc* test with a significance $p<0.05$. The percentages above the bars in each graph correspond to the percent consumption of each food per colony during the experiment.

4.3.5 Genera selected and shared between the gut microbiome from different populations

For the population fed with *S. lycopersicum* were fifty-two unique OTUs to genera target, followed by the population fed only with Artificial Diet with thirty-eight, and *A. thaliana* population that had twenty-eight OTUs to genera target.

The genera that were significantly different among at least one of the populations were Achromobacter (p=0.00394), Bacillus (p=0.00612), Enterococcus (p=0.011), Gemella (p=0.027), Mesorrhizobium (p=0.0315), Propionibacterium (p=0.0467), Streptococcus (p=0.0485), Veillonella (p=0.000687), unclassified family Streptomycetaceae (p=0.027), unclassified family Xenococcaceae (p=0.027), unclassified order iii1-15 (p=0.027), unclassified family Planococcaceae (p=<2e-16) and unclassified family Alcaligenaceae (p=<2e-16).

Furthermore, thirty-four genera were shared between the three diets, twenty-four between the plant and diets, six between *Arabidopsis* and artificial diet, and nineteen between *S. lycopersicum* and artificial diet.

4.3.6 Shared genera between diets and population of *Trichoplusia ni* fed on specific diets

Since most OTUs richness are shared between each population of *Trichoplusia ni* and their respective diet (figure 4b, 4c, 4d), it was possible to analyze what the genera are the same between the gut microbiota of the population of *Trichoplusia ni* and their respective diet which was maintained. This fact may help us to better understand the influence of certain bacterial genera on the establishment of a specific diet even in insects with general behavior such as *Trichoplusia ni*.

It was verified (figure 4b) that the *T. ni* population-maintained fed leaves of *Arabidopsis thaliana* has nineteen genera shared with those that were found in the leaves of *A. thaliana plant*. In this case, eleven OTUs to genera were exclusively shared between those found in leaves of *A. thaliana* and the gut microbiome in the *A. thaliana* population.

Also, for the population fed with *S. lycopersicum* leaves it was verified that fourteen OTUs to genera level are shared with those present in *S. lycopersicum* plant (figure 4c), and seven (7) genera that are exclusive between leaves of *S. lycopersicum* and the microbiota of the respective population (Figure 4c).

The population that was maintained as control, continuously fed with artificial diet had twenty-one families shared with those that were found present in the artificial diet (figure 4d). Twelve genera exclusively present in the artificial diet and the population fed with the respective diet.

All the genera shared between the populations, and their respective diets are in the supplementary table 2. It is interesting to note that some genera shared exclusively between each population and their respective diet may have fundamental roles for the adoption of the dietary diet by *Trichoplusia ni*.

4.4 DISCUSSION

The specialization or food polyphagia of insects has always been of interest to some researchers, the behavior that determines a broad spectrum of hosts choice or diets by the generalist insects is indeed interesting and, allows these species to have an advantage concerning the food choice. An animal with unlimited food choice has advantages over a niche when food can become scarce or easily depleted (25). Many species of herbivores have a generalized diet across the geographic extent, but they can function as specialists with restricted diets in local communities. Thus, the particular specialization of feeding can be produced by biochemical, behavioral, ecological and evolutionary processes (18).

In our study, we propose that generalist insects can function as specialists due to the microbiological load of bacteria acquired through restrictive feeding. Then, specimens of *Thichoplusia ni* originating from the same population were divided into three different groups according to the exclusive diet. Initially, it was observed that the larvae of *Thichoplusia ni*, after the experiments of food preference did not present significant differences in mass average between each of the populations (Fig 1).

Thus, showing relative flexibility to maintain homeostasis of the mass of these animals. However, the three different populations of *Trichoplusia ni* presented a feeding preference, which were submitted to four successive cycles (Fig 2). General herbivores have tools that stimulate the ingestion of different plants by being able to metabolize, detoxify an array of different chemical types that have heterogeneous effects (26, 44). We suggest that this high metabolizing capacity of some chemical compounds may be potentiated if the local population is kept on an exclusive diet with a specific load of microorganisms. Then, some microorganisms can direct food preference even in generalist insects.

Therefore, maintaining the metabolic balance with an item of lower energy expenditure in insects may favor the food preference in each different population. The subject to diet restriction whereas the diet itself can influence through the endogenous microbial load the generalist insect preference even in future generations. This fact has already been suggested, and humans, where some studies report that the load gut microbiome load is strongly influenced by the type of diet and the richness of the microbiome can be shaped depending on the vegetable diet consumed by the hosts (27, 28).

We have obtained a large-scale panorama that allowed us to examine and understand the intestine of *Trichoplusia ni*, an insect pest with considerable economic influence in agriculture. In previous studies, it is described that insects' gut of the most different orders is composed mainly by the phylum Proteobacteria and Firmicutes representing 82.8% of the total sequences (2). The Proteobacteria and Firmicutes were also the predominant phyla in 81 insect gut samples from different orders, comprising 57.4% and 21.7% of sequences, respectively (1).

Our study also showed that the predominant phyla in the guts of the *Trichoplusia ni* examined were Proteobacteria (62.91%) followed by the phylum Firmicute (11.26%) and the phylum Actinobacteria (10.51%). We went further and observed that depending on the specific diet, the populations of *T. ni* varied the predominance between phyla Firmicutes and Actinobacteria (Table 2 and Fig 3). As the phylum Actinobacteria showed to be highly represented in the control population fed continuously with Artificial Diet (13.07%), compared to the population fed with *S. lycopersicum* (9.91%) and *A. thaliana* (8.56%).

The relative abundance of phylum Proteobacteria was significantly different (p=6.15E-07) among the three types of diet to which each population was restricted. The Proteobacteria phylum was highly represented in the samples of Artificial Diet, at a rate, almost four times more than *S. lycopersicum* leaves and six times more than in *A. thaliana* leaves. This point gives us an indication of the caloric diet influence on intestinal microbiome in *T.ni.* The high prevalence of the phylum Actinobacteria in all populations may be due to the origin of the initial population, obtained from the same company (*Frontier Science*), where they were fed with Artificial Diet.

In other words, we can have a parental population that already had a great abundance of phylum Actinobacteria maintained in part in the next generations. This context may explain the great abundance of the phylum Actinobacteria in this work, unlike the insects from other studies (1, 2), that were captured from the natural environment and maintained only herbivorous behavior during the whole study time.

In our study, the *PCoA* revealed a distinct difference in the composition of bacterial communities in the guts of from different *T. ni* population, showing that the variation in gut microbiota among populations is so considerable than on different types of food diet used in the research. We note that the communities of microorganisms present on the plant leaves, and the artificial diet showed differences by *PCoA* clustering (p=0.004) (Fig 3a). It is demonstrating that each type of plant has its microbial community structure (29) that directly influences the diversity and relative abundance of the microorganisms found by its insect's hosts. That is, the diet seems directly to influence the difference in the structure of communities as shown in Figure 3b (p=0.041). Some studies corroborate with our results, indicating that different insect orders have the gut microbiome composition influenced by the host diet because they have niches and differentiated feedings behaviors (1, 2).

Also, we observed that the artificial diet reduced the microbial diversity in *Trichoplusia ni* (Shannon index). Among the populations fed with plant diets, the population fed *S. lycopersicum* diet

had the lowest diversity followed by the *Arabidopsis thaliana* diet. Additionally, some microbiota studies with experimental models of mammals, rats' model (30, 35, 36) and humans (27, 28) that describe the increase of microbial diversity rates in the intestines of animals is favored due to load microbial obtained through feed, being highly abundant in vegetable diets (27, 28, 36, 35).

The microbiome enrichment fact is due to the bacterial load present on the vegetable diet in *Termites* for example (10, 11, 12). Some studies showing that the bacterial richness and diversity could be differentiated between grassy and woody diets, and different supplementing blends of sugars, amino acids, and secondary metabolites at different diets may be related to the lower recalcitrance for different kinds of vegetable biomass degradation in *Termite* species (10, 11).

One of the theories that may explain the diet can also target the *Lepidopteran* insect microbiota is that secondary metabolites in plant biomass can have antimicrobial properties. Plant secondary metabolites, such as essential oils, alkaloids, and phenolic compounds, have been shown to have antimicrobial properties for different bacterial families and could act as inhibitors against some members of the gut microbiome as well (15). Therefore, we also propose that the diet-specific bacterial genera may represent organisms with the capacity to detoxify some of these secondary metabolites, mainly present in *S. lycopersicum* (alkaloids) and *A. thaliana* (glucosidases) that were used as diets in this study.

There is a relationship between different populations of *T.ni* and the respective diets used in this study (figure 5). The figure 5b shows that the gut from the population that was fed only with leaves of *A. thaliana* has nineteen genera also present in the *A. thaliana* leave. The *A. thaliana* is a plant member of the *Brassicaceae* family, as well as the cabbage, has abundant glycosidases in its metabolite's composition.

Some studies have reported the breakdown of glucosidases, found in cruciferous plants like *A. thaliana*, is catalyzed by microbial myosinases in rats (32) and humans (33). Thus, it is possible that specific genera that can be shared between leaves of *A. thaliana* and the specific population fed with this Brassicaceae present a higher relative abundance of genera that may degrade glycosidase compounds. We observed for example that the Propionibacterium, Shinella and Terribacilus genera were expressively more abundant only in the *A. thaliana* fed the population.

Glucosidases present abundantly in *A. thaliana* are potential antimicrobial compounds but Propionibacterium genera, one of the most abundant shared between *A. thaliana* and in the gut of *A. thaliana* population is a bacterial genus already reported as tolerant to toxicity of the glucosidases and which may surprisingly slightly increase their number of colonies when in the presence of glucosidases compounds (37). In our study it was observed that groups that may be pathogenic and have a particular limitation in the resistance against the glucosidases contained in Brassicas (34), as the bacteria of the family *Xanthomoneacea*, present a lower relative abundance when compared with the relative abundances in the populations of insects fed with *S. lycopersicum* and Artificial Diet.

These interesting aspects were also seen in populations fed *S. lycopersicum* leaves. The figure 5c shows that the population that was fed only with leaves of *S. lycopersicum* has as well fourteen genera also shared with the leaves of the vegetable. Here we highlight the Pseudomonas, Agrobacterium and Rhizobium genera. The Agrobacterium and Rhizobium group presented a higher relative abundance (3.9317% and 4.4171%, respectively) when compared to the populations of *T. ni* fed with *A. thaliana* and artificial diet (supplementary table 2). These same genera are also more abundant in *S. lycopersicum* leaves (0.01594% for Agrobacterium and 0.012% for Rhizobium) than in leaves of *A. thaliana* and the Artificial Diet (supplementary table 1).

The Agrobacterium and Rhizobium are genera that can degrade alkaloids (38, 39) and knowing that these cyclic amines are present in large quantities in some groups of plants, among them the family of *Solanaceae (40)* which belongs to *S. lycopersicum.* Then, it is possible that the relative abundance of these two genera of bacteria is higher in the *S. lycopersicum* gut population and shared with *S. lycopersicum* leaves, showing possible adaptations in the gut microbiome from the bacterial load present on specific vegetable diets. Those particular groups of bacteria may degrade some alkaloids that are produced by plants as a defense against insects and even in protection against other bacteria pathogens groups (40, 41).

Curiously, the genus Pseudomonas, for example, that is present in leaves of *S. lycopersicum* (Fig 5C) had a lower relative abundance in the population feeding with *S. lycopersicum* leaves than in those that were fed with *A. thaliana* and artificial diet (supplementary table 2). The Pseudomonas group is widely known to be a pathogen for a variety of invertebrate and humans (42, 43) and the state of having a relatively lower abundance for a population fed *S. lycopersicum* may mean the activity of alkaloids present in this vegetable leaves.

We supposed that the effect of other bacteria genus might be relevant to this study. However, we will limit ourselves in the comments just from the genus upstairs. There is not still a description in the literature of the effects of alkaloids and glucosidases for a large number of families and the genera of bacteria that can be detected by the new generation sequencing technique. This point makes it difficult to describe the possible effects of the genera, to each different population used in this study. Thus, we relate that the population controls maintained with the artificial diet which did not contain any antibiotic keeps a microbiota differentiated from most of the seen in the populations fed with vegetal diet as shown in figure 3. This groups of bacterial, specific for each experimental population, seems to remain in successive generations of *T. ni*.

Furthermore, it is known that diets with a high caloric rate are capable of altering the composition of medium intestines in mammalians (35) and the same seems to happen with invertebrate organisms as in the case of *T. ni*. In rats, for example, it is known that animals fed with highly caloric diets possess the intestinal microbiota different from those that have been fed a balanced diet in plant fibers (36). Again, the highlights that there may be modulation of the gut microbiome of

T. ni, a generalist insect species, according to the restricted diet and that this microbiome may favor the feeding preference of next insect generations.

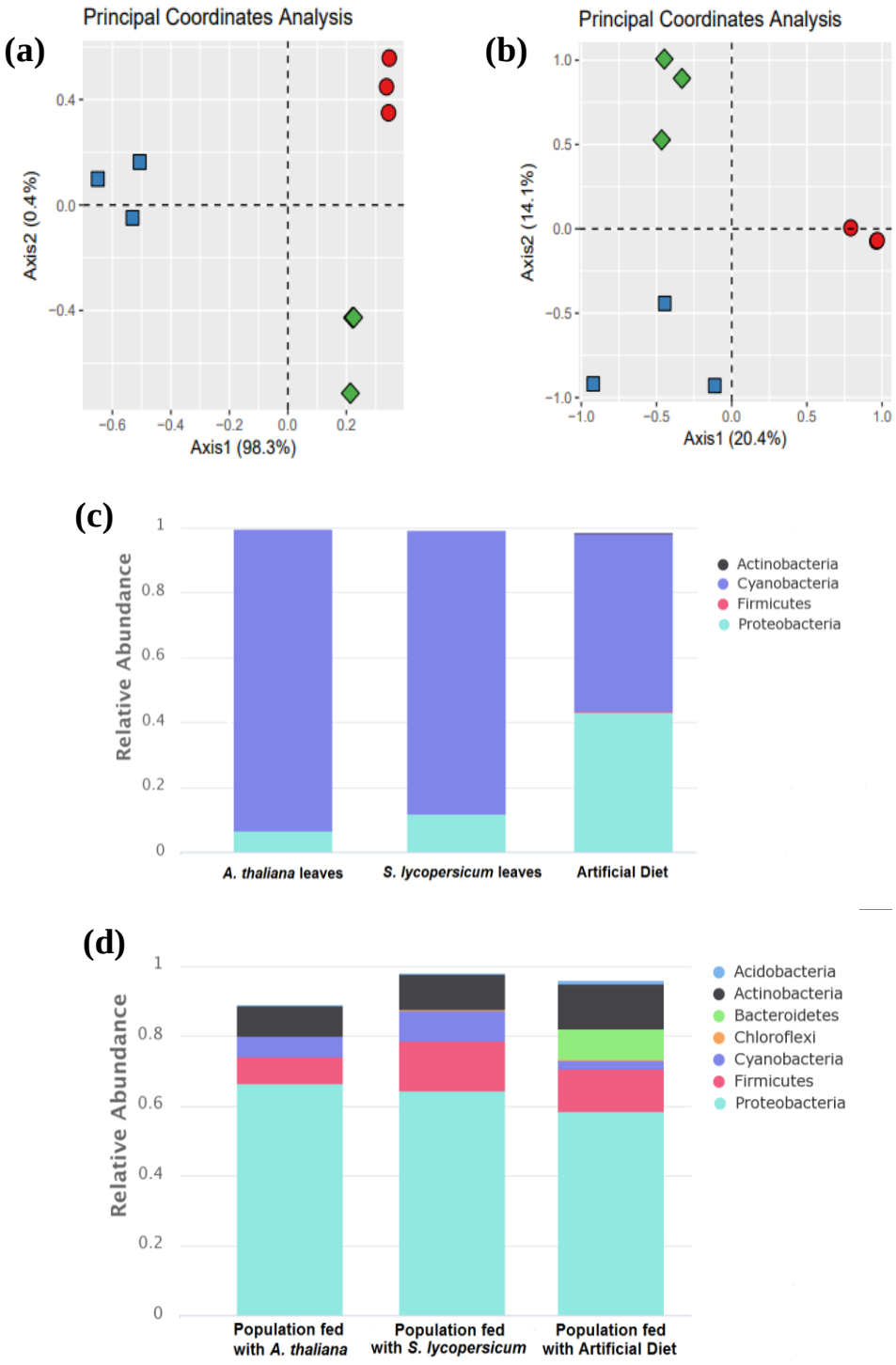

Figure 3. **(a).** Principal coordinates analysis (PCoA) of pairwise dissimilarities (Bray–Curtis index for bacterial communities in *A. thaliana* leaves, *S. lycopersicum* leaves and artificial diet) to OTUs level. **(b).** Principal coordinates analysis (PCoA) of pairwise dissimilarities (Bray–Curtis index for bacterial communities in *Trichoplusia ni* guts derived from different populations fed with different diets) to OTUs level. **(c).** Taxonomic bacterial composition from OTUs by phyla level presents in each diet (*A. thaliana* leaves, *S. lycopersicum* leaves and artificial diet). ANCOVA ($P<0.05$). **(d).** Taxonomic bacterial composition of OTUs by phyla level in *Trichoplusia ni* guts samples from different populations fed with specific diets. The size of each segment in the **(c)** and the **(d)** chart is proportional to the relative abundance from OTUs assigned to the indicated phyla.

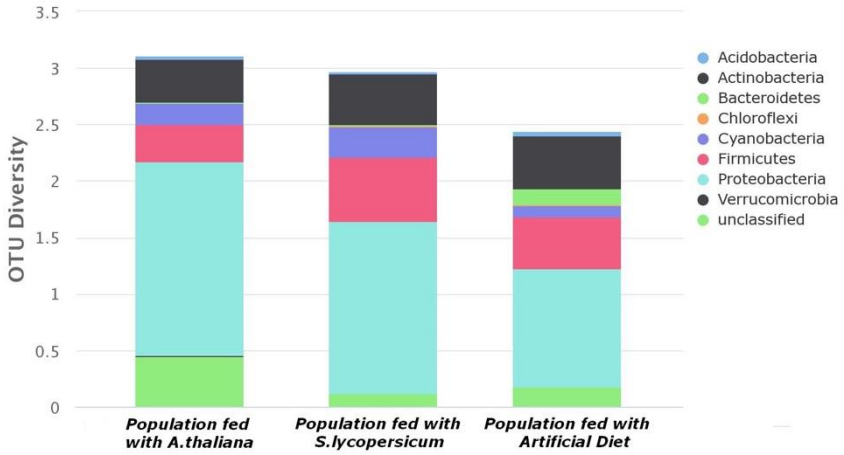

Figure 4: Bacterial diversity in *Thichoplusia ni* gut microbiota to each hosts' diet population. The amount of bacterial diversity was demonstrate comparing the numbers of OTUs by the phyla to Shannon diversity index.

Table 1: Bacterial relative abundance in *plants and artificial diet* microbiota used for fed the different population of *Trichoplusia ni*. The bacterial relative abundance in percentage was determined by comparing the numbers of OTUs by phyla. Asterisks indicate statistically significant differences among all pairs of values (*$P<0,05$).

Phyla	*A. thaliana leaves (%)*	*S. lycopersicum leaves (%)*	*Artificial Diet (%)*	*p-value*
*Cyanobacteria**	93	87	55	5.99E-07
*Proteobacteria**	7	12	43	6.15E-07
*Firmicutes**	0	0	0.3	0.00049
*Actinobacteria**	0	0	0.1	0.000087
*Unclassifiedclassifed**	0	1	1.6	0.000197

*ANCOVA univariate test (P<0.05)

Table 2: Bacterial relative abundance in *Thichoplusia ni* gut microbiome concerning respect to the hosts' diet. The bacterial relative abundance in percentage was determined by comparing the numbers of OTUs by phyla. Asterisks indicate statistically significant differences among all pairs of values (*$P<0,05$).

Phyla	*Population fed with A. thaliana leaves (%)*	*Population fed with S. lycopersicum leaves (%)*	*Population fed with Artificial Diet (%)*	*p-value*
Proteobacteria	66.22	64.25	58.27	0.883
Actinobacteria	8.56	9.91	13.07	0.39
Firmicutes	7.73	14	12.06	0.124
Cyanobacteria	5.63	8.94	2.52	0.58
Acidobacteria	0.37	0.3	0.81	0.608
Bacteroidetes	0	0	8.68	0.402
Chloroflexi	0	0.17	0.15	0.467
Unclassified	11.49	2.43	4.44	0.547

Table 3: Bacterial diversity in *Thichoplusia ni* gut microbiota for the hosts' diet population. The bacterial diversity was determined by comparing the numbers of OTUs by phyla. Asterisks indicate statistically significant differences among all pairs of values (*$P<0,05$).

Plyla	*A. thaliana leaves (%)*	*S. lycopersicum leaves (%)*	*Artificial Diet (%)*	*p-value*
Acidobacteria	0.07109102	0.0564335	0.104361227	0.748
Actinobacteria	1.14693493	1.33673888	1.429378398	0.698
Bacteroidetes	0.01626229	0.05255079	0.411576392	0.348
Chloroflexi	0	0.03304868	0.024227832	0.427
Cyanobacteria	0.5830702	0.7865521	0.286299747	0.552
*Firmicutes**	0.97603421	1.72339406	1.39251779	0.0389
Proteobacteria	5.16512598	4.56814045	3.139027154	0.163
unclassified	1.35376699	0.35839965	0.525643654	0.549
Total	9.31228562	8.91525811	7.31303219	0.945

*ANCOVA univariate test (P<0.05)

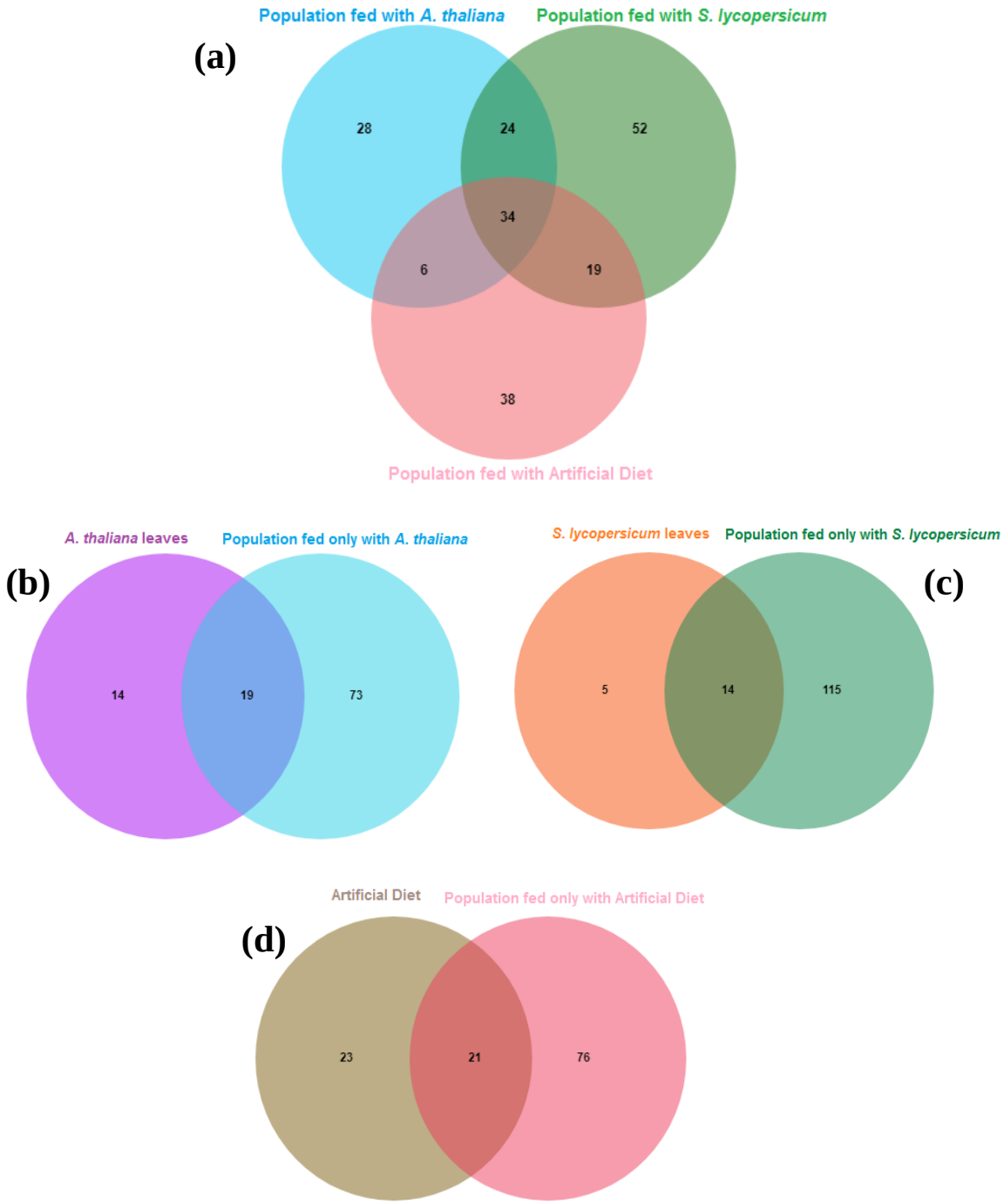

Figure 5. (a). Venn diagram showing distribution of common OTUs assigned to the indicated genera among different populations fed with different diets for three cycles. **(b). (c). (d).** Venn diagram showing distribution of common OTUs assigned to the indicated genera, between each diet (*A. thaliana* leaves, *S. lycopersicum* leaves and art. diet) and guts from specific population isolate by its respective diet. Consensus classifications for genera shared across each gut from the respective population (**a**) and, for genera shared across each diet and its respective populations **(b)/ (c)/ (d)** demonstrated at Venn diagrams, are shown in supplementary table 3.

4.5 CONCLUSION

Populations of *T. ni* fed with different diets by consecutive cycles may have the differentiated intestinal microbiota due to obtaining load microbial through feed, and this modulation may favor the degradation of metabolites that can be harmful to the insect homeostasis.

The intestinal microbiota of each population may have a direct influence on the food preferences of successive generations.

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CONFLICT OF INTEREST

We have no declaration of conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary table 1: Percentage for OTUs at genera level for the dependent variable relative abundance in the Diet Samples, which fed each *Thichoplusia ni* population (**P<0,05*).

Name	*Taxa level*	*A. thaliana (%)*	*S. lycopersicum (%)*	*Artificial Diet (%)*
*Pseudomonas**	genus	0.1232	0.0529	2.6021
*Streptomyces**	genus	0.077	0	0
Shinella	genus	0.0343	0	0
Rhodoplanes	genus	0.0303	0	0.0432
Mesorhizobium	genus	0.0056	0	0.0178
Dokdonella	genus	0.0039	0	0
Cellvibrio	genus	0.0034	0	0
Sphingopyxis	genus	0.0033	0	0
Methylibium	genus	0.0033	0	0
*Propionibacterium**	genus	0.0025	0	0.0485
Acinetobacter	genus	0.002	0	0
Flavobacterium	genus	0.0014	0	0
*Agrobacterium**	genus	0	0.1594	0
*Sphingobium**	genus	0	0.0353	0
Sphingomonas	genus	0	0.0196	0
*Delftia**	genus	0	0.0123	0
Rhizobium	genus	0	0.012	0.0106
*Azospirillum**	genus	0	0.008	0
Chryseobacterium	genus	0	0.0057	0.0091
Limnohabitans	genus	0	0.0051	0
Sphingobacterium	genus	0	0.0029	0.0049
Methylotenera	genus	0	0.0023	0
Bacillus	genus	0	0	0.0421
Staphylococcus	genus	0	0	0.0348
Corynebacterium	genus	0	0	0.0218
Exiguobacterium	genus	0	0	0.0218
Plesiocystis	genus	0	0	0.0216
Paenibacillus	genus	0	0	0.021
Lentzea	genus	0	0	0.0183
Geobacillus	genus	0	0	0.0158
Brevibacillus	genus	0	0	0.0139
Brachybacterium	genus	0	0	0.0136
Actinobaculum	genus	0	0	0.0134
Actinomadura	genus	0	0	0.013
Planctomyces	genus	0	0	0.0116
Rhodanobacter	genus	0	0	0.0106
Serratia	genus	0	0	0.0077
Schlegelella	genus	0	0	0.007
Clostridium	genus	0	0	0.0066
HTCC	genus	0	0	0.0055

Aeromicrobium	genus	0	0	0.0054
Acinetobacter	genus	0	0	0.0049
*mitochondria**	family	6.3115	11.287	36.197
*Moraxellaceae**	family	0.1494	0.1016	4.2123
*Enterobacteriaceae**	family	0.0152	0.2022	0.0115
Xanthomonadaceae	family	0.0119	0.0056	0.0061
Rhizobiaceae	family	0.009	0	0
Nitrosomonadaceae	family	0.0083	0	0
Phyllobacteriaceae	family	0.0071	0	0
Rhodospirillaceae	family	0.0067	0	0
OM27	family	0.0057	0	0
Bradyrhizobiaceae	family	0.0048	0	0
Hyphomicrobiaceae	family	0.0047	0	0
Oxalobacteraceae	family	0.0034	0	0
Methylophilaceae	family	0.0029	0	0
Nocardioidaceae	family	0.0027	0	0.0095
*Caulobacteraceae**	family	0	0.0636	0
Comamonadaceae	family	0	0.0102	0
AKIW874	family	0	0	0.0165
Streptomycetaceae	family	0	0	0.0121
Beijerinckiaceae	family	0	0	0.0099
Peptostreptococcaceae	family	0	0	0.0094
AK1AB1_02E	family	0	0	0.0086
Gaiellaceae	family	0	0	0.0065
Rhodocyclaceae	family	0	0	0.0064
*Streptophyta**	order	92.753	87.314	54.72
Rhodospirillales	order	0.0046	0	0
Rhizobiales	order	0.0037	0	0
MND1	order	0.0033	0	0
Ellin329	order	0.0018	0	0
SC-I-84	order	0.0008	0	0
JG30-KF-CM45	order	0	0	0.0178
Ellin6067	order	0	0	0.0117
Sphingomonadales	order	0	0	0.0073
Gemmatimonadetes	class	0	0	0.0046
*Unclassified**	Unclassified	0.3995	0.7006	1.6972

*ANCOVA univariate test (P<0.05)

Supplementary table 2: Percentage for OTUs at genera level for the dependent variable relative abundance in the gut samples to each different population of *Thichoplusia ni* (**P<0,05*).

Name	*Taxa level*	*Population fed with A. thaliana leaves (%)*	*Population fed with S. lycopersicum leaves (%)*	*Population fed with Artificial Diet (%)*
Serratia	genus	22.925	1.3234	0
Pseudomonas	genus	10.981	9.2787	15.769
Propionibacterium	genus	4.2248	4.0474	3.4434
*Shinella**	genus	1.6956	0.2046	0
Terribacillus	genus	1.349	0	0
Streptococcus	genus	1.0683	6.8859	3.199
Novosphingobium	genus	0.8776	0	0
*Microbacterium**	genus	0.8237	0.0557	0.0881
Bacillus	genus	0.7813	3.3365	0.1843
Stenotrophomonas	genus	0.7788	0	0
Arthrobacter	genus	0.5107	0.075	0
Corynebacterium	genus	0.4849	0.743	0.4625
Agrobacterium	genus	0.4734	3.9317	0.0924
Skermanella	genus	0.4062	0	0
Mesorhizobium	genus	0.3252	0	0
Ochrobactrum	genus	0.2708	0	0
Staphylococcus	genus	0.2558	0.7664	0.607
Deinococcus	genus	0.2438	0.056	0.0237
Paracoccus	genus	0.2427	0.242	0.1307
Roseococcus	genus	0.2281	0	0
Sphingobium	genus	0.1529	0	0
Pseudonocardia	genus	0.1398	0.0828	0
Actinobaculum	genus	0.1332	0.3456	0.3125
Haemophilus	genus	0.1315	0.0517	0.0139
Rhodococcus	genus	0.1305	0.044	0.0823
Cryocola	genus	0.1285	0	0
Nocardioides	genus	0.1249	0.1946	0.0164
Methylobacterium	genus	0.1201	0	0
Sphingomonas	genus	0.1164	0.3061	0.3028
Lactobacillus	genus	0.1091	0.0316	0
Rhodoplanes	genus	0.1055	0.2115	0
Variovorax	genus	0.1054	0	0
Friedmanniella	genus	0.1035	0.0241	0
Enhydrobacter	genus	0.096	0.0472	0
Acinetobacter	genus	0.0897	0.3447	0.0008
Lactococcus	genus	0.0828	0	0.0939
Janibacter	genus	0.0783	0	0
Mycobacterium	genus	0.0771	0.3871	0.4187
*Enterococcus**	genus	0.0699	0	1.9556
Streptomyces	genus	0.0628	1.0424	5.2551
Kocuria	genus	0.0578	0	0

Glycomyces	genus	0.0506	0	0
Hymenobacter	genus	0.0485	0.0476	0
Hyphomicrobium	genus	0.0419	0	0
Moryella	genus	0.0378	0.0485	0
Devosia	genus	0.0349	0.3424	0
Kaistia	genus	0.0329	0	0
Cellulomonas	genus	0.0321	0.0723	0
Eikenella	genus	0.0196	0.084	0.0023
Paenibacillus	genus	0.0148	0	0
Tepidimonas	genus	0.0119	0	0
Sporomusa	genus	0.0044	0	0
Rhizobium	genus	0	4.4178	0
Rothia	genus	0	1.2889	0.3302
Luteimonas	genus	0	0.4915	0
*Veillonella**	genus	0	0.3304	0.0286
Methylobacterium	genus	0	0.2514	0
Gemella	genus	0	0.1943	0.3954
Anaerococcus	genus	0	0.1911	0
Jeotgalicoccus	genus	0	0.1498	0
Dokdonella	genus	0	0.1323	0
Candidatus_Solibacter	genus	0	0.1308	0
Actinomyces	genus	0	0.1299	0
Cellulosimicrobium	genus	0	0.1261	0
Labrys	genus	0	0.1246	0
Dermacoccus	genus	0	0.1138	0
Granulicatella	genus	0	0.1055	0.03
Exiguobacterium	genus	0	0.1007	0
Burkholderia	genus	0	0.0985	0
Aggregatibacter	genus	0	0.0845	0
Kaistobacter	genus	0	0.0746	0.1361
Acetobacter	genus	0	0.073	0
Leuconostoc	genus	0	0.0651	0
Asticcacaulis	genus	0	0.0628	0
Finegoldia	genus	0	0.0579	0
Solibacillus	genus	0	0.057	0
Chryseobacterium	genus	0	0.0563	0
Rhodanobacter	genus	0	0.0561	0.5192
Mycoplana	genus	0	0.0559	0
Prevotella	genus	0	0.0545	0.1144
Facklamia	genus	0	0.0485	0.2965
Sphingopyxis	genus	0	0.0467	0
Thermomonas	genus	0	0.0465	0
Actinocatenispora	genus	0	0.0398	0
Paludibacter	genus	0	0.0387	0
Steroidobacter	genus	0	0.0379	0
Dermabacter	genus	0	0.0302	0

Clavibacter	genus	0	0.0282	0
Denitrobacter	genus	0	0.0278	0
Amaricoccus	genus	0	0.0276	0
Roseomonas	genus	0	0.0275	0
Tetrathiobacter	genus	0	0.0275	0
*Mesorhizobium**	genus	0	0.0253	0
Lautropia	genus	0	0.0203	0
Brevibacterium	genus	0	0.0195	0
Porphyromonas	genus	0	0.0175	0
Rubrivivax	genus	0	0.0171	0
Amycolatopsis	genus	0	0.0162	0
Flavobacterium	genus	0	0.0146	0.0649
Delftia	genus	0	0.0143	0
Shewanella	genus	0	0.0115	0
Peptoniphilus	genus	0	0.0105	0
Brevundimonas	genus	0	0.0013	0
*Achromobacter**	genus	0	0	0.3156
Blastococcus	genus	0	0	0.1878
Limnohabitans	genus	0	0	0.1538
Balneimonas	genus	0	0	0.1188
Gluconobacter	genus	0	0	0.1138
Clavibacter	genus	0	0	0.1066
Rathayibacter	genus	0	0	0.0922
Erwinia	genus	0	0	0.0875
Geobacillus	genus	0	0	0.0808
Couchioplanes	genus	0	0	0.0788
Salinicoccus	genus	0	0	0.0731
Cellvibrio	genus	0	0	0.0712
Luteolibacter	genus	0	0	0.0502
Flavisolibacter	genus	0	0	0.0469
Agrococcus	genus	0	0	0.0437
Oribacterium	genus	0	0	0.0354
Ramlibacter	genus	0	0	0.0331
Dietzia	genus	0	0	0.0318
Dyella	genus	0	0	0.0193
Actinomadura	genus	0	0	0.0093
Lysobacter	genus	0	0	0.0046
Aeromicrobium	genus	0	0	0
Moraxellaceae	family	17.801	14.419	24.854
Enterobacteriaceae	family	3.8221	20.8	13.036
*Planococcaceae**	family	3.2553	0	4.8961
Xenococcaceae	family	2.7706	0.0382	0
Sphingomonadaceae	family	1.9033	0	0
Erythrobacteraceae	family	0.4412	0.0571	0.0407
mitochondria	family	0.3769	4.44	0
Rhizobiaceae	family	0.3584	0.0602	0

C111	family	0.2965	0.036	0
Micrococcaceae	family	0.2717	0.0876	0.5461
Hyphomicrobiaceae	family	0.22	0	0
*Alcaligenaceae**	family	0.1804	0	0
Rhodospirillaceae	family	0.1633	0.3075	0.2929
Gaiellaceae	family	0.1627	0.0137	0.0909
*Nocardioidaceae**	family	0.1595	0.3921	0.0344
Bradyrhizobiaceae	family	0.1503	0.2656	0.0107
Pseudomonadaceae	family	0.0972	0.1239	0.1147
Oxalobacteraceae	family	0.087	0.1197	0.279
Promicromonosporaceae	family	0.0837	0	0
Microbacteriaceae	family	0.0753	0.0856	0.1231
Frankiaceae	family	0.0707	0	0
RFP12	family	0.0531	0	0
Intrasporangiaceae	family	0.0439	0.08	0
*Comamonadaceae**	family	0.0337	0.0828	0.0019
Bacillaceae	family	0.0315	0.0632	0
Neisseriaceae	family	0.0306	0.0576	0.083
Weeksellaceae	family	0.0268	0.0136	0
Conexibacteraceae	family	0.0266	0	0
Pirellulaceae	family	0.0045	0	0
Streptococcaceae	family	0	1.3994	0
Solirubrobacteraceae	family	0	0.2069	0.0158
Caulobacteraceae	family	0	0.1939	0.0047
Halomonadaceae	family	0	0.1445	0
Acetobacteraceae	family	0	0.1287	0
Methylobacteriaceae	family	0	0.1009	0
Sphingomonadaceae	family	0	0.0928	0
Alicyclobacillaceae	family	0	0.0693	0
*Streptomycetaceae**	family	0	0.0669	0.4823
Geodermatophilaceae	family	0	0.0443	0.1018
0319-6A21	family	0	0.0384	0
Xanthomonadaceae	family	0	0.0348	1.2347
Clostridiaceae	family	0	0.0295	0
Ellin6075	family	0	0.0264	0
Phyllobacteriaceae	family	0	0.0247	0
Chitinophagaceae	family	0	0	8.4041
Acidobacteriaceae	family	0	0	0.6705
Nitrosomonadaceae	family	0	0	0.1973
Actinospicaceae	family	0	0	0.1884
Thermomonosporaceae	family	0	0	0.077
Syntrophobacteraceae	family	0	0	0.0613
Sphingobacteriaceae	family	0	0	0.0529
Micromonosporaceae	family	0	0	0.052
Patulibacteraceae	family	0	0	0.0514

Aerococcaceae	family	0	0	0.0389
Burkholderiaceae	family	0	0	0.0386
Sinobacteraceae	family	0	0	0.0356
AKIW874	family	0	0	0.0336
Sporichthyaceae	family	0	0	0.0205
Streptophyta	order	2.8608	8.8638	2.5269
Bacillales	order	0.6657	0.0273	0
CCU21	order	0.1972	0	0
Rhizobiales	order	0.1708	0	0.0773
Actinomycetales	order	0.1145	0	0.0795
Acidimicrobiales	order	0.0879	0	0
iii1-15	order	0.0843	0	0
RB41	order	0.0579	0	0.0977
Ellin329	order	0	0.2261	0.024
iii1-15	order	0	0.1509	0
WD2101	order	0	0.0764	0.0567
JG30-KF-CM45	order	0	0.0717	0
Stramenopiles	order	0	0.0378	0
Solirubrobacterales	order	0	0	0.3707
Phycisphaerales	order	0	0	0.0731
Bacteroidales	order	0	0	0.0079
Betaproteobacteria	class	0.1468	0	0
Acidobacteria-5	class	0.0385	0	0.0441
Ellin6529	class	0	0.1025	0.1496
Gemm-1	class	0	0.018	0
Gemmatimonadetes	class	0	0	0.1005
Unclassified	Unclassified	11.086	1.957	4.1025

*ANCOVA univariate test ($P<0.05$)

5 FINAL CONCLUSIONS

First chapter:

1) Herbivorous insect attack may be able to modulate the bacterial and fungal community structure in distinct ways in the *A. thaliana* rhizosphere.

2) Plant developmental stages contribute to differences in structure and abundance in bacterial and fungal communities in the *A. thaliana* rhizosphere.

Second chapter:

1) Structure of the rhizosphere microbiota altered by the insect attack depends on the host plant;

2) Specific groups of bacteria are recruited into the rhizosphere microbiota for each type of host plant;

3) Modulation of the rhizosphere microbiota by insect attack might negatively influence the biomass of subsequent plant generations.

Third chapter:

1) Populations of generalist insects fed with consecutive cycles of different diets show distinct intestinal microbiota.

2) Populations of gut microbiota directly influence the food preferences of successive generations.