University of São Paulo
"Luiz de Queiroz" College of Agriculture

# Gut microbiota of the rice and corn strains of *Spodoptera frugiperda*: diversity and function

**Nathalia Cavichiolli de Oliveira**

Thesis presented to obtain the degree of Doctor in Science. Program: International Plant Cell and Molecular Biology

Piracicaba
2021

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*To my parents, Antoniel and Cristina*

*To my brother Arthur and my sister in-law Isabella*

*To my grandparents Walter and Isola*

*To God*

**I DEDICATE**

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*You are not an encapsulated bag of skin*

*Dragging around a dreary little ego.*

*You are an evolutionary wonder,*

*A trillion cells singing together in a vast chorale,*

*An organism – environment,*

*A symbiosis of cell and soul.*

*Jean Houston*

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# RESUMO


## Microbiota intestinal das raças milho e arroz de *Spodoptera frugiperda*: diversidade e função

A microbiota intestinal de insetos é um importante fator que contribui para vários aspectos da fisiologia e ecologia do seu hospedeiro. Neste estudo nós testamos a hipótese de que os simbiontes associados ao intestino de *Spodoptera frugiperda*, uma importante praga agrícola, podem estar desempenhando um papel relevante no processo de adaptação às plantas hospedeiras de suas raças geneticamente distintas, as raças "milho" e "arroz". Para este fim, caracterizamos a microbiota intestinal das raças utilizando sequenciamento de nova geração do 16S rRNA de espécimes coletados em condições de campo para a avaliação do efeito da raça, da planta hospedeira e da origem da população. Nós também analisamos a composição da microbiota intestinal sob condições controladas, incluindo neste último, a análise do intestino posterior. Também procuramos compreender a origem da microbiota associada às lagartas de duas formas: avaliando a microbiota presente nos alimentos ingeridos e buscando investigar a presença de bactérias nos ovos e tecidos reprodutivos de *S. frugiperda* usando FISH e microscopia confocal. Para explorar os aspectos funcionais destas interações, utilizamos uma abordagem metatranscritômica, para definir o perfil de genes diferentemente expressos no intestino das raças de *S. frugiperda* e o perfil transcricional das bactérias associadas ao intestino dessas raças. Finalmente, utilizamos a abordagem metabolômica de forma holística, comparando o perfil metabólico das raças ao fornecê-las diferentes fontes alimentares. Nós não encontramos diferenças na composição e na estrutura da microbiota intestinal das raças. Verificamos que a microbiota intestinal de *S. frugiperda* é principalmente modulada pelo alimento ingerido, mas não é reflexo dele. Adicionalmente nós fornecemos indicações de transmissão vertical de bactérias por meio da detecção de bactérias nos ovos e oócitos de *S. frugiperda.* Ao nível funcional, encontramos uma comunidade bacteriana metabolicamente ativa que funcionou da mesma forma nas raças de *S. frugiperda*, com exceção de quando a dieta artificial foi oferecida com alimento as lagartas. Em contrapartida, verificamos que as raças respondem diferentemente ao nível transcricional ao alimento ingerido. Da mesma forma, o perfil metabólico do intestino médio das raças também diferiu em cada substrato alimentar. Finalmente, nossas descobertas fornecem apoio adicional para *Enterococcus* como membro central da comunidade bacteriana associada ao intestino larval de *S. frugiperda*, pois este gênero foi encontrado metabolicamente ativo e consistentemente associado ao intestino de *S. frugiperda* em todas as condições analisadas, apoiando a hipótese de que estas bactérias mantêm relações verdadeiras de mutualismo com *S. frugiperda*.


Palavras-chave: Simbiose, Lagarta do cartucho, Ecologia microbiana, 16S rRNA, Metaboloma, Metatranscritoma

# ABSTRACT


## Gut microbiota of rice and corn strains of *Spodoptera frugiperda:* diversity and function

The insect gut microbiota is an important factor that contributes to various aspects of the physiology and ecology of their host. In this study we tested the hypothesis that the gut-associated symbionts of *Spodoptera frugiperda,* an important agricultural pest, may be playing a relevant role in the process of adaptation to the host plants of genetically distinct host-adapted strains, known as corn and rice strains. To this end, we characterized the gut microbiota composition of the strains using next generation 16S rRNA sequencing by sampling larvae in field conditions, evaluating the effect of strain, host plant and population origin. We also analyzed the composition of the gut microbiota under controlled conditions, including in the latter the analysis of the hindgut. We also sought to understand the origin of the microbiota associated with the caterpillars in two ways: by assessing the microbiota present in the ingested food, and by investigating the presence of bacteria in the eggs and reproductive tissues of *S. frugiperda* using FISH and confocal microscopy. To explore the functional aspects of these interactions, we used a metatranscriptomic approach to profile differentially expressed genes between strains in the gut of *S. frugiperda* and the transcriptional profile of the bacteria associated with the strains. Finally, we also used metabolomics to compare the metabolic profile of strains fed on different food sources. We found that the composition and structure of the gut microbiota between the strains was not different in the tests performed. We found that the gut microbiota of *S. frugiperda* is greatly modulated by the food ingested but is not a reflex of it. Additionally, we provided indications of vertical transmission of bacteria by detecting bacteria in eggs and oocytes of *S. frugiperda*. At the functional level, we found a metabolically active bacterial community that functioned equally in both strains of *S. frugiperda*, except when the food source was the artificial diet. In contrast, we found that the strains responded differently at the transcriptional level to the ingested food. Similarly, the metabolic profile of the midgut of the strains also differed for each food source tested. Finally, our findings provide additional support for *Enterococcus* as a core member of the bacterial community associated with the larval gut of *S. frugiperda*, as this genus was found metabolically active and consistently associated with the gut of *S. frugiperda* under all conditions analyzed, supporting the hypothesis that *Enterococcus* maintain true symbiotic mutualistic relationships with *S. frugiperda*.


Keywords: Fall armyworm, Symbiosis, Microbial ecology, 16S rRNA, Metabolomics, Metatranscriptomics

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## 1. INTRODUCTION

The fascinating complexity of the interactions between living things has instigated scientists from all fields to seek a better understanding of the causes and effects of these relationships in the organisms and their surroundings. Symbiosis (from the Greek syn 'together' and bios 'life') is a term widely used to describe the most variable interactions between organisms of different species (De Bary 1879). The symbiosis between microorganisms and multicellular organisms is increasingly receiving attention due to the discovery of the essential roles that microbial symbionts play in the physiology and ecology of host (Charroux and Royet 2012; Clemente et al. 2012), and that the diversity of microbes depends on genetic or environmental factors. Thus, a more comprehensive research of the individual and its responses to the environment required a holistic investigation that includes the relationship between the individual and its associated symbionts. Within this context the term holobiont was originated, which defines the individual and its symbionts as a single selection unit (Gilbert et al. 2012; Guerrero et al. 2013; Margulis 1993; Mindell 1992; Zilber-Rosenberg and Rosenberg 2008). This concept that is still controversial and causes intense debate in its definitions (Bordenstein and Theis 2015; Douglas and Werren 2016; Roughgarden et al. 2018; Skillings 2016). However, even if the heritable aspect of the holobiont is disregarded, the microbial community and its functions to the host may still provide time for the host genome to evolve during periods of changes in the environment (Zilber-Rosenberg and Rosenberg 2008).

Insects are excellent models for the study of microbial symbiosis. Due to its great diversity, it is quite easy to find groups with short life cycles, easy management and rich in microorganism associations(Charroux and Royet, 2012). Symbiosis in insects is a vast and very interesting topic. There are symbionts that inhabit specialized host cells and organs, called bacteriocytes and bacteriome, respectively (Moran and Telang, 1998). These are called primary symbiotes that have intimate associations with their hosts to the point of being vital for their survival (Bourtzis and Miller 2003; Douglas 2009; Moran and Telang 1998; Wernegreen 2002). Other symbiotes are found adhered externally to the host cells or in the hemocoel, the most notable ones are those that inhabit the intestines of insects. In some cases, this association became so intimate that insects developed specialized cells to harbor bacteria, known as bacteriocytes. These cells are free in the hemocel or assembled into a specific structure called bacteriome(Smith and Douglas 1987).

Symbionts associated with insects are partly responsible for the wide distribution of these arthropods through a range of habitats. Symbionts allowed insect hosts to explore restricted food sources by supplementing them with essential nutrients, considering their availability or not in the host's diet (Gilbert et al. 2018; Shropshire and Bordenstein 2016). In addition, several symbionts protect insect hosts from xenobiotic contained in their food sources (Tartar et al. 2009), from infection by pathogens(Eichler and Schaub 2002; Oliver and Moran 2009) and parasitism by parasitic wasps (Oliver et al. 2012), besides improving insect resistance to other sources of stress (Montllor et al. 2002), allowing the exploitation of different food resources and consequently new habitats. Moreover, symbionts may also serve as a source of new genes for their hosts through horizontal/lateral gene transfer, potentially providing new functions to their eukaryotic hosts (Frost et al. 2020; Wybouw et al. 2014). Additionally, several of them are important from a medical, veterinary, and agricultural point of view. Consequently, the use of insects as models in addition to providing meaningful ecological answers can at the same time provide solutions to economically important issues.

The insect gut is inhabited by a diverse and abundant microbial community. The gut microbiota can vary depending on specialized structures in the gut, pH values, redox conditions, digestive enzymes, food type and host

habitat(Yun et al. 2014). The gut microbiota can contribute with food digestion via production of digestive enzymes(Anand et al. 2010; Tartar et al. 2009), detoxification of allelochemicals from plants and synthetic insecticides (Adams et al. 2013; Almeida et al. 2017; Kikuchi et al. 2012). Besides, gut microbiota can allow insect hosts to explore suboptimal dietary sources through the production and release of vitamins and essential amino acids (Douglas 2006) and the cycling of nitrogen (French et al. 1976; Hongoh et al. 2008; Ohkuma et al. 1996). One of such example is the case of the western corn rootworm, in which the gut microbiota shifted in response to crop rotation increasing the abundance of *Klebsiella* and *Stenotrophomonas*. This change in the microbiota led to an increase of bacterial enzymes in the gut that could aid food digestion and were insensitive to the soybean cysteine protease inhibitors. In addition to their nutritional contributions, gut microbes can influence the process of species differentiation. Gut microbes of *Drosophila melanogaster* were demonstrated to interfere with the mating choice as they induced changes in the cuticular hydrocarbons that serve as contact sex pheromones(Sharon et al. 2011). Another study with the parasitoid *Nasonia* showed the gut microbiota interferes with the lethality of hybrids in this parasitic wasp (Brucker and Bordenstein 2013).

Lepidoptera is considered one of the most widespread and diverse groups of insects. Furthermore, many lepidopterans are considered important agricultural pests causing huge economic losses every year in several crops, as they own a broad range of host plants(Scoble 1992). The gut microbial communities in lepidopterans are simple and dynamic. Even considering that symbionts are important players in the interactions between insects and plants, currently it is controversial the relevance of microorganisms in lepidopterans. Some investigations point out that due to the group simple food tube morphology, without specialized structures, the high alkalinity of the midgut and rapid food digestion, intestinal bacteria are only temporary and non-resident, reflecting the microbiota of the food ingested (Anand et al. 2010; Hammer et al. 2017). In addition, studies have shown that these bacteria are not functionally important, because caterpillars can survive without bacteria in their gut (Hammer et al. 2017). On the other hand, reports have proved the presence of a microbial core gut that is distinct from the food ingested and capable of actively colonize the gut of lepidopterans (Mason et al. 2020; Teh et al. 2016). Some functional roles in the host have also been found in immunity, nutrition, and suppression of plant defense (Shao et al. 2017). In addition, evidence of vertical transmission has also been observed (Freitak et al. 2014; Teh et al. 2016). Therefore, given the importance of this group, more studies are fundamental to a better understand of microbial associations to develop possible new methods of interventions, and it may shed light upon symbiont–host co-adaptation and how insects acquire their microbial partners.

Within Lepidoptera, the genus *Spodoptera* is widely studied due to its broad geographical distribution, as it can be found in almost all continents (Kergoat et al. 2012). *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae), the fall armyworm, is a widespread and well-known agricultural pest in the Western hemisphere (Johnson 1987) and a recent invasive species to Africa, Asia and Oceania (Goergen et al. 2016, Otim et al. 2018, Johnson 1987, Padhee and Prasanna 2019, Piggott et al. 2021). *S. frugiperda* stands out as a highly polyphagous species that can feed on more than 300 host plants (Montezano et al. 2018). *S. frugiperda* is however widely known as a pest of grasses even though it does not possess a suitable buccal apparatus for this type of plant, due to the high quantity of silica in grasses. Mandibles of the *S. frugiperda* has serrate-like processes adapted to the consumption of dicots or monocots that do not accumulate silica (Pogue 2002). Therefore *S. frugiperda* is primitively a polyphagous, but because of the mandible-type it is thought to have started exploiting cultivated grasses as host plants only recently (Kergoat et al. 2021, Kergoat et al. 2012). This species adds another level of complexity as two host-adapted strains, known as "corn" (CS) and "rice" strains (RS) are recognized. The molecular dating analyses suggested these strains diverged more than 2

Myr ago(Kergoat et al. 2021) The strains are morphologically similar but they differ in several aspects such as their genomes and genetic expression, preferences and performance in host plants, sexual behavior and susceptibility to insecticides (Orsucci et al. 2020, Silva-Brandão et al. 2017, Schöfl, Heckel and Groot 2009, Ríos-Díez and Saldamando-Benjumea 2011, Dumas et al. 2015, Pashley, Hammond and Hardy 1992, Unbehend et al. 2013, Meagher, Nagoshi and Stuhl 2011, Veenstra, Pashley and Ottea 1995, Pashley, Hardy and Hammond 1995).

Considering the holistic view that a multicellular individual interacts directly and/or indirectly with its associated microbiome and that there are previous reports on the involvement of the gut microbiota in the process of host plant adaptation and speciation in insects, it is likely that symbionts associated with the gut of *S. frugiperda* are playing a role or reflecting the process of strain differentiation in this species. In order to gain a better understanding of this complex system of interactions, we used a variety of molecular methods and techniques.

First, we aimed to characterize the gut microbiota of the strains using 16S rRNA high-throughput sequencing in order to assess whether they harbor a gut microbiota with distinct composition and structure. For this purpose, we evaluated field-collected larvae feeding on different host plants, maize, millet and cotton in the same landscape. Then we compared the microbiota of strains from different populations throughout the Americas on the same host plant, maize, and also on rice plants, and analyzed populations under controlled laboratory conditions using maize and artificial diet as food sources. In the last study, we also sought to better understand the origin of this microbiota in two ways: by characterizing not only the bacteria present in the larval gut but also in the food ingested by them, and by detecting the presence of bacteria in the eggs and reproductive tissues of *S. frugiperda* using FISH and confocal microscopy in order to have clues whether the microbiota was only a reflection of the food ingested or coming from vertical transmission. To explore the functional aspects of these interactions, we used metatranscriptomics to define the transcriptional profile of genes differentially expressed between the strains in the gut of *S. frugiperda* and the transcriptional profile of the associated bacteria. Finally, we used metabolomics to compare the metabolic profile of the strains when feeding on different food sources.

## 1.1. HYPOTHESIS AND PREDICTIONS

The gut microbiota of *Spodoptera frugiperda* strains is involved in the process of host plant adaptation

1. The gut microbiota will differ from strain to strain when using the same host plant. And changes in the gut microbiota within a strain from one host plant to another will be less conspicuous than changes in between strains in the same host plants;
2. The variation in the composition of the gut microbiota would be higher in between strains than within different populations of a same strain;
3. The functional contribution of the gut microbiota will differ in between strains when using the same host plants;
4. Members of the gut microbiota would be vertically transmitted from mother to offspring.

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# 2. DYSBIOSIS OF THE LARVAL GUT MICROBIOTA OF *Spodoptera frugiperda* STRAINS ON DIFFERENT HOST-PLANTS

## ABSTRACT

The gut microbiota plays important roles in the bioecology of insects, including host plant adaptation and speciation. *Spodoptera frugiperda* has two well-established host-adapted strains with marked differences at the genetic and host plant utilization levels. We investigated whether differences in the gut microbiota would occur between the "corn" (*CS*) and "rice" (*RS*) strains of *S. frugiperda* when feeding on different crops. The gut microbiota of larvae fed on corn and millet was predominantly represented by *Firmicutes* followed by *Proteobacteria*, with an opposite pattern in larvae fed on cotton. No differences were observed between the *CS* and *RS* using PERMANOVA. PCoA analyses resulted in distinct bacterial clusters based on the host plant. Comparisons of strains gut microbiota at the phylum level resulted in differences only for larvae fed on cotton, but differences in the relative abundance of minor representatives at the genus level between strains were observed regardless of the food source used. We also found differences in the potential for functional contribution of bacteria between the strains. In conclusion the gut microbiota of *S. frugiperda* is strongly modulated by the host plant while strains seemed to play a minor role in changing the abundance of members of the gut bacterial community.

**Keywords:** Fall armyworm; gut microbes; host preference; host utilization; symbiosis, nutritional ecology.

## 2.1 INTRODUCTION

It is increasingly recognized that microbes, particularly bacteria, play a crucial role in a wide range of aspects of the host physiology, ecology and evolution (Gilbert et al., 2018; E. T. Miller, Svanbäck, & Bohannan, 2018; Shropshire & Bordenstein, 2016). Thus, it is expected that hosts harboring beneficial microbes will have advantages over their peers, and their joint exposure to processes of natural selection will also act on characteristics that will contribute to select the most suitable microbiota to deliver the services required by the host, maintaining therefore the established association (Shapira, 2016). The best symbiont-service providers will increase the fitness of their host and of their own, providing a basis for coevolution (Shapira, 2016). Thus, the host-microbiota coevolution predicts the existence of species-specific gut microbiota composed of beneficial microbes adapted to the host (Shapira, 2016).

The insect gut may harbor a diverse and abundant microbial community. The composition of the gut microbiota is prone to variations depending on the existence of specialized structures in the gut, gut pH, redox conditions, digestive enzymes, antimicrobial peptides, food type, and host habitat among others (Ryu et al., 2008; Yun et al., 2014). The gut microbiota can contribute with food digestion through the synthesis and release of digestive enzymes (Anand et al., 2010; Krishnan et al., 2014), and the detoxification of plant allelochemicals and synthetic insecticides (Adams et al., 2013; Almeida, Moraes, Trigo, Omoto, & Cônsoli, 2017; Kikuchi et al., 2012). Moreover, the gut microbiota can provide the host with vitamins and essential amino acids (A. E. Douglas, 2006; Nikoh, Hosokawa, Oshima, Hattori, & Fukatsu, 2011), as well as recycle waste nitrogen (French, Turner, & Bradbury, 1976; Ohkuma, Noda, Usami, Horikoshi, & Kudo, 1996), allowing the host to establish new associations with suboptimum food sources. In addition to their nutritional contributions, gut microbes can influence the process of species differentiation. Gut microbes of Drosophila melanogaster interfere with the mating choice as they influence the hydrocarbon composition of the cuticle that serve as contact sex pheromones (Sharon, Segal, Zilber-

Rosenberg, & Rosenberg, 2011)]. Gut microbes were also shown to influence species differentiation by inducing hybrid lethality in the parasitic wasp *Nasonia* (Brucker & Bordenstein, 2013).

The fall armyworm *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae) is a severe, widespread and well-known agricultural pest that was restricted to the Americas (Johnson, 1987), but has recently invaded the Old-World through Africa (Goergen, Kumar, Sankung, Togola, & Tamò, 2016; Otim et al., 2018)], and has now reached the far east Asia (H. Liu et al., 2019; Padhee & Prasanna, 2019)]. There are two well-characterized host-adapted strains of *S. frugiperda* that regardless of the genetic differences (Dumas et al., 2015; Gouin et al., 2017; Roy et al., 2016) and postzygotic mechanisms of reproductive isolation (Kost, Heckel, Yoshido, Marec, & Groot, 2016)] identified so far, are still defined as strains carrying different bioecological traits belonging to a single species. Molecular dating analyses indicate these strains diverged more than 2 Myr ago (Kergoat et al., 2012)]. The two strains are identified as the rice (*RS*) and the corn (*CS*) strains, and they show a high level of genetic differentiation (Gouin et al., 2017)], with differences in host plant utilization (Pashley, Hardy, & Hammond, 1995; Veenstra, Pashley, & Ottea, 1995). The *CS* feeds preferentially on corn, millet, cotton and sorghum, whereas the *RS* on rice and several pasture grasses (Cano-Calle, Arango-Isaza, & Saldamando-Benjumea, 2015; Pashley, 1986; Pashley, Johnson, & Sparks, 1985)]. *CS* and *RS* also have different rates of development and fitness depending on the host-plant used (Busato et al., 2005; Meagher, Nagoshi, Stuhl, & Mitchell, 2004; Pashley et al., 1995; Veenstra et al., 1995). They also differ in mating behavior, such as mating allochronism (Pashley, Hammond, & Hardy, 1992; Schöfl, Dill, Heckel, & Groot, 2011)] and pheromone composition (Groot et al., 2008; Lima & McNeil, 2009)]. A broad study of different populations from Brazil recently recognized several molecular markers and loci under selection when comparing the different strains feeding on different host plants (Silva-Brandão et al., 2018)].

Considering the participation of the gut microbiota in the processes of host adaptation to new food resources and of speciation, we hypothesized the gut microbiota may be involved in the process of host-strain adaptation in *S. frugiperda*. To our hypothesis hold true, we predict that the gut microbiota of the strains differs from each other and therefore we also expect a different functional contribution from each other in exploiting similar host plants. In addition, we predicted that alterations in the gut microbiota within one strain from one host plant to another would be less conspicuous than the changes in the microbiota between strains in the same host plant.

Our investigation addressed field-collected insects, which carry a much higher variation in the gut microbiota than those maintained under controlled laboratory conditions (Gomes, Omoto, & Cônsoli, 2020). Assessing the variation available under field conditions can provide essential information on potential symbionts that could be ecologically important to their hosts in their natural habitats. The selection pressure in natural and laboratory conditions are quite different and can lead to the selection of distinct traits, including the interaction with symbiotic bacteria (Paniagua Voirol, Frago, Kaltenpoth, Hilker, & Fatouros, 2018).

## 2.2 MATERIAL AND METHODS

### 2.2.1 Sampling

Larvae of the fall armyworm were collected from corn, cotton and millet crops cultivated in the same landscape in the western region of Bahia state, in the district of Roda Velha, Brazil (12°42'0" S 45°50'0" W). In this area there is crop rotation and some of them also cooccur, moreover there is no evidence indicating occurrences of

migration of *S. frugiperda* in the area, therefore we assume that the caterpillars collected on the different host plants analyzed in this study correspond to the same population of *S. frugiperda.* The larvae were collected and directly fixed in RNAlater™ (Thermo Fisher), taken to the laboratory where the width of their head capsules was individually measured under a stereoscopic. Only larvae with head capsules in the range of 1.6 – 2.9 mm, corresponding to the late instar were used in the experiments.

### 2.2.2 *Spodoptera frugiperda* host strains identification

Strain identification was performed using the DNA extracted in the same way described below (session 2.3) from part of the larvae tegument. Then, RFLP-PCR analysis of a partial sequence of the mitochondrial COI gene using the primers set JM76 (5´-GAGCTGAATTAGGRACTCCAGG-3´) and JM77 (5´-ATCACCTCCWCCTGCAGGATC-3´) (Levy, Garcia-Maruniak, & Maruniak, 2002)].The PCR mixture contained 100-150 ng of DNA, 1.5 mM of $MgCl_2$, 1x PCR buffer, 0.2 mM of each dNTP, 0.32 μM of each primer and 0.63U of GoTaq® DNA Polymerase (Promega) in a total volume of 25 μL. The thermocycling condition was 94°C x 1 min (1x) followed by 33 cycles at 92°C x 45 s, 56°C x 45 s, 72°C x 1 min, and a final extension at 72°C x 3 min (1x).

Amplicons were then subjected to restriction analysis using the *Msp*I (HpaII) restriction endonuclease. This enzyme produces two fragments of 497pb and 72pb for amplicons of the *CS*, and no digestion of the 569-bp amplicon for *RS* is observed. We used 10 μL of the PCR product added to 18 μL nuclease-free water, 2 μL 10x Buffer Tango and 1 unit of *Msp*I. Samples were gently mixed, spun down for a few seconds on a tabletop centrifuge and incubated overnight at 37°C. Subsequently, the resulting products of digestion were verified using 1.8% agarose gel electrophoresis following standard procedures (Sambrook, 2001)].

### 2.2.3 Gut isolation and genomic DNA extraction

Midgut dissection was performed under aseptic conditions under a laminar flow hood after larval surface sterilization in cooled 70% ethanol solution added with 0.2% sodium hypochlorite (5 min). Larvae were washed once in sterile water and transferred to sterile saline solution (125 mM NaCl, 4°C) for Midgut dissection. Tissues were stored in absolute ethanol at -20°C until DNA extraction. The midguts of individual larvae were grouped according to strain and host plant. Three true biological replicates were established for each treatment (1 replicate = pooled guts of 3 larvae). The homogenization was performed in liquid nitrogen.

DNA was extracted using the protocol for genomic DNA preparation from RNAlater™ (Thermo Fisher) preserved tissues with some modifications. The macerate of the pooled midguts was placed in 750 μL digestion buffer (60 mM Tris pH 8.0, 100 mM EDTA, 0.5% SDS). Proteinase K was added to a final concentration of 500 μg/mL and mixed well by inversion. Samples were incubated overnight at 55°C. Afterwards, 750 μL of phenol: chloroform (1:1) was added and rapidly inverted for 2 min. Samples were centrifuged at a toptable centrifuge for 10 min. The aqueous layer was collected and the process of phenol: chloroform extraction was repeated twice before a final extraction with chloroform. The aqueous layer was collected and added to 0.1 volume of 3 M sodium acetate, pH 5.2, and 1 volume 95% ethanol. Samples were mixed by inversion, incubated for 40 min at -80°C, and centrifuged (27,238 *g* x 30 min x 4°C). The pellet obtained was washed twice in 1 mL of 85% ice-cold ethanol, centrifuged for 10 min after each wash and dried at 60°C during 5-10 min in a SpeedVac. Finally, the pellet was

resuspended in DEPC water. DNA concentration and quality were estimated using a Nanodrop UV spectrophotometer.

### 2.2.4 16S rDNA sequencing and analysis

DNA samples were PCR-amplified using primers targeting the V3-V4 region of the 16S rRNA gene (Klindworth et al., 2013). The reaction was programmed at 95°C for 3 min (1 cycle), followed by 25 cycles at 95°C for 30 s, 55°C for 30s and 72°C for 30 s, with a final extension (1 cycle) at 72°C for 5 min. DNA samples was sent to the Animal Biology Laboratory (ESALQ / USP, Piracicaba, SP) for library preparation using Illumina kit, following their recommendation, and for sequencing. Genomic DNA was amplified by PCR with primers targeting the hypervariable V3–V4 region of the 16S rRNA gene. Paired-end reads (2x – 250 bp) were generated with multiple barcodes and sequenced on the Illumina MiSeq platform.

The sequences were processed using QIIME2 (2017.4 release) (Caporaso et al., 2010) according to the developers recommendations. The demultiplexed reads were quality filtered, had singletons removed, denoised of truncated reads (250 bp length), and filtered for phiX and chimera sequences with the q2-dada2 plugin. A feature table was generated with a summary of amplicons sequence variants (ASV), a method shown to have higher resolution than the OTU method, with biological meaning independently from a reference database (Callahan, McMurdie, & Holmes, 2017)]. All ASVs were aligned with the mafft program (via q2-alignment plugin), and the construction of the phylogenetic tree was performed with FastTree. The sequences were rarefied to a depth equivalent to the smaller total count in our samples in order to maintain all of them in our analysis. Rarefied feature tables were used as input for alpha and beta diversity analyses and statistics using the core-metrics-phylogenetic method. We examined alpha diversity using Shannon and Simpson's index, both estimator of species richness and evenness, being the first more weight on species richness and the second on species evenness. And Chao1 index, an abundance-based estimator that gives more weight to the low abundance species. For the beta-diversity we used Jaccard distance that does not take the ASVs abundances into account, just the presence and absence in the samples, and Jackknifed weighted UniFrac distance, that considers the phylogenetic relationships and the abundance of the ASVs. We used them as a basis for hierarchical clustering with Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and PCoA plots. The variation degree among the repetitions was evaluate using the Jackknife technique, and displayed by confidence ellipsoids around the samples. Pairwise PERMANOVA (999 permutations) was used to detect differences in composition (β-diversity) between groups. Taxonomy was assigned to ASVs using pre-trained (V3-V4) classifier via q2-feature-classifier trained on the Silva_132_99_16S.fna file and clustered at 97% similarities (341-806 region, seven-level taxonomy). Functional profiles from 16S rRNA data were predicted using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2) plugin for QIIME2. For each sample, the composition of The Kyoto KEGG pathway abundances from the predicted KEGG ORTHOLOGY (KO) abundances were made following the default protocols of the PICRUSt2 GitHub page (https://github.com/picrust/picrust2/wiki).

We used the Statistical Analysis of Metagenomic Profiles (STAMP) software (Parks, Tyson, Hugenholtz, & Beiko, 2014)] to identify taxa that differ significantly between groups. White's non-parametric t-test was used for taxonomic comparisons and the bootstrap method was used to calculate the confidence intervals. Functional comparisons were done using the Welch's t-test to test statistical hypothesis and Welch's inverted as a confidence interval method.

## 2.3 RESULTS

Our sampling effort was adequate to access the diversity of the microbiota in the gut of both strains of *S. frugiperda* as the rarefaction curves based on the Shannon index did not change after sampling 8,000 sequences, much below the near 30,000 sequences obtained for the lowest represented 16S rRNA library (Anexo A).

Alpha diversity was similar between strains when disregarding the host plant in all calculated indices (Fig. 1). When the alpha diversity was compared within the groups of host plants, the corn strain showed higher Shannon and Simpson indices when the caterpillars fed on millet. On the other host plants, the gut microbiota showed similar values of Shannon, chao1 and Simpson indices (Fig. 1). The beta diversity of the gut bacterial communities of the *RS* and *CS* strains were strongly influenced by the host plants tested – corn, millet and cotton, but no differences in the diversity of the gut bacteria between *RS* and *CS* were detected when feeding on the same host plant (Tab. 1, Fig. 2).

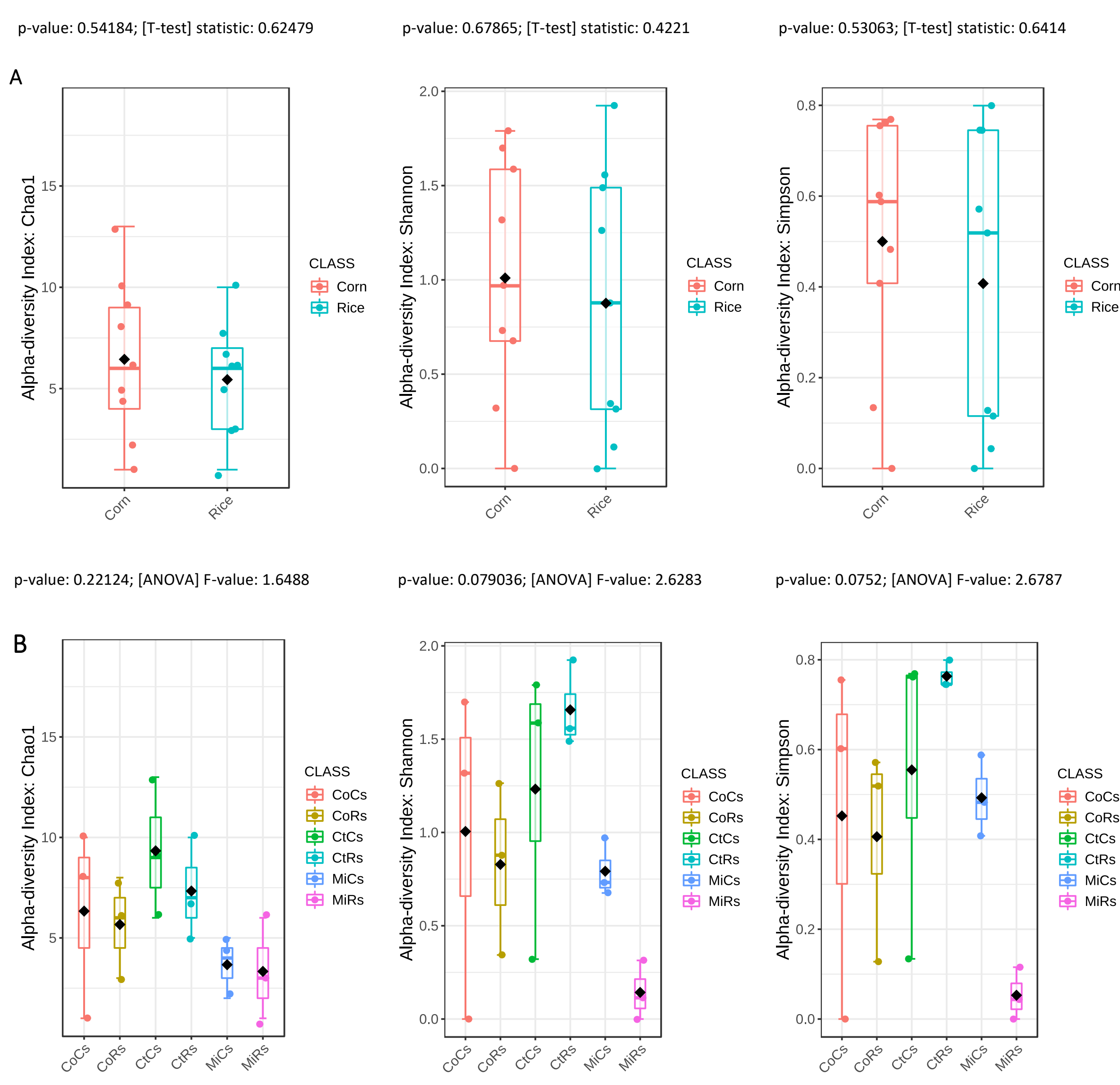


**Figure 1.** Shannon, Chao1 and Simpson Diversity index in (A) samples from midgut of *Spodoptera frugiperda* strains (RS or CS) regardless diet. (B) Samples from midgut of *Spodoptera frugiperda* strains (RS or CS) fed on different diets (Corn=Co, Cotton=Ct and millet=Mi). The samples are represented on X-axis and their estimated diversity on Y-axis. The p-values from the Test T are shown in which box of comparison.

**Table 1.** Pairwise PERMANOVA comparisons based on weighted UniFrac distance matrices among the different treatments after 999 permutations. Abbreviations: CS= Corn Strain; RS= Rice strain

| Group 1 | Group 2 | Sample size | pseudo-F | p-value | q-value |
|---|---|---|---|---|---|
| ***CS*** **- Corn Plant** | ***RS*** **– Corn Plant** | 6 | 0.615 | 0.886 | 0.886 |
| ***CS*** **– Cotton Plant** | ***RS*** **- Cotton Plant** | 6 | 1.126 | 0.284 | 0.525 |
| ***CS*** **– Millet Plant** | ***RS*** **– Millet Plant** | 6 | 0.329 | 0.816 | 0.874 |
| **Corn** | **Cotton** | 12 | 4.087 | 0.012 | 0.024 |
| **Corn** | **Millet** | 12 | 5.098 | 0.052 | 0.052 |
| **Cotton** | **Millet** | 12 | 9.117 | 0.016 | 0.024 |

UPGMA clustering analysis based on the V3-V4 region of the 16S rRNA gene yielded three clusters, each of them grouping most of the samples of each host plant. The replicates of the gut microbiota of the cotton-fed RS larvae were the most distinct, all of them resolved in one of the clusters formed. The cotton-fed CS replicates grouped with the millet samples and the other with the corn-fed caterpillars. The corn-fed caterpillar replicates all resolved together except for one RS replicate that clustered with the millet replicates. Likewise, the millet replicates were in the same cluster except for one RS that clustered with the corn replicates (Fig. 2).

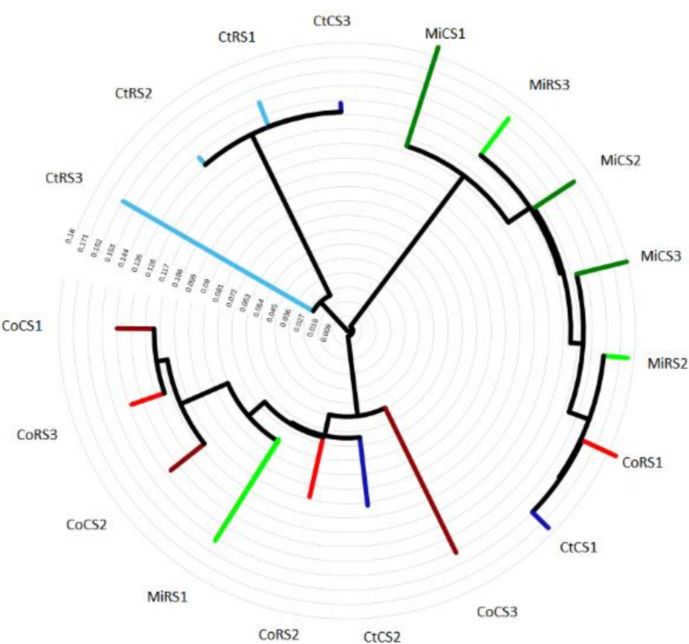


**Figure 2.** Jackknifed Weighted Pair Group Method with Arithmetic mean (UPGMA) cluster tree of *S. frugiperda* corn strain (*CS*) and rice strain (*RS*) gut microbiota when feeding on corn (Co), millet (Mi) and cotton (Ct).

We identified 17 phyla of bacteria in the midgut of *S. frugiperda* (Anexo B). The gut microbiota of larvae feeding on the monocots corn and millet was dominated by *Firmicutes,* followed by *Proteobacteria* regardless of the host plant strain (*RS* or *CS*) (Fig. 3). But a significant increase in the relative proportion of *Proteobacteria* was observed in both *RS* and *CS* larvae when feeding on the dicot cotton (Fig. 4).

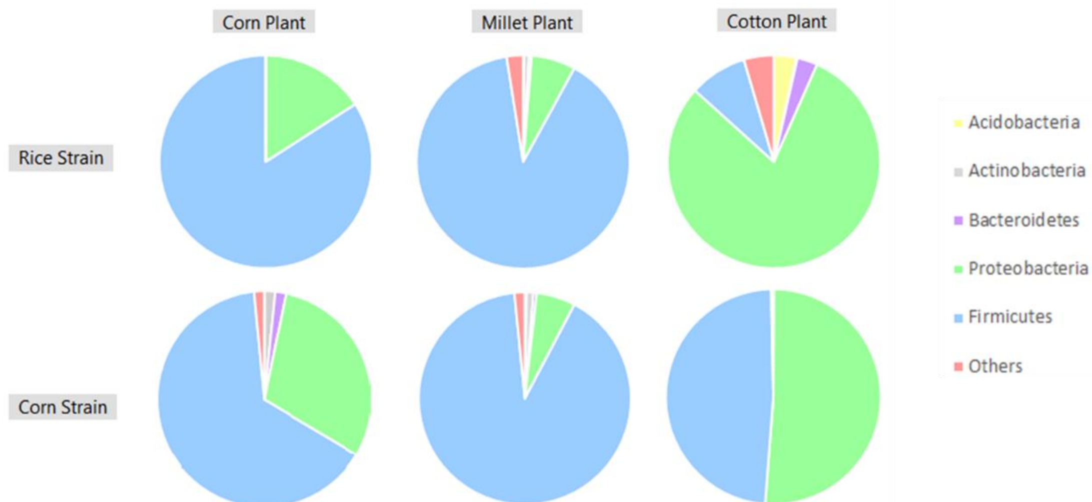


**Figure 3.** Pie chart of the relative proportion (%) of bacterial phyla abundance between the gut microbiota associated with *S. frugiperda* corn and rice strain when feeding on corn, millet, and cotton.

*Enterobacter, Enterobacteriaceae, Klebsiella* and *Acinetobacter* (γ-*Proteobacteria, Enterobacteriaceae*) represented most of the diversity of enterobacteria in the midgut of *S. frugiperda*. *Firmicutes* was mostly represented by *Enterococcus* (*Bacilli, Enterococcaceae*) and *Erysipelatoclostridium* (*Erysipelotrichia, Erysipelotrichaceae*). The unidentified *Enterobacteriaceae* and *Enterobacter* represented most of the diversity of the larval midgut microbiota from cotton; *Enterococcus* and *Erysipelatoclostridium* from millet; and *Enterococcus* and *Klebsiella* from corn (Fig. 5).

Comparisons of the relative abundance of the most common ASVs in the midgut of *RS* and *CS* larvae of *S. frugiperda* at the genus level, indicated that the *Enterobacteriaceae* was more abundant in *RS* than in *CS*, but only when larvae were collected in cotton ($p$ = 0.046). Larvae fed on millet and corn presented differences only in the minor representatives of the gut microbiota. In the corn plant , *RS* larvae exhibited a lower relative abundance in *Stenotrophomonas* ($p$ = 0.031) and a higher abundance of *Erysipelotrichaceae* ($p$ = 0.034) than the *CS* larvae. In the millet plant, the abundance of *Sphingobium* ($p$ < 0.005) and *Acinetobacter* ($p$ = 0.012) in the midgut of *RS* differed from that in the midgut of *CS* larvae (Fig. 6).

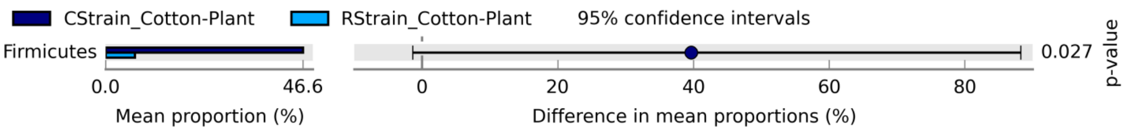


**Figure 4.** Differences in relative proportion (%) of bacterial phyla abundance between the gut microbiota associated with *S. frugiperda* corn and rice strain when feeding on cotton.

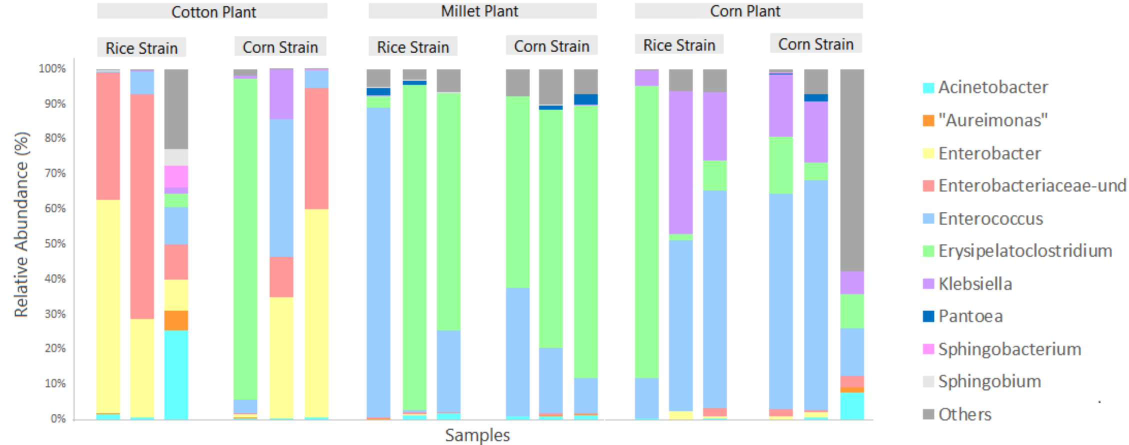


**Figure 5.** Taxa plot of bacterial genera of the gut microbiota associated with *S. frugiperda* corn strain and rice strain when feeding on corn, millet, and cotton.

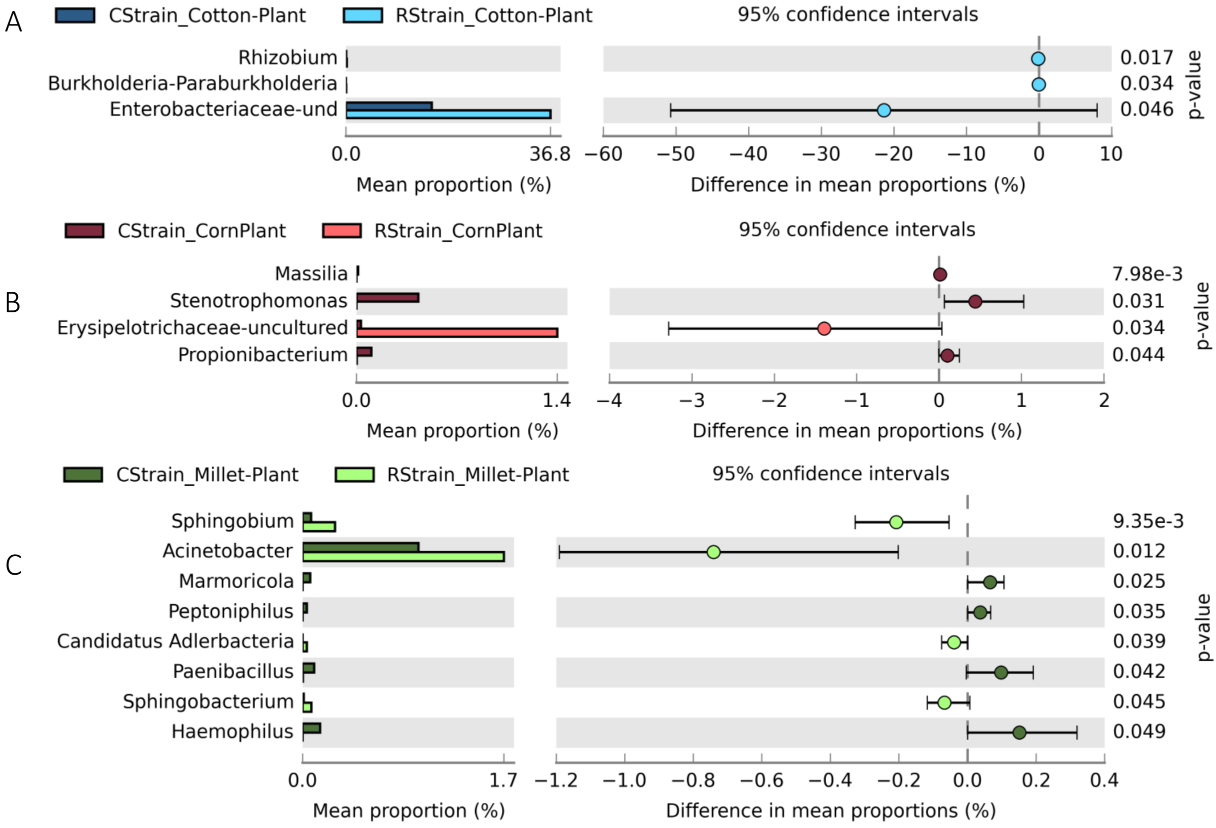


**Figure 6.** Differences in relative proportion (%) of bacterial genus abundance in the gut microbiota associated with *S. frugiperda* corn and rice strain when feeding on (A) corn, (B) millet, and (C) cotton

The small differences in the diversity of the midgut microbiota between the strains were enough to allow the detection of changes in the potential functional contribution of the gut microbiota of *RS* and *CS*. But although highly significant, the variation in the proportional changes of the potential functional contribution was very minor, and possibly with no physiological impact. Differences in energy metabolism between *CS* and *RS* were detected but only for potential contribution of the *norF* gene, which belongs to a gene cluster involved in nitrogen metabolism and microbial defense against nitric oxide toxicity (p=0.045). The gut microbiota of the *CS* had a higher potential contribution for the *norF* protein (Fig. 7). Pairwise comparisons of *CS* and *RS* in each host plant detected that the

midgut microbiota of the *RS* had a higher potential contribution to metabolism (*wcaE* - glycosyltransferases, beta-glucuronidase - secondary metabolites) and the processing of genetic information (integrase) in millet, while the contribution of the gut microbiota of the *CS* was much higher to the signaling and cellular processes (*hpaX*, *fliJ*, *hoxN*, *flgL*, *dmsB*, *fliS*, *fliH*, *cheV*, *flgE*, *fliM*, *flgD*, *fliNY*, *fliE*, *fliI*, *fliP*) (Fig. 8). The potential contribution of the gut microbiota to signaling and cellular processes (*pilX*, *terZ*, *TC.AAA*) was also higher in the *CS* than the *RS* strain when feeding on corn. In cotton, the gut microbiota of both host-adapted strains had increased contribution to metabolism, but while the contribution of the microbiota of the *CS* to metabolism was due to the higher abundance of glyceride 2-quinase (carbohydrate metabolism) and L-serine dehydrate (amino acid metabolism), the potential contribution of the gut microbiota of the *RS* to metabolism was due to the increased abundance of oxidases (glycine and vitamin cofactors) and amylosucrase (carbohydrate metabolism). The gut microbiota of the *RS* also had a higher potential of contribution to the processing of genetic information (*pinR*) and signaling and cellular processes (ATP-binding cassette) than that of the *CS* strain when feeding on cotton (Fig. 8).

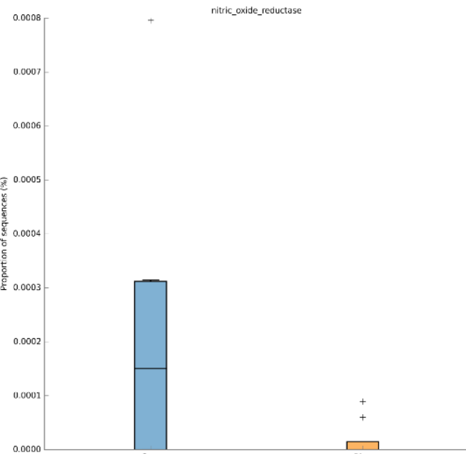


**Figure 7.** Box plot of the difference in PICRUSt functional prediction between the gut microbiota associated with *S. frugiperda* corn and rice strains regardless of host plant.

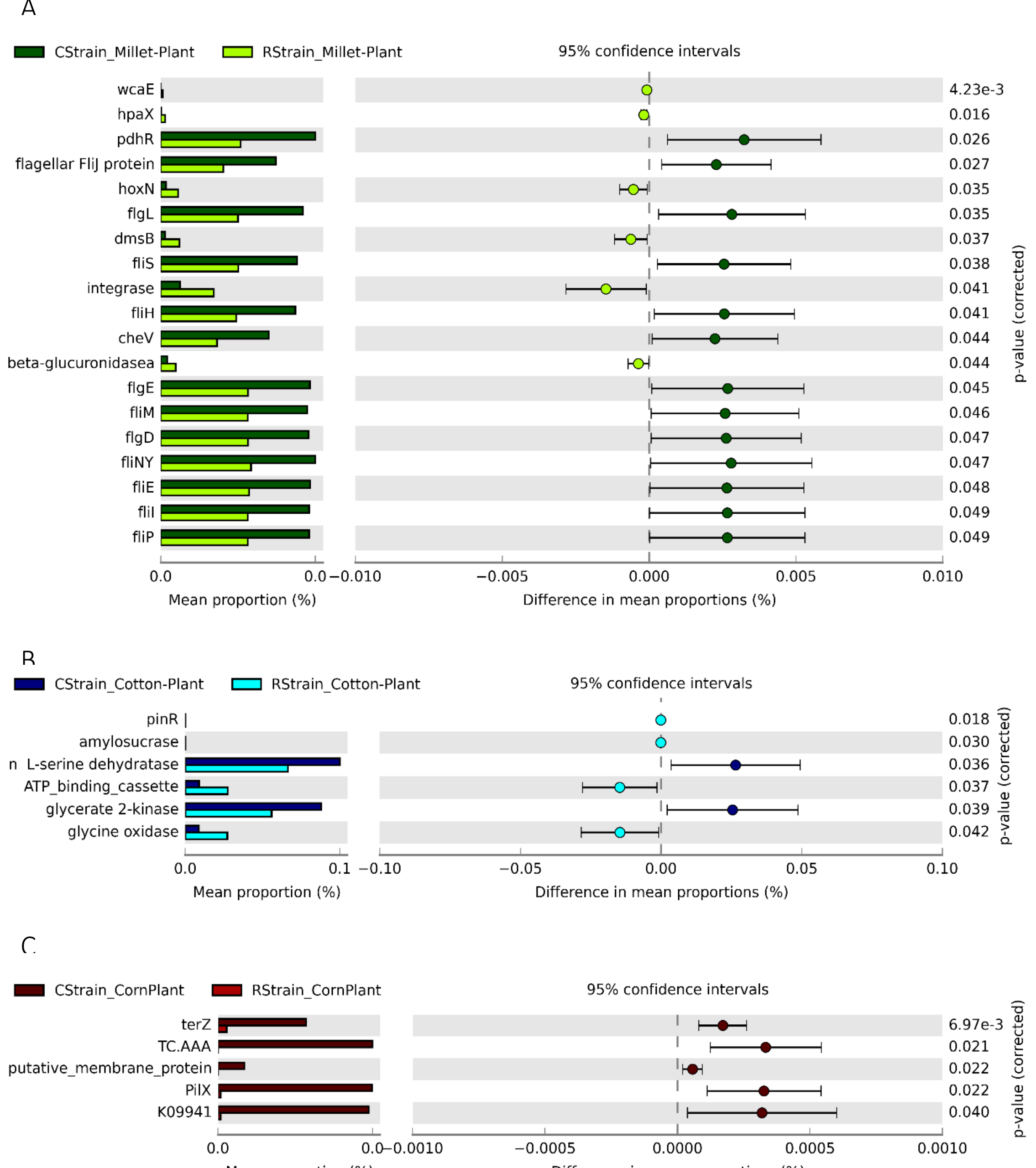


**Figure 8.** Difference in relative proportion (%) of PICRUSt functional prediction of the the gut microbiota associated with *S. frugiperda* when feeding on corn and rice strain when feeding on (A) millet (B), and cotton, (C) corn.

## 2.4 DISCUSSION

The *Spodoptera frugiperda* strains share an overall similar microbial diversity when feeding on the same host plants, although there is a significant genetic divergence between the host-adapted strains. The midgut microbiota of *CS* and *RS* larvae went through similar changes when the larvae were feeding on the different host plants analyzed. These results suggest that the diet, but not the strains play a more important role in shaping the gut bacteria community structure of these larvae. These findings are similar to those found in humans, where host genetics

played a minor role in determining microbial composition than environmental factors such as diet (Rothschild et al., 2018)].

There are convincing molecular and biological data demonstrating the *RS* and *CS* strains of *S. frugiperda* are in a process of speciation and host plant adaptation (Dumas et al., 2015; Gouin et al., 2017; Saldamando & Vélez-Arango, 2010)]. Although we can clearly detect a shift in the midgut microbiota in response to the different host plants tested, it was not possible to detect selection of specific microbe species that would characterize the diversity of the gut microbiota of each strain.

Thus, we first argue that the lack of a clear difference in the midgut microbiota of *RS* and *CS* could be explained by the recency of their speciation process (Kergoat et al., 2012). In fact, the strains are still interbreeding and producing viable offspring (Dumas et al., 2015), although postzygotic mechanisms of reproductive isolation are acting as facilitators of speciation in *S. frugiperda* (Kost et al., 2016). Therefore, the short evolutionary period may not be enough for selection of specific members of the microbial by the host. In addition, the gut microbiota composition is regulated by several factors, such as immune system (e.g. lysozyme, reactive oxygen species and antimicrobial peptides) (Chapelle, Girard, Cousserans, Volkoff, & Duvic, 2009), physical and chemical properties of the gut (e.g. pH and redox conditions), presence of digestive enzymes and development of specialized structures in the gut. Perhaps these processes that are involved in controlling and selecting the components of the gut microbiota have not differentiated enough between the strains to cause differences in the midgut bacterial community of the strains.

Alternatively, we also argue that the existing differences among bacterial members of the midgut of each strain could not be adequately accessed using the 16S metabarcoding procedure, which is based on a very short sequence of the 16S rRNA gene. It is clear that gut symbionts become host specialized during the process of host evolution (Frese, Benson, Tannock, Loach, & Kim, 2011; Kwong, Engel, Koch, & Moran, 2014), and data from single cell genome sequencing have demonstrated the existence of significant genomic differences even within bacterial cells that share highly homologous 16S rDNA sequences (Engel, Stepanauskas, & Moran, 2014).

Our third argument is based on the presence of individuals from mixed crossings from *CS* and *RS*, as our field samples also had low *RS* larvae represented (corn=28%; millet: 25%; cotton:15%; data not shown). As *RS* x *CS* crossings naturally occur in the field (Nagoshi & Meagher, 2003), and that the bacterial composition of the gut of hybrids can differ from that of parental (S. G. Miller & Miller, 1996)], we cannot ruled out that the existence of *CS*-*RS* or *RS*-*CS* hybrids in our samples may have diluted the differences between *RS*-*RS* and *CS*-*CS* midgut bacterial communities.

Finally, we examined only the larval midgut in view of its important function in food digestion and assimilation (Billingsley, 1996; J. A. T. Dow, 1987). However, this gut region is predisposed to be an adverse environment for microorganisms due to enzymes, antimicrobial peptides, and high alkaline pH (J. A. Dow, 1984). In addition, another possible obstacle bacteria face to colonize the midgut of lepidopterans is the presence of a type 1 peritrophic membrane, which is produced and continuously replaced, moving subsequently with the food bolus along the digestive tract (Hegedus, Toprak, & Erlandson, 2019; Jiang, Vilcinskas, & Kanost, 2010)]. Perhaps some resident symbionts could colonize the hindgut, which is known to be more favorable to bacteria due to the lack of digestive enzymes, presence of favorable morphological structures, and of ions and metabolites released in the urine (Angela E. Douglas, 2015)].

The midgut of the *CS* and *RS* larvae of *S. frugiperda* carries basically the same group of bacteria in each host plant analyzed. *Erysipelatoclostridium, Enterococcus,* one unidentified genus of *Enterobacteriaceae* (*Enterobacteriaceae*-

und)*, Klebsiella* and *Acinetobacter*. The relative abundance of these bacteria was altered depending on the host plant, but changes observed in each host plant were quite similar between *RS* and *CS* larvae.

*Enterobacteriaceae*-und and *Enterobacter* were the most abundant in the gut microbiota of cotton-fed larvae. *Enterobacter* is commonly found in the gut , and was shown to reproduce in the insect midgut (Tanada & Kaya, 2012; Watanabe, Abe, & Sato, 2000)].

*Erysipelatoclostridium*, the most abundant in the bacterial community of the midgut of larvae from millet is reported as one of the major genus in the human gut, where it utilizes proteins and saccharides as substrates, producing acetate, carbon dioxide, hydrogen, formate and lactate (Oliphant & Allen-Vercoe, 2019)]. Food-induced changes in the *Firmicutes*: *Bacteroidetes* ratio in the gut of humans were noticed by alterations in the abundance of *Erysipelatoclostridium* (Smith-Brown, Morrison, Krause, & Davies, 2016)]. In model vertebrate animals, *Erysipelatoclostridium* has been associated with the upregulation of glucose and fat transporters in the gut (Günther, Remer, Kroke, & Buyken, 2007)]. Increased abundance of *Erysipelatoclostridium* in the gut microbiota of rats has been positively correlated with the fatty acid isovalerate (by-product of leucine fermentation), and be affected by the digestibility of the food source (Han et al., 2018)]. In insects, *Erysipelatoclostridium* has been reported as an unculturable gut symbiont of field populations of *Spodoptera litura*, but nothing is known on the role of this bacterium in the gut of insects (Yalashetti, Yandigeri, Rudrappa, Muthugounder, & Gopalasamy, 2017)].

*S. frugiperda* fed on corn present a high abundance of the Gram-negative bacterium *Klebsiella* sp. This genus has been isolated from corn leaves and has also been identified in the gut of many Lepidoptera species, demonstrating the ability of these bacteria to colonize the digestive tract of these insects (Chen et al., 2016; Snyman, Gupta, Bezuidenhout, Claassens, & van den Berg, 2016)]. In addition, *Klebsiella* can play a role as a mediator of insect-plant interactions. The presence of this bacteria in oral secretions of *S. frugiperda* larvae can regulate the expression of the herbivorous induced proteinase inhibitor gene (*mpi*) in corn (Acevedo et al., 2016)].

*Enterococcus* was present in all our samples but with higher abundance in samples from corn and millet. Nearly 40 species of *Enterococcus* are known and are predominantly reported as commensals of the gastrointestinal tract (Ramsey, Hartke, & Huycke, 2014). Studies with *Spodoptera littoralis* suggested the existence of a clear symbiotic relationship with *Enterococcus mundtii,* a biofilm-like structure maker that contributes with the secretion of antimicrobial peptides (AMP) supposedly contributing to the host as an additional chemical barrier against pathogens (Yongqi Shao, Arias-Cordero, Guo, Bartram, & Boland, 2014; Y. Shao et al., 2017)].

Another aspect that we observed in our study was the importance of diet in shaping the midgut microbiota as already documented in the literature (Gayatri Priya, Ojha, Kajla, Raj, & Rajagopal, 2012; Tang et al., 2012; Yun et al., 2014)]. Our samples were collected from a single, well-defined landscape; thus, we did not expect much variation in the genome of the sampled insect population, a factor that could also interfere with the gut microbiota. Therefore, variation lies mainly in the host plant and strain. Analyzing the gut microbiota composition, we observed a dysbiosis in the gut driven by diet. These changes in the community structure may benefit *S. frugiperda* as an agriculture pest. One example of this is the case of the western corn rootworm, in which the gut microbiota shifted in response to crop rotation, increasing the abundance of *Klebsiella* and *Stenotrophomonas*. This change in the microbiota led to an increase of bacterial enzymes in the gut that aided in food digestion, as these microbial enzymes were insensitive to soybean cysteine protease inhibitors (Chu, Spencer, Curzi, Zavala, & Seufferheld, 2013)].

The analysis of the potential functional contribution of the midgut microbiota of *S. frugiperda* resulted in only a single difference between the strains when we disregard the host plant. The higher potential contribution of the *CS* midgut bacteria to denitrification due the higher abundance of species carrying putative *norF* genes, which has

been described as part of a cluster of genes encoding nitric-oxide reductases. Analysis of mutants of this cluster of genes indicated *norF* is involved in the regulation of nitric oxide reductase activity (De Boer et al., 1996)]. Differences in the potential functional contribution between bacterial communities arise either by the presence of different bacteria members or the exposure to diverse environmental conditions. Since we did not find differences in the composition of the bacterial communities of the midgut between strains when we disregarded the host plant, we can assume that changes detected in the potential functional contribution of the midgut microbiota is likely a result of different biochemical conditions in the gut of each strain. Environments with low oxygen concentration allows the expression of genes involved in the process of denitrification, enhancing the bacterial survival and growth capability in anaerobic environments (Delgado, Casella, & Bedmar, 2007)].

The major facilitator transporter 4-hydroxyphenylacetate permease (*hpaX*) is a transmembrane transporter of 4-hydroxyphenylacetate (HPC), which is the first product of the degradation process of 4-hydroxyphenyl-acetic acid (HPA). HPA has been linked to the overgrowth of bacteria in the gut (Chalmers, Valman, & Liberman, 2019)]. HPC is also known to be a fermentation product of amino acids, and could be anaerobically degraded by denitrifying bacteria (Seyfried, Tschech, & Fuchs, 1991)]The dimethyl sulfoxide reductase subunit B (*dmsB*) is a subunit of the terminal anaerobic electron transfer enzyme DMSO reductase, which is also involved in anaerobic metabolism. The high affinity nickel permease (*hoxN*) is involved in the incorporation of nickel into hydrogenase and urease enzymes. The acquisition, delivery and incorporation of nickel into target enzymes (e.g. urease and hydrogenase) are essential for the catalytically activity of nickel-dependent enzymes, and some bacteria, such as *Escherichia coli, Helicobacter pylori*, *Yersinia* species, *Salmonella, Shigella* and *Mycobacterium tuberculosis* rely on the system of nickel trafficking for their survival and pathogenicity (Degen & Eitinger, 2002; Higgins, Carr, & Maroney, 2012; Mulrooney & Hausinger, 2003)].

The midgut microbiota of *CS* fed on millet had a higher number of genes involved in motility and host colonization such as *fli* and *flg*, which encodes for flagellar proteins, as well as *cheV*. Some of these genes (*flgD, flgE, flg*L, *fliI, fliM*, *fliP*) are common to most bacterial taxa, while others (*fliE, fliJ*, *fliS*, *fliH*) are sporadically distributed (R. Liu & Ochman, 2007)]. Besides the expected contribution of bacterial flagellum to movement, flagellum also affects cell adhesion, biofilm formation and host invasion (Macnab, 2003)]. *cheV* is a chemotactic protein consisting of a *cheW* domain fused to a phosphorylatable receiver domain. Evolutionary genomics studies suggested *CheV* as an additional adaptor for the accommodation of specific chemoreceptors within the chemotaxis signaling complex (Ortega & Zhulin, 2016)]. Bacterial chemotaxis is fundamental to allow bacteria to detect and follow chemical gradients in their environment (Baker, Wolanin, & Stock, 2006; L. D. Miller, Russell, & Alexandre, 2009)]. Therefore, taking all this together, there is a possibility that the *CS* has a microbiota with greater capacity to colonize the host midgut than the *RS*.

In samples from cotton, the *CS* bacterial community contain species commonly encoding higher levels of L-serine dehydratase, that was shown to be essential for colonization of the avian gut by *Campylobacter jejuni* (Velayudhan, Jones, Barrow, & Kelly, 2004)]. Additionally, the gut microbiota of *CS* contributes with higher levels of glycerate-2- kinase, that degrade glucose via a nonphosphorylative Entner-Doudoroff pathway, a pathway central for energy and carbon metabolism (Conway, 1992)]

Finally, the genes predicted for the bacterial community in the midgut of larvae feeding on corn were predominantly related to signaling and cellular processes (*TC.AAA, tellurium resistance protein-*TerZ, *pilX*). For all these predicted genes, *CS* had a higher abundance, suggesting a microbial community better suited to communicate with its environment and/or to better respond to temporal variations of external signals they experience.

This study has certain limitations. First, we used only three replicates for each treatment. In order to compensate for this limitation, we used a pool of three intestines in each replicate to increase the representativeness of the gut microbiota of the samples collected in the field. However, using more replicates would increase the power of this study and allow the exclusion of outliers. Second, our study is based on 16S rRNA Gene Amplicon, that captures the taxonomic diversity existing in the digestive tract of *S. frugiperda*, which does not mean that the taxa are metabolically active, limiting our ability to distinguish the autochthonous gut microbiota from the one associated with the insect diet. Finally, although Picrust is useful for predicting the potential functional contributions of the gut microbiota, the use of this tool brings limitations that should be highlighted such as those associated with the availability of appropriate references, biased primers, and gaps or inaccuracies in pathway annotation or gene function assignments. Future work to address these limitations could test the real contribution of the gut microbiota of *S. frugiperda* and verify if they differ between strains

In conclusion, our data demonstrate that the larval midgut of *Spodoptera frugiperda* harbor a bacterial community that varies according to the host plant. We also demonstrate that the midgut bacterial community consisted predominantly of Firmicutes followed by Proteobacteria when the larva feeds on corn and millet, with an opposite pattern when the larva feeds on cotton, regardless of the host strain of *S. frugiperda*. Differences at the genus level between the bacterial community of the *CS* and *RS* and predicted functional groups of low abundance were also detected. Studies of the gut microbes of this important agricultural pest can provide new knowledge not only for their control, but also for a better understanding of processes of host adaptation and evolution in insects.

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# 3. HOST-ADAPTED STRAINS OF *Spodoptera frugiperda* HOLD AND SHARE A CORE MICROBIAL COMMUNITY ACROSS THE WESTERN HEMISPHERE

## ABSTRACT

The Fall armyworm *Spodoptera frugiperda* is an important polyphagous agricultural pest in the Western Hemisphere and currently invasive to countries of the Eastern Hemisphere. This species has two host-adapted strains named "rice" and "corn" strains. Our goal was to identify the occurrence of core members in the gut bacterial community of fall armyworm larvae from distinct geographical distribution and/or host strain. We used next-generation sequencing to identify the microbial communities of *S. frugiperda* from corn fields in Brazil, Colombia, Mexico, Panama, Paraguay, and Peru, and rice fields from Panama. The larval gut microbiota of *S. frugiperda* larvae did not differ between the host strains neither was it affected by the geographical distribution of the populations investigated. Our findings provide additional support for *Enterococcus* and *Pseudomonas* as core members of the bacterial community associated with the larval gut of *S. frugiperda,* regardless of the site of collection or strain, supporting the hypothesis that these bacteria hold true mutualistic symbiotic relationships with fall armyworm.

KEYWORDS: Microbial ecology, dysbiosis, symbiosis, Fall armyworm

## 3.1 INTRODUCTION

The complexity and wide variety of host-microbe interactions are increasingly evident through new molecular techniques and the improvement of bioinformatic analysis tools. The advancement of understanding of this topic has brought support to some hypotheses and challenged others. An example is the discussion about whether the gut microbiota is relevant for all animals (Hammer, Sanders, & Fierer, 2019). The gut is a rich environment for holding a variety of host – microorganism associations, and the gut microbiota has been shown to play crucial roles in a wide range of aspects of host physiology, morphology and ecology. The insect gut microbiota can influence intra and interspecific interactions, such as sexual behavior (Sharon et al., 2010; Sharon, Segal, Zilber-Rosenberg, & Rosenberg, 2011) and the relationship between host plants and natural enemies (Frago, Dicke, & Godfray, 2012). It also plays a key role in insect adaptation to their environment by providing essential nutrients (A. E. Douglas, 2009; Engel & Moran, 2013) and/or boosting the host immune response to parasites and pathogens (Azambuja, Feder, & Garcia, 2004; Cavichiolli de Oliveira & Cônsoli, 2020). In addition, microbial symbionts can act in detoxifying xenobiotics as insecticides (Almeida, Moraes, Trigo, Omoto, & Cônsoli, 2017; Chen et al., 2020; Gomes, Omoto, & Cônsoli, 2020; Kikuchi, Hosokawa, & Fukatsu, 2011).

Such range of beneficial contributions has led to the establishment of true mutualistic associations in several groups of hemipterans, dipterans, blattids, and coleopterans, among others (Cheng et al., 2017; Chu, Spencer, Curzi, Zavala, & Seufferheld, 2013; Hosokawa, Kikuchi, Nikoh, Shimada, & Fukatsu, 2006; Kikuchi et al., 2011; Koch & Schmid-Hempel, 2011; Salcedo-Porras, Umaña-Diaz, Bitencourt, & Lowenberger, 2020). Lepidopteran larvae, however, have been thought not to have established mutualistic associations with their gut-associated bacteria. Some studies demonstrated the survival, development time, and weight gain were not affected in antibiotic-fed larvae (Hammer, Janzen, Hallwachs, Jaffe, & Fierer, 2017). Additionally, the lack of special regions in the gut to house microorganisms has been argued as a strong limitation for the establishment of true associations with free-living microbes (Appel, 2017). The harshness of the extremely alkaline conditions of the gut to most microorganisms also represents an unfavorable condition for establishing microbial associations (Dow, 1984). Finally, the high variation in the composition of the microbial community driven by host plants would difficult the occurrence of associations

that could hold through the required evolutionary time in order to allow the selection and establishment of true gut residents (Gayatri Priya, Ojha, Kajla, Raj, & Rajagopal, 2012). Nevertheless, other studies have shown that even in hostile environments as the midgut of lepidopteran larvae, there are evidence of gut colonization by certain bacterial groups (Mason et al., 2020; Mazumdar et al., 2020; Teh, Apel, Shao, & Boland, 2016). In addition, gut-resident bacteria of lepidopteran larvae were demonstrated to play important physiological roles for their hosts (Yongqi Shao, Arias-Cordero, Guo, Bartram, & Boland, 2014; Xia, Lan, Tao, Lin, & You, 2020); besides, the continuous association with their hosts for some of these microbes has been proved as they are horizontally transmitted (Y. Shao et al., 2017).

Controversial topics in the scientific literature are always an invitation to new studies aiming at better understanding and clarification of the topic. The debated existence of true gut-associates in lepidoptera is a subject that needs further clarification due to two important contexts it is placed in. First, its remarkable relevancy to the understanding of how microbial associations can influence host phenotypes (Moya, Pereto, Gil, & Latorre, 2008), and insects have provided simple models for the clarification of fundamental principles in host-microbe interactions (Angela E. Douglas, 2011; Kostic, Howitt, & Garrett, 2013), with a great potential to assist in unravelling complex systems such as in mammalians. Second, lepidopterans are yet the major group of agricultural pests, causing severe losses in food production, posing a serious threat to food security (McCaffery, 1998; Riegler, 2018; Scoble, 1992), and understanding the diversity and function of gut microbes associations can lead to the development of new strategies for herbivore control.

In the present study we have chosen a lepidopteran species that is important both in the ecological and in the economic context to investigate the existence of true gut associates of lepidopteran larvae. *Spodoptera frugiperda* is an important agricultural pest in the Western Hemisphere and is currently invasive to countries in Africa, Asia, and Oceania (Goergen, Kumar, Sankung, Togola, & Tamò, 2016; Johnson, 1987; Otim et al., 2018; Padhee & Prasanna, 2019; Piggott, Tadle, Patel, Gomez, & Thistleton, 2021). *Spodoptera frugiperda* is highly polyphagous, feeding on more than 300 host plants (Débora G. Montezano et al., 2018). This species is actually a complex composed of two distinct strains known as the rice (RS) and corn (CS) strains. The two strains are morphologically identical, with clear differences in host preference, susceptibility to insecticides and transgenic crops (*Bacillus thuringiensis*), composition of sex pheromone and mating behavior (Adamczyk Jr, Holloway, Leonard, & Graves, 1997; Cruz-Esteban, Rojas, Sánchez-Guillén, Cruz-López, & Malo, 2018; Ingber, Mason, & Flexner, 2018; Lima & McNeil, 2009; Orsucci et al., 2020; Pashley, Hardy, & Hammond, 1995; Schöfl, Heckel, & Groot, 2009; Veenstra, Pashley, & Ottea, 1995). Genomic analysis of the host-adapted races of *S. frugiperda* identified several genes involved in the chemodetection of non-volatile molecules and detoxification of xenobiotics showing signatures of positive selection, suggesting their contribution to their host plant preferences (Gouin et al., 2017). Some of these genomic variations between host races of *S. frugiperda* were also detected at the transcriptional level, including those involved in xenobiotic metabolism (Silva-Brandão et al., 2017).

Genetic studies suggest that population structure of *S. frugiperda* in the Western Hemisphere shows more variation within *S. frugiperda* populations than between populations of different locations, indicating a significant gene flow (Clark et al., 2007; Kondidie, 2011). The Mexican populations, on the other hand, have proven to be the most different, suggesting limited migratory interactions with foreign populations (Nagoshi et al., 2015; Tay et al., 2020). The population genetic structure of Brazilian populations of *S. frugiperda* is partially based on host plants, with rice populations, which are basically represent by rice strain individuals, having a strong effect on the overall genetic structure of fall armyworm populations in Brazil (Silva-Brandão et al., 2018).

Therefore, in this study we aim to verify the existence of bacterial groups that remain associated with the gut microbial community of *S. frugiperda* larvae regardless of the geographical region or host plant used. So, we sampled and sequenced the gut microbiota of fall armyworm larvae from corn and rice fields across the American continent. Larvae were genotyped as rice or corn strain, and the structure of the bacterial gut community was checked based on geographical origin of the larvae, host-adapted strain and/or host plant used. Despite the variation expected due to uncontrolled and unforeseen environmental factors, the field conditions may provide essential information on potential symbionts that could be ecologically important to their hosts in their natural habitats.

## 3.2 MATERIAL AND METHODS

### 3.2.1 Sampling and strains identification

Larvae of *Spodoptera frugiperda* with 2.5-3.0 cm in length were collected from corn and/or rice fields during 2016-17 in Brazil (13.8224° S, 56.0835° W), Colombia (4.5709° N, 74.2973° W), Mexico (23.6345° N, 102.5528° W), Panama (8.5380° N, 80.7821° W), Paraguay(23.4425° S, 58.4438° W), and Peru (9.1900° S, 75.0152° W), and stored in absolute ethanol. Once in the laboratory, larvae had the width of the head capsule measured, and only those larvae with head capsule width within the limits of size of 5$^{th}$ and 6$^{th}$ instars (Débora Goulart Montezano et al., 2019) were further dissected for gut collection. Dissections were carried after surface sterilization under aseptic conditions in a laminar flow hood. The larval digestive tract was carefully removed, washed in sterile saline and further used in metabarcoding analysis of the gut microbiota. The remaining carcass was used for host strain identification.

*Spodoptera frugiperda* were genotyped for strain identification using the mitochondrial *cytochrome oxidase I* (COI) gene as a marker. DNA was extracted using the genomic DNA preparation protocol from RNALater™, with modifications. The carcass obtained from dissected larvae was placed in 2 mL tubes with 750 µL digestion buffer (60 mM Tris pH 8.0, 100 mM EDTA, 0.5% SDS) and proteinase K (500 µg/mL), macerated using pestle, and mixed well by inversion. Samples were incubated overnight at 55°C. Afterwards, 750 µL of phenol:chloroform (1:1) was added and rapidly inverted for 2 min. Samples were centrifuged at high speed for 10 minutes. The aqueous layer was collected and phenol:chloroform extraction was repeated twice before a final extraction with chloroform. The aqueous layer was collected, added to 0.1 volume of 3M sodium acetate (pH 5.2) and an equal volume of 95% ethanol. Samples were then mixed by inversion, incubated for 40 min at -80°C before centrifugation (27,238 g x 30 min x 4°C). The pellet obtained was washed twice with 1 mL of 85% ice-cold ethanol, centrifuged for 10 min after each wash, and dried at 60°C during 5-10 min in a SpeedVac. Finally, the pellet was resuspended in nuclease-free water. DNA concentration and quality were estimated by spectrophotometry and standard DNA agarose gel electrophoresis (Sambrook, 2001).

Polymerase chain reactions (PCR) for partial amplification of the mitochondrial COI gene was conducted using the primer set JM 76 (5´-GAGCTGAATTAGGRACTCCAGG-3´) and JM 77 (5´-ATCACCTCCWCCTGCAGGATC-3´), to produce an expected amplicon of 569 base pairs (bp) (Levy, Garcia-Maruniak, & Maruniak, 2002). The PCR mixture contained 100-150 ng of gDNA, 1.5 mM of $MgCl_2$, 1x PCR buffer, 0.2 mM of each dNTP, 0.32 µM of each primer and 0.5U of GoTaq® DNA Polymerase (Promega) in a total volume of 25 µL. The thermocycling condition was 94°C x 1 min (1x), followed by 33 cycles at 92°C x 45 s, 56°C x 45 s, and 72°C x 1 min, and one cycle at 72°C x 3 min for final extension. Amplicons were then subjected to restriction

analysis using the *Msp*I (HpaII) restriction endonuclease. Samples were gently mixed, centrifuged for a few seconds and incubated overnight at 37°C. Subsequently, digestion and the resulting products were verified using a 1.5% agarose gel electrophoresis. The corn strain (*CS*) was identified from restriction analyses yielding two fragments (497bp and 72bp), while restriction analyses that produced no digestion identified the rice strain (*RS*) (Levy et al., 2002).

### 3.2.2 DNA extraction, amplification and 16S rDNA sequencing

The midgut obtained from dissected larvae were individually powdered in liquid nitrogen, and genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega), following the manufacturer's recommendations. The quality, integrity and purity of the DNA obtained was measured by spectrophotometry and agarose gel electrophoresis as before. DNA samples were stored in -20°C and sent for library construction, normalization and sequencing in the Center for Functional Genomics (http://www.esalq.usp.br/genomicafuncional/), one of the multiusers laboratories of our institution. Paired-end reads were generated after amplifying the v3-v4 region of 16S rRNA gene (approximately 550 bp) using the Nextera XT DNA Library Preparation Kit (Illumina) for paired-end (2x 300 bp) sequencing in the Illumina MiSeq platform.

### 3.2.3 Sequences analyses

Illumina adapters at the 3' end of the reads were removed using Cutadapt (Martin, 2011). The bioinformatics analyses of the gut microbiome were performed with QIIME2 v. 2020.2.0 (Bolyen et al., 2019). Raw sequence data were quality filtered with *q2-dada2* plugin for filtering phiX reads and chimeric sequences (Callahan et al., 2016). In order to remove low quality regions from quality filter reads, *dada2 denoise-single* method trimmed off the first 18 nucleotides of the forward reads and 22 nucleotides from the reverse reads. It also truncated each sequence at position 290 in the forward and 220 in the reversed reads. These positions were chosen based on visual inspection of plotted quality scores from demultiplexed reads. A phylogeny was estimated with SEPP (Mirarab, Nguyen, & Warnow, 2012) as implemented in the *q2-fragment-insertion* QIIME2 plugin. All amplicon sequence variants (ASVs) were aligned with *feature-classifier classify-sklearn* against the SILVA-132-99 database (Quast et al., 2012) that was trained with a Naïve Bayes classifier (Bokulich et al., 2018) on the Illumina 16S rRNA gene primers targeting the V3–V4 region (F- CCTACGGGNGGCWGCAG; R- GACTACHVGGGTATCTAATCC).

The downstream analysis was performed in the MicrobiomeAnalyst web platform (https://www.microbiomeanalyst.ca/) (Chong, Liu, Zhou, & Xia, 2020) and in R (version 4.0.4) (Team, 2020). Data were filtered keeping ASV with minimum count 4 per library and low count filter based on 20% prevalence across samples. Data were rarefied to the minimum library size (1155 reads), before any statistical comparisons. Rarefaction curves were based on the relationship between number of ASVs and number of sequences. Alpha diversity analysis was measured by the observed species and Shannon index. The results were plotted across samples and showed as box plots for each group. Beta diversity was investigated through principal components analysis (PCoA) using weighted UniFrac distances, and through hierarchical clustering analysis using unweighted UniFrac distances.

We used PERMANOVA to test the strength and statistical significance of sample groupings based on generalized UniFrac distances. This distance contains an extra parameter $\alpha$ (set at $\alpha$=0.5) to control the weight of

abundant lineages, so the distance is not dominated by highly abundant lineages. When differences were found between samples distances, a post-hoc analysis was performed with the package *pairwise.adonis* to identify differences among treatments and verify the adjusted *p* value (Arbizu, 2019). As PERMANOVA assumes homogeneity of variances, we used *betadisper*, a multivariate analogue of Levene's test, as implemented in R to verify whether differences between groups in terms of their centroids are not due to differences in variances. Analysis of similarity (ANOSIM) was used when there was heterogeneity of variance among groups. In our sample set we had basically 3 groups: (i) countries that presented both strains in corn plants, (ii) countries with only the corn strain in corn plants and (iii) Panama with both strains in corn plants and only the rice strain in the rice plant. Since our design is unbalanced, we performed separate analyses to properly grasp our data. First, we excluded the samples that had rice as host plant, thus only the variables "strain" and "country" were considered. And to test the effect of country and host plant, we excluded the corn strain from the analysis, considering only the rice strain.

To visualize taxa abundance across the different groups, taxa plots were constructed based on phyla and genera. The core microbiome analysis was defined as the genera present in 50% or more of the samples and showing a relative abundance of 0.05% in each library. The differential abundance analysis was also analyzed using DESeq2 methods (Love, Huber, & Anders, 2014). Pattern Search was used to identify which features were correlated with the core microbiome in the gut microbial community. Pearson r was the distance measure used using the MicrobiomeAnalyst tool (Chong et al., 2020).

To cluster our samples groups into distinct 'metacommunities', we performed Dirichlet multinomial mixtures using the *get.communitytype* function (Holmes, Harris, & Quince, 2012) after exportation of biom ASV table from qiime2 to Mothur (v.1.44.3) and the selection of subsamples with *subsample=1000*, excluding low abundance samples that might be a result of artifact operational units and/or variation due to rare taxons ("singletons"). The best fitting number of metacommunities was obtained by selecting the minimum local Laplace value obtained after running this command five times.

## 3.3 RESULTS

A total of 63 *S. frugiperda* individuals, 8 *RS* and 45 *CS* were used in our analyses. Except for 8 specimens from Panama that were collected on rice, all other samples were collected in corn fields. Out of the 63 specimens analyzed, 21 were from Brazil (*CS*=18; *RS*=3), nine from Colombia (*CS*=8; *RS*=1), eight from Mexico (*CS*=8), six from Paraguay (*CS*=3; *RS*=3), five from Peru (*CS*=3; *RS*=2), and 13 from Panama (6 from corn fields; *CS*=5, *RS*=1; and 8 from rice fields; *RS*=8).

Rarefaction analysis (Anexo C) showed that the sampling effort provided the needed sequencing depth for an accurate characterization of the diversity and richness of the larval gut microbiota of *S. frugiperda*. There was no difference in alpha-diversity values between strains or among countries (Fig. 9).

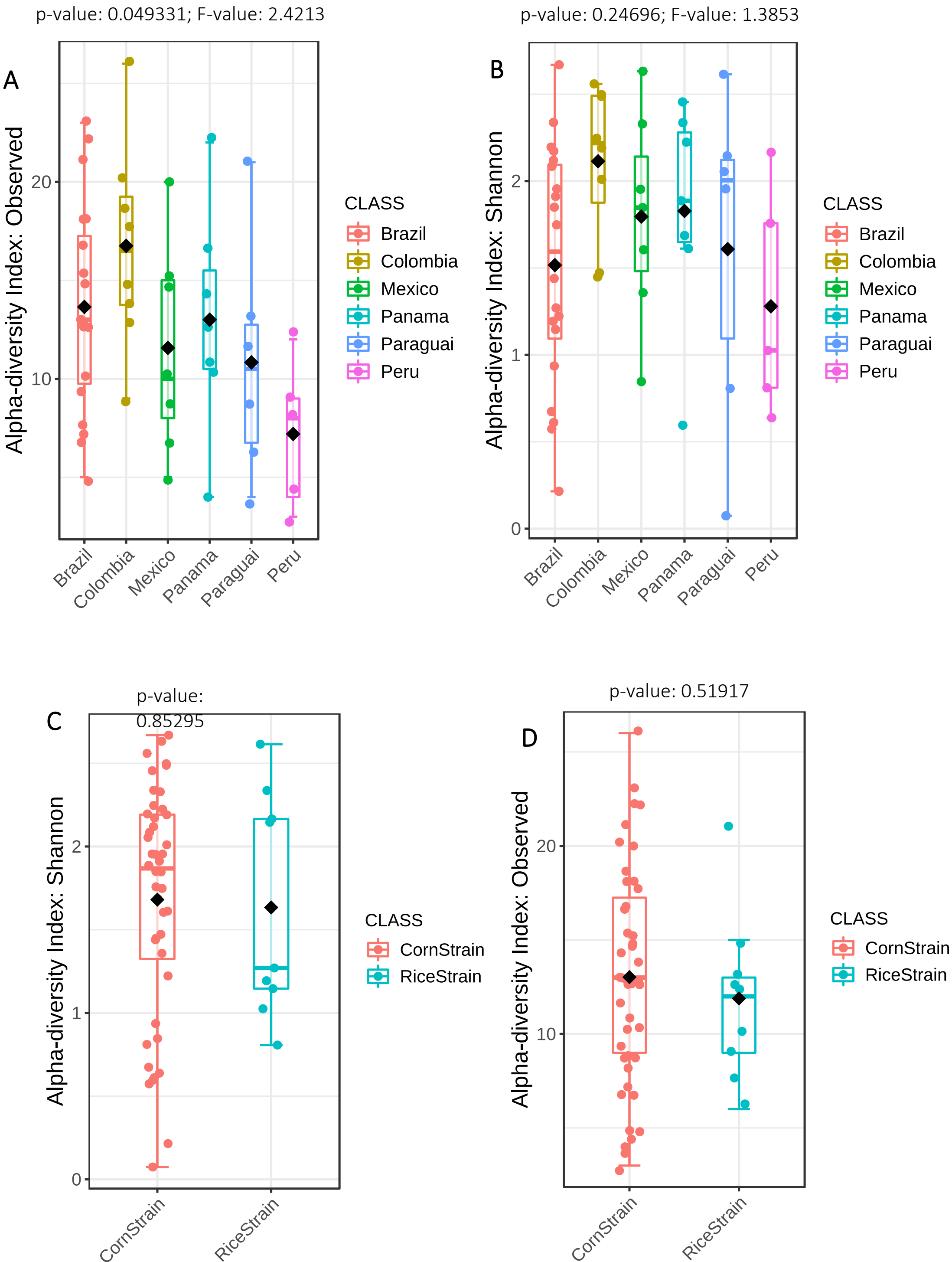


**Figure 9.** Alpha diversity index of observed taxa (A,C) and Shannon index (B,D) obtained for samples from the gut microbiota of the corn and rice strains of *Spodoptera frugiperda* larvae (C,D) from different countries (A,B). The p-values from the Test T (pairwise comparison) and ANOVA (group comparison) are shown in which box.

The beta diversity measured by weighted Unifrac distances did not exhibit specific clustering based on the country of origin or strains (Fig. 10).

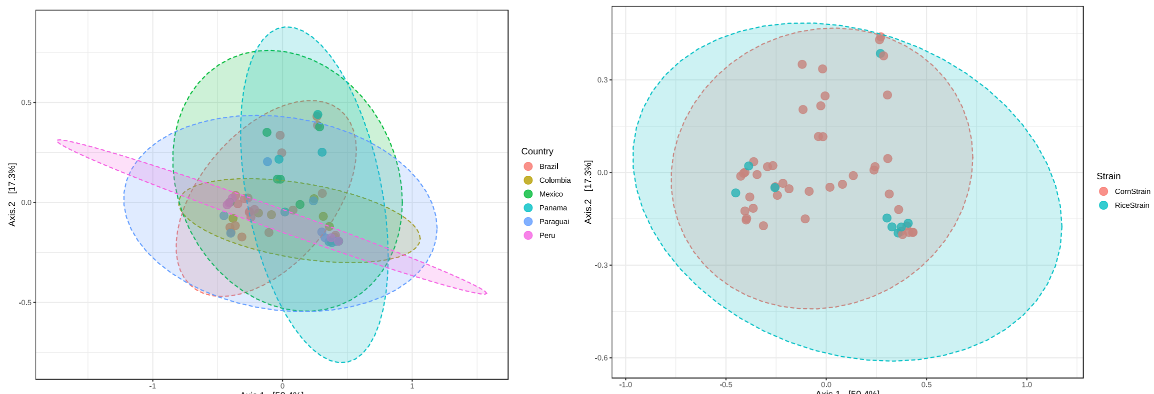


**Figure 10.** Principal coordinates analysis (PCoA) based on weighted unifrac analysis of the midgut microbial community of the corn and rice strains of *Spodoptera frugiperda* larvae (B) from different countries (A).

When considering samples collected in maize, no differences in the composition of the gut microbial community between strains ($p$=0.215) (Table.2) nor among different countries considering the adjusted $p$ values ($p$ values < 0.05) were detected (Table.3). Betadisper showed that groups had the same dispersion, failing to reject the null hypothesis of homogeneous multivariate dispersions, meeting the assumption for Adonis (Table.2). It thus provide confidence to the PERMANOVA results, meaning the values obtained are not an artifact of heterogeneity of dispersions. Likewise, no differences were found between host plants ($p$=0.344) nor country ($p$=0.0709) when considering only the rice strain (Table 4). Additionally, all replicates of metacommunity analyses resulted in the same pattern (K=1), meaning that according to the Dirichlet model there is not a clear pattern of grouping ASVs across samples.

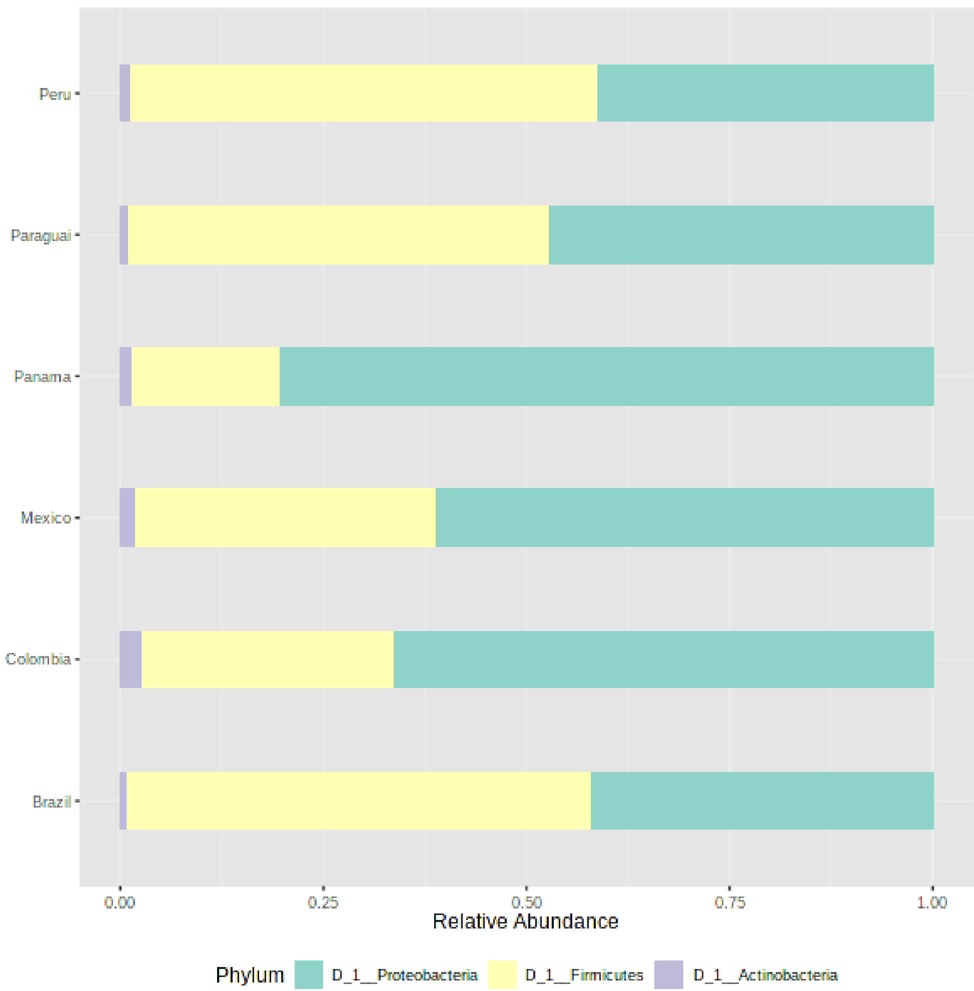


**Figure 11.** Taxonomic composition of the microbial community associated with the midgut of corn and rice strains of *Spodoptera frugiperda* larvae sampled in different countries at the phylum level.

**Table 2.** PERMANOVA and ANOSIM results from comparisons of the gut microbial communities among countries and *Spodoptera frugiperda* strains (corn and rice strains) using UniFrac (alpha 0.5) values.

| **Maize plant (Rice and corn strain)** | **PERMANOVA** | | **ANOSIM** | | **BETADISPER** | |
|---|---|---|---|---|---|---|
| | $R^2$ | *p* value | R | *p* value | F value | Pr(>F) |
| **Country** | 0.11698 | 0.044 * | - | - | 0.2444 | 0.9406 |
| **Strain** | 0.02181 | 0.215 | - | - | 3.3965 | 0.07093 . |
| Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1 | | | | | | |

**Table 3.** Post-hoc analysis results of the comparisons of the gut microbial communities of *Spodoptera frugiperda* strains among countries.

| Pairs | Df | SumsOfSqs | F.Model | R2 | p.value | p.adjusted |
|---|---|---|---|---|---|---|
| Colombia vs Brazil | 1 | 0.3806171 | 2.0309445 | 0.07245366 | 0.011 | 0.165 |
| Colombia vs Mexico | 1 | 0.2728639 | 1.3648260 | 0.08882795 | 0.080 | 1.000 |
| Colombia vs Panama | 1 | 0.2279787 | 1.1800870 | 0.07773915 | 0.196 | 1.000 |
| Colombia vs Paraguai | 1 | 0.3238154 | 1.6384779 | 0.12013642 | 0.095 | 1.000 |
| Colombia vs Peru | 1 | 0.3136700 | 1.6244547 | 0.12867524 | 0.060 | 0.900 |
| Brazil vs Mexico | 1 | 0.2544575 | 1.2752619 | 0.04675526 | 0.147 | 1.000 |
| Brazil vs Panama | 1 | 0.3235731 | 1.6516793 | 0.05973161 | 0.044 | 0.660 |
| Brazil vs Paraguai | 1 | 0.1472816 | 0.7425192 | 0.03000985 | 0.722 | 1.000 |
| Brazil vs Peru | 1 | 0.2553736 | 1.3015037 | 0.05355651 | 0.151 | 1.000 |
| Mexico vs Panama | 1 | 0.2217735 | 1.0281323 | 0.06841384 | 0.390 | 1.000 |
| Mexico vs Paraguai | 1 | 0.2483650 | 1.1092582 | 0.08461640 | 0.292 | 1.000 |
| Mexico vs Peru | 1 | 0.2227645 | 1.0045716 | 0.08368242 | 0.383 | 1.000 |
| Panama vs Paraguai | 1 | 0.2516586 | 1.1648641 | 0.08848281 | 0.254 | 1.000 |
| Panama vs Peru | 1 | 0.2287484 | 1.0730526 | 0.08887998 | 0.328 | 1.000 |
| Paraguai vs Peru | 1 | 0.1878847 | 0.8404890 | 0.08541130 | 0.553 | 1.000 |

**Table 4.** PERMANOVA and ANOSIM results from comparisons of the gut microbial communities of *Spodoptera frugiperda* strains (corn and rice strains) among countries and host plants using UniFrac (alpha 0.5) values.

| Rice Strain (maize and rice plant) | PERMANOVA | | ANOSIM | | BETADISPER | |
|---|---|---|---|---|---|---|
| | $R^2$ | *p* value | R | *p* value | F value | Pr(>F) |
| **Country** | - | - | 0.2664 | 0.070929 | 5.6096 | 0.00755 ** |
| **Host Plant** | 0.06149 | 0.344 | - | - | 2.1328 | 0.1635 |
| Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1 | | | | | | |

At the phylum level, the midgut of *S. frugiperda* was composed by *Proteobacteria*, *Firmicutes* and *Actinobacteria* (Fig. 11). There was no significant difference at the phylum level among countries or between strains. Taxa bar plots at the genus level indicated that individuals from the same country exhibited a high degree of variability in terms of bacteria taxa abundance (Fig. 12). *Klebsiella* and *Erysipelatoclostridium* were the taxa that differed among countries (Fig. 13), and the abundance of *Erysipelatoclostridium* also differed between *RS* and *CS* (Fig.14).

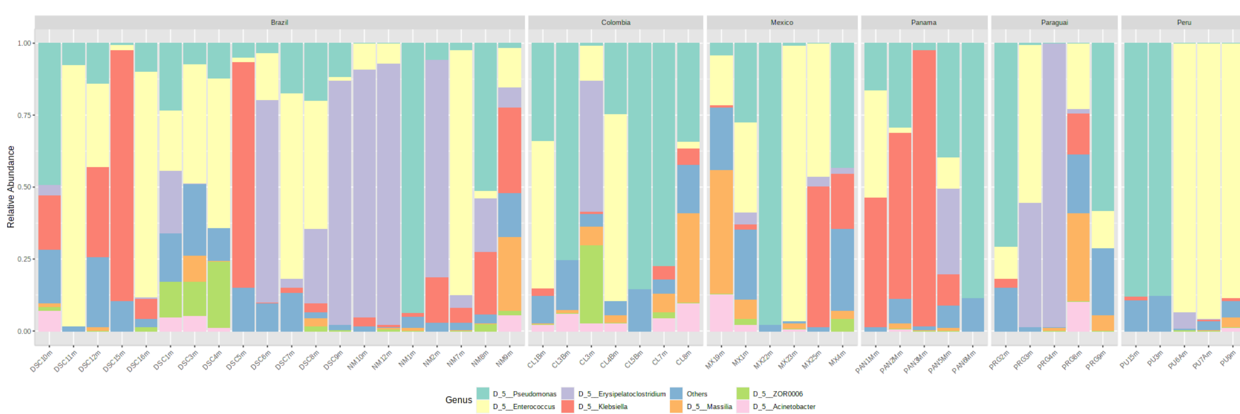


**Figure 12.** Taxonomic composition of the microbial community of the larval midgut of corn and rie strains of *Spodoptera frugiperda* at the genus level. Taxa with less than 200 counts were merged in a group as "others".

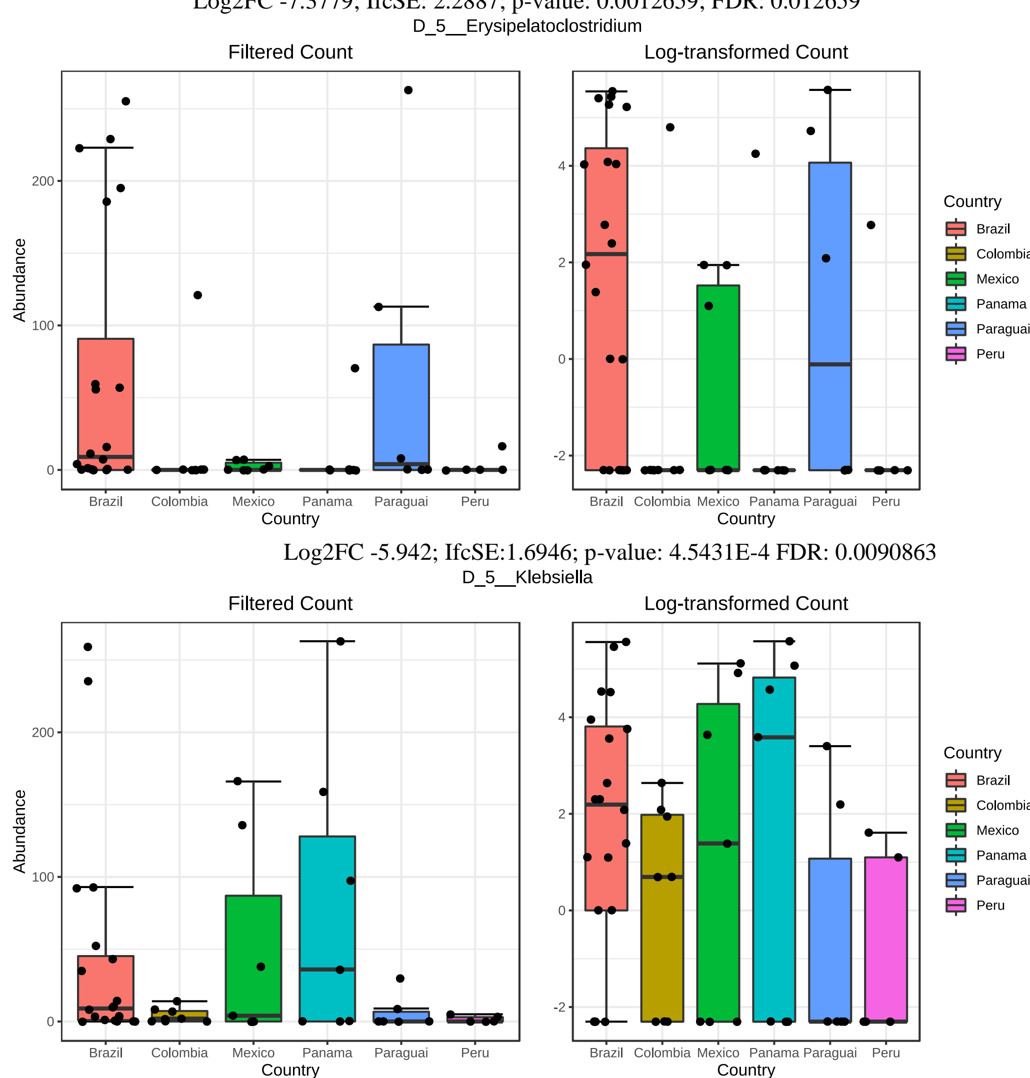


**Figure 13.** The abundance of *Klebsiella* and *Erysipelatoclostridium* as a differential feature of the microbiota associated with the larval midgut of *Spodoptera frugiperda* from different countries.

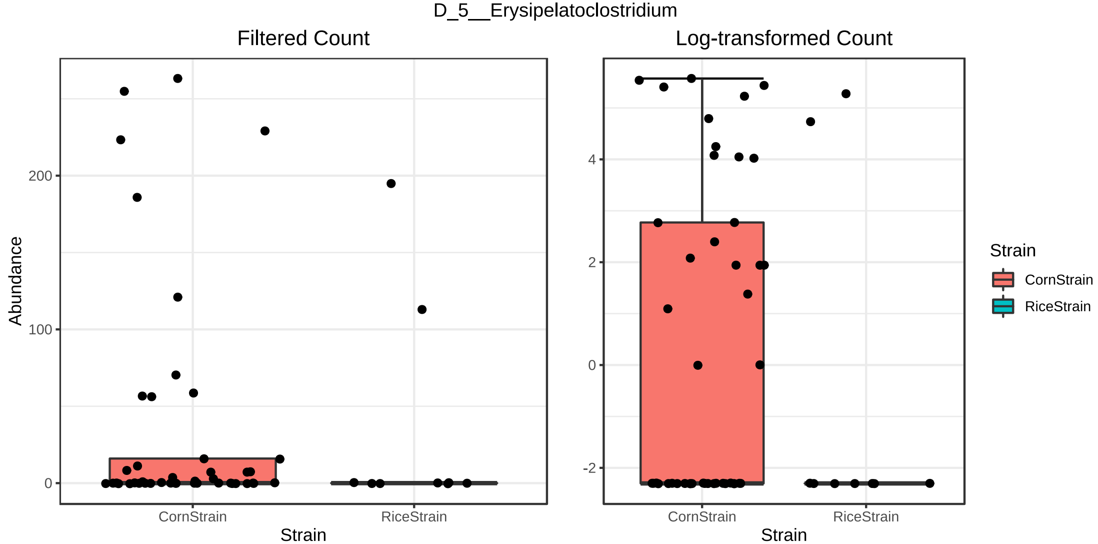


**Figure 14.** The abundance of *Erysipelatoclostridium* as a differential feature of the microbiota associated with the larval midgut of the corn and rice strains of *Spodoptera frugiperda* from different countries.

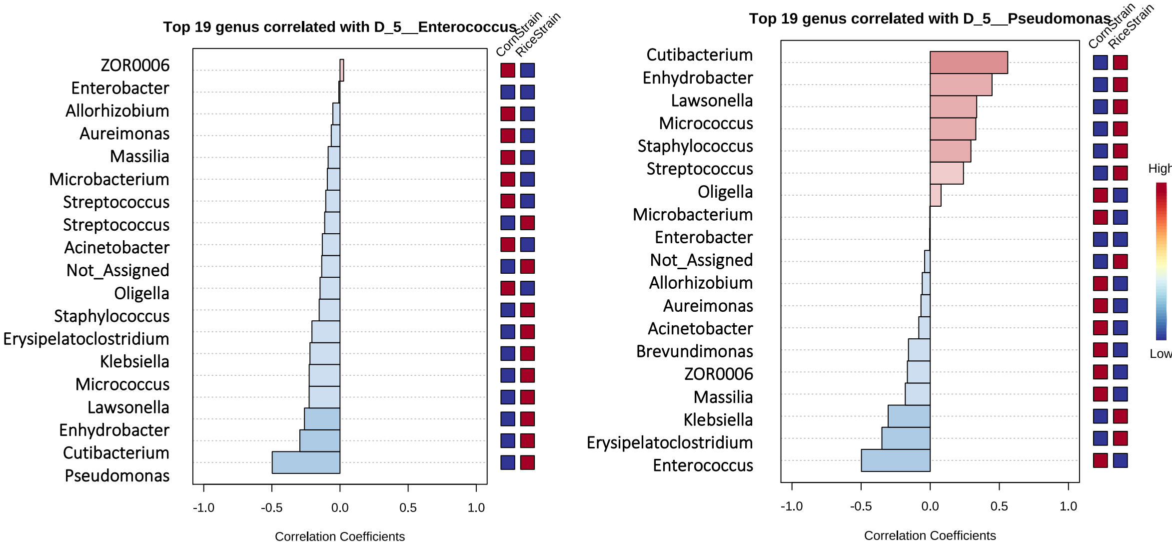


**Figure 15.** Pattern correlation analysis of bacteria genera in *Spodoptera frugiperda* gut. Red indicates positive correlation and blue indicate negative correlations with the presence of *Pseudomonas* (A) or *Enterococcus* (B).

The bacterial core of the larval midgut of *S. frugiperda* at the genus level was composed by *Pseudomonas* and *Enterococcus*. Correlation analysis identified 10 genera that were positively correlated and 10 genera negatively correlated with *Pseudomonas*. However, only three genera were positively correlated, while 18 were negatively correlated with *Enterococcus* (Fig. 15).

## 3.4 DISCUSSION

The bacterial community of the Fall armyworm larval midgut did not differ between strains collected from the same country nor among countries. These findings follow the pattern of the population genetic structure of *S. frugiperda* in the Western Hemisphere, where the majority of the genetic variability is within individual populations and not between populations, suggesting that populations of *S. frugiperda* functions as a panmictic population (Clark et al., 2007; Kondidie, 2011).

As expected, we detected high variations in the composition of the gut microbiota among larvae. Such differences are likely to occur due to differences in corn varieties and associated endophytes, and soil type and associated microbiota, which also interact with plants and affect the plant endophyte community, ultimately interfering with the microbial composition of herbivores (Correa-Galeote, Bedmar, & Arone, 2018; Liu et al., 2020; Meliani, Bensoltane, & Mederbel, 2012). Variation in the microbiota from individual samples within treatments is commonly reported to several organisms, including species of Lepidoptera (Hisada, Endoh, & Kuriki, 2015; Mach et al., 2020; Martínez-Solís, Collado, & Herrero, 2020). In humans, sample to sample variation in the populations of gut microbes can be higher than 90% (Dorrestein, Mazmanian, & Knight, 2014). But regardless the high variation observed in the gut microbiota associated with the larval midgut of *S. frugiperda*, our analysis identified a core of bacteria despite the geographical origin of fall armyworm samples. The maintenance of a core independently of any interfering systemic effects points to the existence of bacterial associates with specific functions.  In addition, the high variability in the composition of the midgut microbiota may allow for rapid host adaptation through rapid

selection of microbiota suitable for contributing to the host under different stress conditions, such as abiotic factors, dietary resources, and risk of natural enemy attack (Paniagua Voirol, Frago, Kaltenpoth, Hilker, & Fatouros, 2018).

The ASVs Pseudomonas and *Enterococcus* identified in this study as core members of the microbiota of the fall armyworm were also identified before as part of the core taxa associated with the gut of *S. frugiperda* larvae from corn fields (Gichuhi et al., 2020; Gomes et al., 2020; Jones, Mason, Felton, & Hoover, 2019; Paniagua Voirol et al., 2018; Ugwu, Liu, Sun, & Asiegbu, 2020). The high abundance of Pseudomonas in our samples suggests that this genus of bacteria could assist *S. frugiperda* larvae to overcome environmental stressors, particularly by aiding larvae to degrade natural and/or synthetic toxic xenobiotics. Pseudomonas capable to degrade several pesticides were recovered from the gut of laboratory-selected resistant lines (Almeida, Moraes, Trigo, Omoto, & Cônsoli, 2017), but also from field populations of *S. frugiperda* collected from several corn-producing areas in Brazil (Gomes, Omoto, & Cônsoli, 2020). Pseudomonas have also been demonstrated to degrade secondary metabolites in the gut of a coleopteran host (Ceja-Navarro et al., 2015). Additionally, Pseudomonas abundance increased in the gut of Plutella xylostella resistant to prothiofos when compared to susceptible larvae, and was also shown to have antagonistic activity to several species of entomopathogenic fungi through siderophore production as demonstrated in culture plates (Indiragandhi et al., 2007).

It is noteworthy that *Enterococcus* is the most prevalent and abundant group identified in the gut microbiota of Spodoptera species (Chen et al., 2016; Gichuhi et al., 2020; Jones et al., 2019), and also the most active in the gut of *S. frugiperda* (Rozadilla, Cabrera, Virla, Greco, & McCarthy, 2020). Additionally, it has been demonstrated that *Enterococcus mundtii* is effective in colonizing and forming biofilm in the gut of Spodoptera littoralis (Mazumdar et al., 2020; Y. Shao et al., 2017). There is also evidence that E. mundtii can be inherited by S. littoralis through vertical transmission (Teh et al., 2016). Some species of *Enterococcus* produce antimicrobial peptides with high level of inhibitory activity against potential bacterial competitors (Y. Shao et al., 2017), which may explain its prevalence when compared to other phylotypes in *S. frugiperda* gut communities, but also the high negative correlation of *Enterococcus* with the other bacterial species of the gut microbiota community of *S. frugiperda* in this study.

Overall, this study provided an extended view of the fall armyworm gut microbiota and supported the hypothesis that bacterial taxonomic compositions across different localities in the Western Hemisphere are similar to each other, presenting high inter-individual variance, and that there are no significant differences in gut microbiota composition between the host-adapted strains of *S. frugiperda*. Nevertheless, our findings provide further evidence that Pseudomonas and *Enterococcus* are true symbionts of *S. frugiperda* as they were identified in the gut microbiota of *S. frugiperda* larvae regardless the host plant and site of collection. Further investigations on the functional contribution of these species as members of the gut bacterial community of fall armyworm larvae is required for a deeper understanding of the nature of this relationship.

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# 4. DOES THE GUT MICROBIOTA OF THE FALL ARMYWORM STRAINS IS JUST A REFLEX OF THEIR FOOD MICROBIOTA?

## ABSTRACT

Insect-associated microorganisms can affect several aspects concerning the physiology and ecology of their hosts. However, the importance of the gut microbiota in Lepidoptera is still controversial. Here we aimed to verify the strength of the association of bacteria to the midgut and hindgut of the corn and rice strains of *Spodoptera frugiperda,* the Fall armyworm (FAW) by comparing the bacterial community hosted in the larval gut with the microbiota present in the offered food, maize leaves and artificial diet. In addition, we investigated whether vertical transmission of bacteria would occur in FAW. We used high-throughput sequencing of the16S rRNA gene to characterize the composition of the microbiota, and FISH to verify the presence of bacteria in the eggs and reproductive tissues of FAW adults. Diet played an important role in altering the composition of the gut microbiota, but the diversity and structure of the larval gut microbiota was distinctive of the that of the food source. We found no differences in gut microbiota composition between FAW strains when feeding on the same food source, but did observe differences in the bacterial communities between the midgut and hindgut of FAW. Our findings indicate the FAW harbors a microbiota that is independent of the microbiota of the food source used, and that these bacteria is likely inherited through vertical transmission.

**KEYWORDS:** Symbionts, host plant adaptation, maize, *Spodoptera frugiperda*.

### 4.1 INTRODUCTION

The advances in molecular and sequencing techniques have greatly expanded our understanding of the microorganisms associated with multicellular organisms and with the environment. The incredible diversity and abundance of these microorganisms and their numerous and pivotal roles in the digestion, defense, behavior, ecology and evolution of their hosts (Charroux and Royet, 2012, Clemente*, et al.*, 2012, Malard*, et al.*, 2021), has led us to shift from a primarily host-centric view to a more holistic perspective that considers the host and its symbionts as a single entity (Gilbert*, et al.*, 2012, Roughgarden*, et al.*, 2018). Insects stand out among the models used to study these interactions because of their great genotypic diversity, easy management, short life cycles, and rich associations with microorganisms (Charroux and Royet, 2012, Ludington and Ja, 2020). Moreover, several of them are important from a medical, veterinary, and agricultural point of view. Consequently, using insects as models provide meaningful ecological information and solutions to economically important issues.

Lepidoptera is considered one of the most widespread and diverse groups of insects and are also considered important agricultural pests (Powell, 2009, Scoble, 1992). The gut microbial communities of lepidopterans are simple and dynamic. Even considering that symbionts are important players in insect-plant interactions, the relevance of microorganisms in larval lepidopterans is currently controversial. Some investigations pointed the simple morphology of the food canal, with the lack of specialized regions to harbor bacteria, and the high alkalinity of the midgut and rapid food transit, would only allow the hosting of temporary, non-resident bacteria, leading to a microbiota that would be similar to the microbiota of the food ingested (Anand*, et al.*, 2010, Hammer*, et al.*, 2017). In addition, lepidopterans-gut inhabiting bacteria were reported to provide non-essential contributions to the larval host, once larvae survived without bacteria in their gut (Hammer, et al., 2017). In controversy, others proved lepidopterans larvae hosted a microbial core community in their guts that is distinctive from that obtained from the food source (Mason*, et al.*, 2020, Teh*, et al.*, 2016). Later investigations demonstrated gut microbes of lepidopteran larvae contribute to host immunity, nutrition, and suppression of plant defenses (Shao*, et al.*, 2017), and can be vertically transmitted (Freitak*, et al.*, 2014, Teh, et al., 2016). However, given the importance

that lepidopteran species play as pests of agricultural crops and their products and the potential for biotechnological exploitation of the associations with their gut microbiota in order to develop new methods of intervention, there are still limited studies aimed at understanding the existence of true symbionts associated with lepidoptera.

*Spodoptera frugiperda* (Lepidoptera, Noctuidae) is a highly polyphagous (Montezano*, et al.*, 2018) and currently represented by two morphologically indistinct host adapted races, the corn (CS) and the rice (RS) strains, but with distinctive ecology and mitochondrial and chromosomal genome information (Dumas*, et al.*, 2015, Orsucci*, et al.*, 2020, Pashley*, et al.*, 1992, Pashley*, et al.*, 1995, Schöfl*, et al.*, 2011, Silva-Brandão*, et al.*, 2018, Veenstra*, et al.*, 1995). *Spodoptera frugiperda* is native to the Americas, but in the last 6-years has spread to Africa, Asia, and Oceania, becoming a serious worldwide threat to food security (Goergen*, et al.*, 2016, Johnson, 1987, Otim*, et al.*, 2018, Padhee and Prasanna, 2019, Piggott*, et al.*, 2021) due to its polyphagia and capacity to evolve resistance to organic pesticides and Bt-transgenic crops(Carvalho*, et al.*, 2013, Huang, 2020, Ríos-Díez and Saldamando-Benjumea, 2011, Yu, 1991).

Thus, given the economic and ecological relevance of *S. frugiperda*, and the potential of exploitation of the associated microbiota in developing strategies for pest control (Almeida*, et al.*, 2017, Crotti*, et al.*, 2012), it is important to investigate the existing controversy on the true association of gut microbes to lepidopterans larvae for this species. Here we characterized the microbiota associated with the midgut and hindgut of *S. frugiperda* larvae of the RS and CS strains under controlled laboratory conditions and compared with the microbiota associated with food sources used in larval rearing, maize leaves, and artificial diet. In addition, in order to provide additional information on the close association of bacteria and *S. frugiperda*, we also investigated the occurrence of bacterial vertical transmission

## 4.2 MATERIAL AND METHODS

### 4.2.1 Insect rearing and strains identification

Colonies of *S. frugiperda* strains were initiated from field-collected populations. The RS colony was obtained from rice fields in Santa Maria, RS, Brazil (29°68'68"S, 53°81'49"W), while the CS colony originated from specimens collected in a maize field in Piracicaba, SP, Brazil (22°43'30"S, 47°38'56"W). Larvae were individualized reared into plastic coffee cups containing an artificial diet based on wheat germ, beans and brewer's yeast (Greene*, et al.*, 1976). Once pupation occurred, pupae were collected and transferred to clean cups lined with filter paper for adult emergence. Finally, newly emerged adults were then transferred to 30-cm high x 10 cm internal diameter PVC tubes lined with paper for mating and egg laying. Egg masses were collected, surface sterilized, and transferred to artificial diet for later larval development (Parra, 1999).

The identification of host-adapted strains of *S. frugiperda* followed (Levy*, et al.*, 2002). DNA was individually extracted from specimens using the genomic DNA preparation from RNAlater™ preserved tissues protocol, with some modifications. The larval exuviae of field-collected specimens was placed in 750 µL digestion buffer (60 mM Tris pH 8.0, 100 mM EDTA, 0.5% SDS) and proteinase K at a final concentration of 500 µg/mL, macerated with a disposable pestle, mixed well by inversion and incubated overnight at 55°C. Afterwards, 750 µL of phenol:chloroform (1:1) was added and rapidly inverted for 2 min. Samples were centrifuged at high speed for 10 minutes. The aqueous phase was collected and phenol:chloroform extraction was repeated twice before a final extraction with chloroform. The aqueous phase was collected and added to 0.1 volume of 3M sodium acetate (pH 5.2) and an equal volume 95% ethanol. Samples were then mixed by inversion, incubated for 40 min at -80°C, and

centrifuged (27,238 g x 30 min x 4°C). The pellet obtained was washed twice with 1 mL of 85% ice-cold ethanol, centrifuged for 10 min after each wash and dried at 60°C during 5-10 min in a SpeedVac. Finally, the pellet was resuspended in nuclease-free water. DNA concentration and quality were estimated by spectrophotometry and standard DNA agarose gel electrophoresis.

Polymerase chain reaction (PCR) amplification of the mitochondrial COI gene was conducted using the primers set JM76 (5´-GAGCTGAATTAGGRACTCCAGG-3) and JM77 (5´- ATCACCTCCWCCTGCAGGATC-3´) (Levy, et al., 2002) to produce an expected amplicon of 569 base pairs (bp)-long. The PCR mixture contained 100-150 ng of gDNA, 1.5 mM of $MgCl_2$, 1 x PCR buffer, 0.2 mM of each dNTP, 0.32 µM of each primer and 0.5U of GoTaq® DNA Polymerase (Promega) in a total volume of 25 µL. The thermocycling program was 94°C x 1 min (1x) followed by 33 cycles at 92°C x 45 s, 56°C x 45 s, 72°C x 1 min, with a final extension at 72°C x 3 min (1x). Amplicons (10 µL) were then subjected to restriction analysis using the *Msp*I (HpaII) restriction endonuclease overnight at 37°C, and the resulting products were verified by 1.5% agarose gel electrophoresis. Restriction of the COI fragment amplified would produce two fragments (497pb and 72pb) for amplicons of the *CS* strain, while no restriction would be observed for those of the *RS* strain.

### 4.2.2 Essay with natural diet and gut dissection

Maize (*Zea mays*, family: Poaceae) var. "Conventional impact" was seeded in 500 mL plastic pots filled with soil conditioner. All plants were maintained in a greenhouse. Phase v3 and v4 of Maize leaves were collected and immersed in a container with distilled water for 30 minutes to maintain turgidity. Newly emerged larvae of RS and CS strains were placed in 25 mL plastic cups containing a piece of the host plant leaf, with area of approximately 3cm, on top of a moistened cotton disk. The leaves were replaced and supplied with fresh ones according to the needs of each stage of caterpillar development, in order to have no leaves older than two days and no shortage of food. Insects were kept under controlled laboratory conditions (25 ± 1°C; 60 ± 10%; 14-hour photophase).

The experimental groups were larvae of the rice strain feeding on maize (CoRS) and artificial diet (DiRS) and larvae of the corn strain feeding on the same diets (CoCS and DiCS). We used five replicates for each treatment, with each replicate corresponding to a pool of midgut collected from five larvae. Sixth-instar larvae were surface-sterilized in cooled 0.2% sodium hypochlorite in 70% ethanol, washed in cold sterile water, and transferred to autoclaved water for gut dissection under sterile conditions. Tissues were flash-frozen in liquid nitrogen and stored at -80°C until metabolite extraction.

### 4.2.3 DNA extraction, amplification and 16S rDNA sequencing

All samples collected were pulverized in liquid nitrogen and subjected to genomic DNA using the Wizard® Genomic DNA Purification Kit, following the manufacturer´s recommendation. The quality, integrity and purity of the DNA obtained was assessed by spectrophotometry and agarose gel electrophoresis. DNA samples were sent to the Animal Biology Laboratory (ESALQ / USP, Piracicaba, SP) for library preparation and sequencing of the hypervariable V3–V4 region of the 16S rRNA gene, following a paired-end approach (2 x 300bp) in the Illumina MiSeq platform.

### 4.2.4 Sequences analyses

The Illumina adapters at the 3' end of the reads were removed using Cutadapt (Martin, 2011). The microbiome bioinformatic analysis was performed with QIIME2 v. 2020.2.0 (Bolyen*, et al.*, 2019). Raw sequence data were quality filtered followed by denoising with DADA2 (Callahan*, et al.*, 2016) (via plugin *q2-dada2*). A phylogeny was estimated with SEPP (Mirarab*, et al.*, 2012) as implemented in the *q2-fragment-insertion* Qiime2 plugin. All amplicon sequence variants (ASVs) were aligned with *feature-classifier classify-sklearn* against the SILVA-132-99 database (Quast*, et al.*, 2012) trained with a Naïve Bayes classifier (Bokulich*, et al.*, 2018) on the Illumina primers of the 16S rRNA gene V3–V4 region (F-CCTACGGGNGGCWGCAG; R-GACTACHVGGGTATCTAATCC). Downstream analyses were performed using R version 4.0.2 and the MicrobiomeAnalyst web platform (https://www.microbiomeanalyst.ca/) (Chong*, et al.*, 2020). Data were filtered using options *minimum count 4* and *low count filter* based on 20% prevalence in samples. Data were rarefied to the minimum library size before any statistical comparisons. Rarefaction curves were based on the relationship between number of ASVs and number of sequences. We examined alpha diversity using Shannon and Simpson's indexes, both of each are estimators of species richness and evenness, Shannon is more weight on species richness and Simpson more weight on species evenness. Chao1 index, an abundance-based estimator that gives more weight to the low abundance species was also estimated. The results were plotted across samples and reviewed as box plots for each group or experimental factor. Beta diversity was calculated based on weighted UniFrac distance metrics and used to perform Principal Coordinates Analysis (PCoA). Prior the testing of similarity/distance matrices, betadisper was used to test for variance homogeneity between the group of interests to ensure the reliability of the results. Permutational variable analysis of variance (PERMANOVA) were performed if the data met the assumption of homogeneous dispersion (betadisper: p value>0.05), and analysis of similarity (ANOSIM) was used when there was heterogeneity of variance among groups (betadisper: p value<0.05). PERMANOVA (using "Adonis" function) and ANOSIM analyses were conducted in the Vegan package in R with 999 permutations. Taxa plots were constructed based on phyla and genera to visualize taxa abundance across the different groups.

### 4.2.5 Identification of the transmission mode of gut symbionts

In order to verify the origin of the microbiota associated with F. frugiperda we tested the hypothesis of vertical transmission of bacteria from the mother to the offspring. For this purpose, eggs, ovaries of adults and intestines of larvae were dissected under the same conditions as described above for the gut and fixed overnight in Carnoy's solution. (60% ethanol, 30% chloroform and 10% glacial acetic acid). Afterwards, samples were storage in ethanol 100%. Samples were rehydrated 5 minutes in an ethanol series (90, 70, 50%) and then twice in distilled water. Then, samples were transferred to 18-well (eggs and ovarioles) chamber slides (Ibidi GmbH, Martinsried, Germany). Hybridization buffer (1 M Tris-HCl pH 8,0; 5 M NaCl; 10% SDS p/v; 30% formamide v/v) and 10 μM of eubacteria probe were add, slides were covered with glass cover slips and incubated overnight at 46°C in the hybridization oven. To keep the samples moistened, the slides were kept in a hybridization chamber that was set up in a very shallow tupperware, containing wet Kimwipes disposable wipers at the bottom. Shortly after, samples were washed with a stringent buffer (1 M Tris-HCl pH 8; 5 M NaCl, 0.5 M EDTA and 10% SDS) (2x - 15 min at 48ºC) to remove non-specific binding, then washed with Milli-Q water (2x) before treatment with 1μM DAPI (4',6-diamidino-

2-phenylindole) during 5 minutes. The analyses were performed under a Leica TCS SP6 confocal scanning microscope. at the Molecular & Cellular Imaging Center (OARDC/OSU Wooster, OH).

## 4.3 RESULTS

Rarefaction curves for each sample group (Anexo D) indicated that most of bacterial taxa associated with the larval mid and hindguts of *S. frugiperda* was accessed. The apha diversity index Shannon, Chao1 and Simpson indexes were higher for the bacterial community found in the artificial diet than in any of the larval gut regions analyzed. An opposite pattern was found when comparing the larval gut microbiota with the microbiota of maize leaves. The apha diversity index of the larval microbiota in the midgut and hindgut of *S. frugiperda* was higher than that observed for the microbiota associated with leaves of maize (Fig 16). Comparative analysis of the Shannon indices of the gut microbiota of host-adapted strains of *S. frugiperda* found no differences in both regions of the gut analyzed, regardless of the diet used (Fig 17).

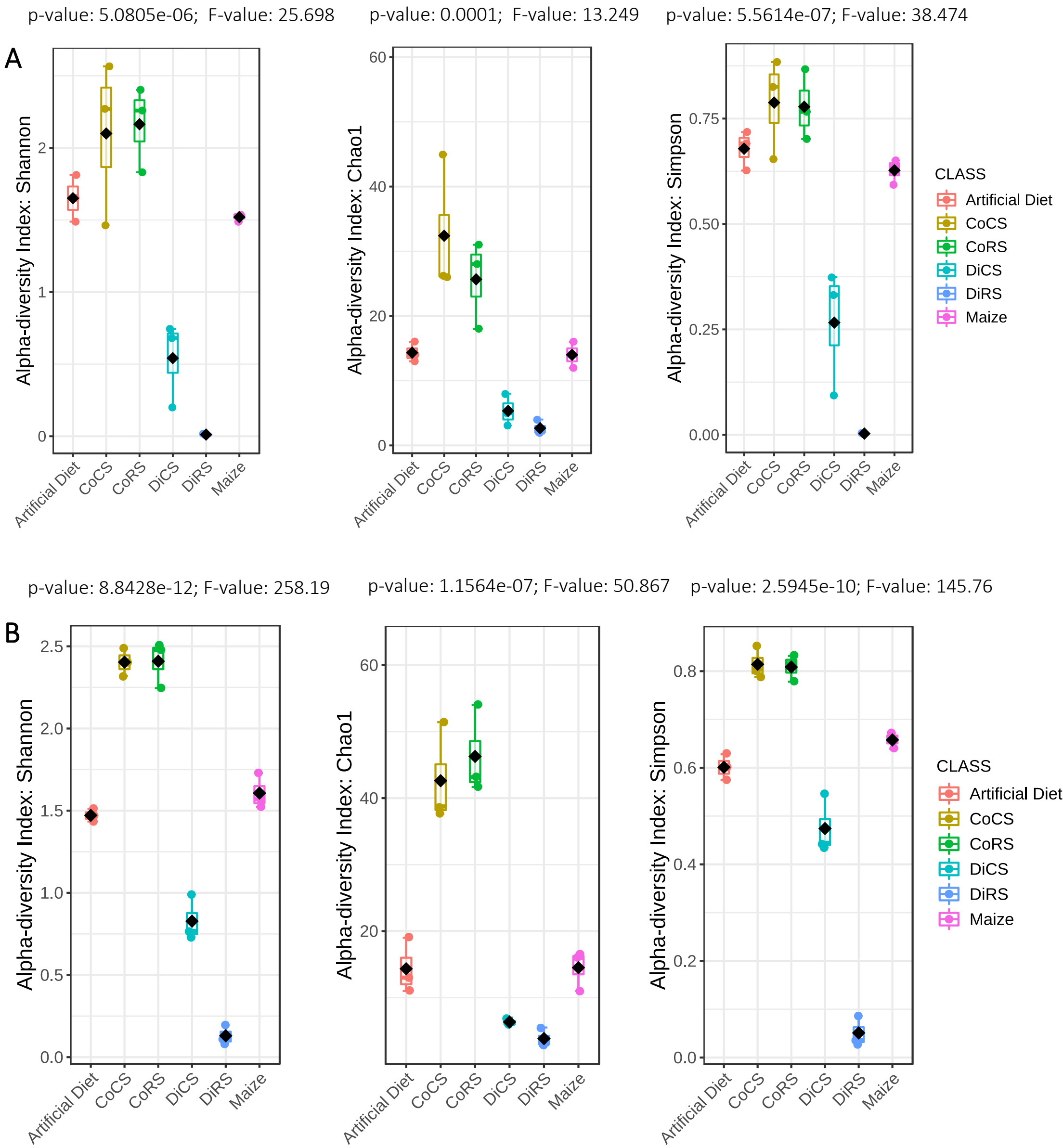


**Figure 16.** Shannon, Chao1 and Simpson Diversity index in samples from midgut (A) and hindgut (B) of *Spodoptera frugiperda* strains (RS or CS) fed on different diets (Corn=Co and artificial diet=Di). The samples are represented on X-axis and their estimated diversity on Y-axis. The p-values and F value from the ANOVA are shown in which box of comparison.

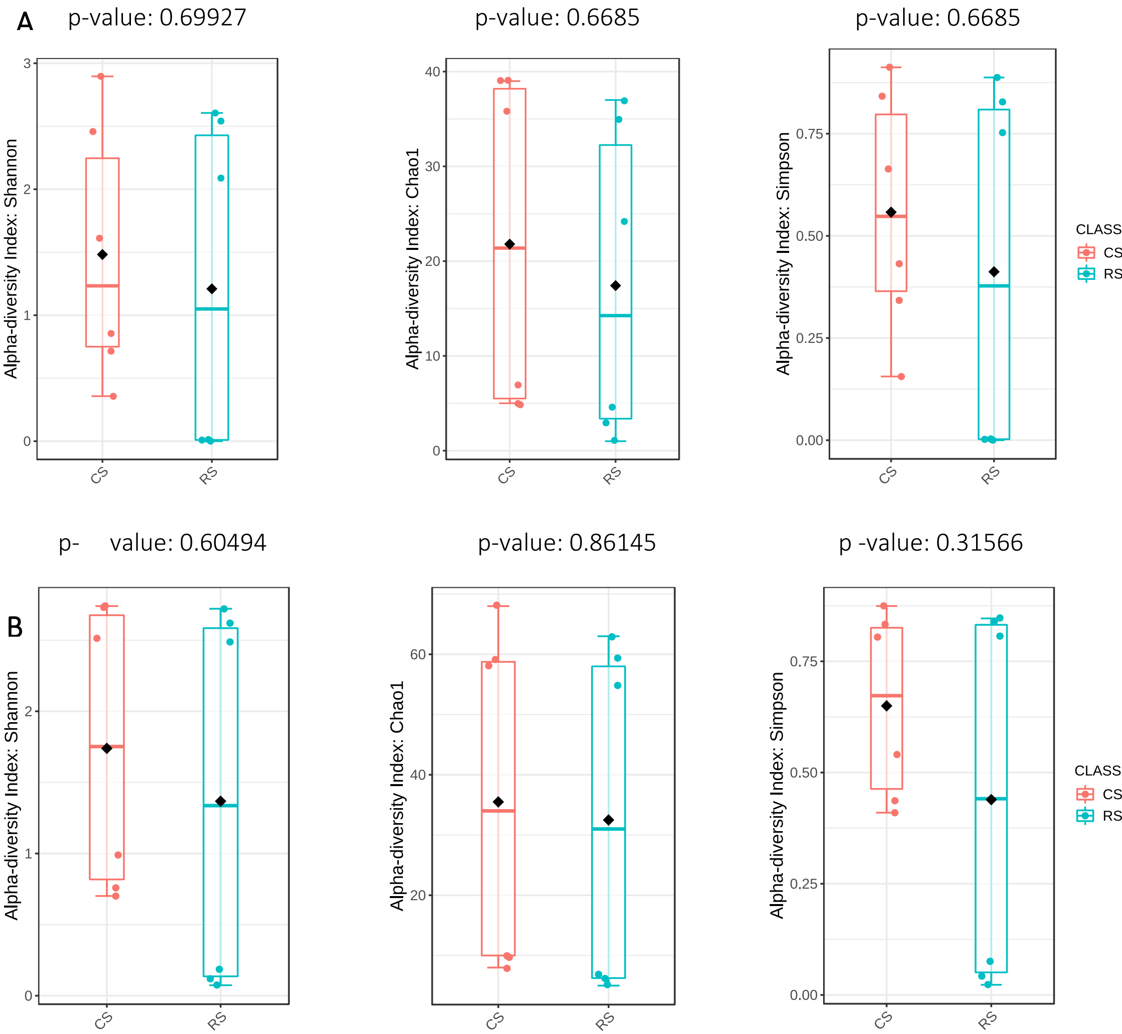


**Figure 17.** Boxplot comparing the Shannon, Chao1 and Simpson diversity index in samples from midgut (A) and hindgut (B) of *Spodoptera frugiperda* strains (RS or CS) regardless diet. The p-values from the Test T are shown in which box of comparison.

The midgut microbiota of the host-adapted strains of *S. frugiperda* fed on artificial diet resolved into a single group, distant from the group formed by the microbiota present in their food source. The same pattern was observed for the hindgut samples. Samples obtained for the larval microbiota fed on maize did not clustered both strains for both gut regions. Nonetheless, the gut microbiota associated with larvae of each strain resolved in clusters far from the cluster formed by the microbiota associated with maize leaves (Fig 18).

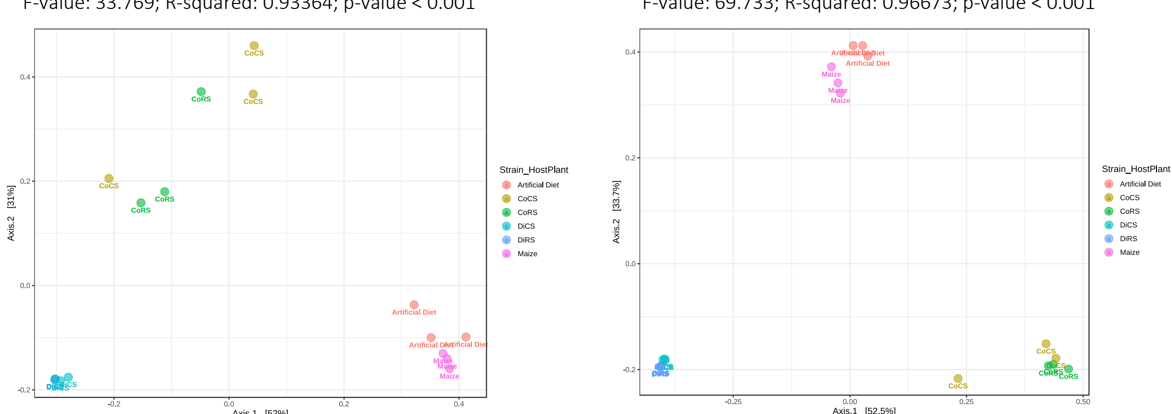


**Figure 18.** Bray-Curtis-based Principal coordinates analysis (PCoA) of the of microbial community in the midgut and (B) hindgut of *Spodoptera frugiperda* strains ( RS= dotted line, CS= continuous line) fed on different diets (Corn=red, cotton= green, artificial diet= blue and rice= purple).

Multivariate analyses based on Unifrac distances (alpha 0.5) revealed no significant differences between the gut microbiota of *S. frugiperda* strains fed on the tested diets. The betadisper test was non-significant for the midgut (p= 0.7744) and hindgut (p= 0.7923) microbiota of *S. frugiperda* strains, meeting the assumptions of homogeneous dispersion for PERMANOVA use. PERMANOVA revealed no significant variation in the midgut (R2 = 0.05078, p = 0.102) and hindgut (R2 = 0.5395, p = 0.094) (Tab.5). microbial community composition between *S. frugiperda* strains. However, we performed ANOSIM test for host plant factor due to the heterogeneity of data demonstrated by betadisper for both gut regions (midgut p=0.01534; hindgut=0.03051). The result of ANOSIM showed highly significant differences in the microbial community composition of the midgut (R = 0.8222, P = 0.0039) and hindgut (R =0.8222, p = 0.0019) of *S. frugiperda* in between both diets offered (Tab.5).

**Table 5.** PERMANOVA and ANOSIM results from comparisons between midgut and hindgut associated microbial communities between *Spodoptera frugiperda* strains (corn and rice strains) feed on different food sources using UniFrac (alpha 0.5) values.

| | **PERMANOVA** | | **BETADISPER** | |
|---|---|---|---|---|
| | **$R^2$** | ***p* value** | **F value** | **Pr(>F)** |
| **Strain - Midgut** | 0.05078 | 0.102 | 0.0867 | 0.7744 |
| **Strain - Hindgut** | 0.05395 | 0.094 | 0.0732 | 0.7923 |
| | **ANOSIM** | | | |
| | **R** | ***p* value** | | |
| **Diet - Midgut** | 0.8222 | 0.003996** | 8.5168 | 0.01534 * |
| **Diet - Hindgut** | 0.8222 | 0.001998** | 6.3388 | 0.03051 * |
| Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1 | | | | |

The gut microbial community *S. frugiperda* when feeding on maize is composed predominantly of Proteobacteria followed by Firmicutes and Bacteroidetes (Fig. 19). Proteobacteria was also the dominant phylum in the microbiota of maize leaves, followed by Actinobacteria. Larvae fed on artificial diet is dominated by Firmicutes whereas Proteobacteria and Actinobacteria are the most abundant in the microbiota of the artificial diet. (Fig. 19).

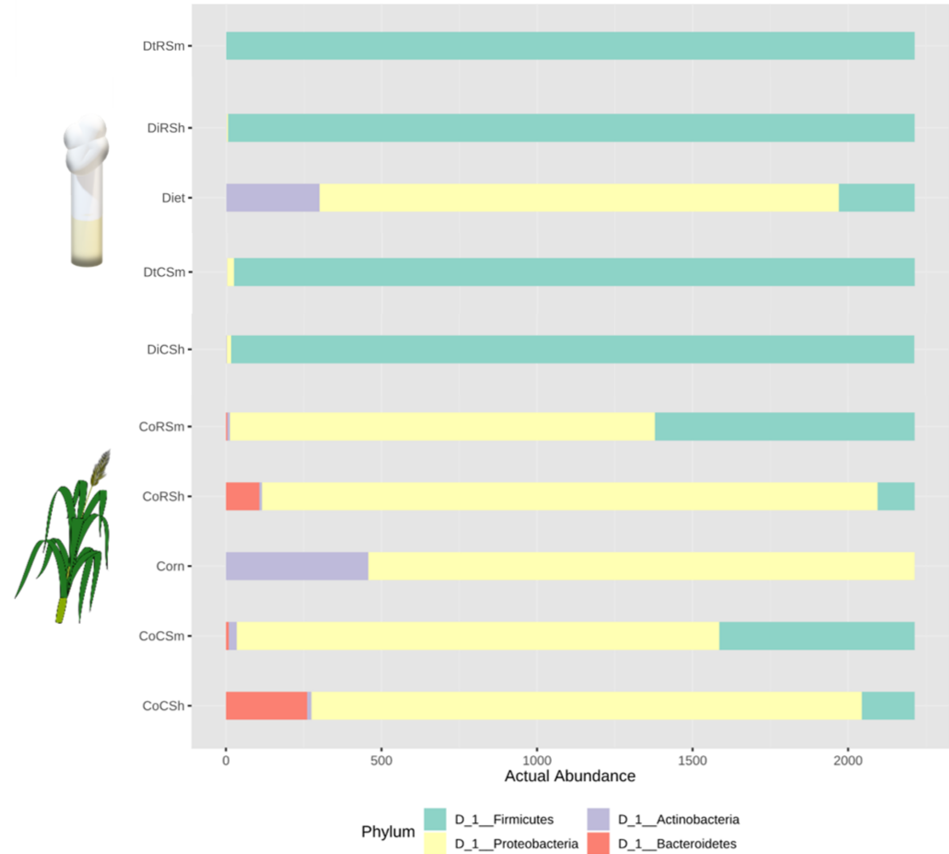


**Figure 19.** Taxonomic composition of midgut (m) and hindgut (h) microbial community of *Spodoptera frugiperda* corn (CR) and rice strains (RS) and at Phylum level using Stacked Bar plot when fed on artificial diet (Di) and Corn (Co). The microbiota of the diet offered is also represented (corn and diet).

At the genus level, the gut microbiota of maize-fed larvae was composed by the ASVs corresponding to the Firmicutes *Enterococcus* and the Proteobacteria *Acinetobacter Klebsiella, Rhizobium,* and *Enterobacter*. The hindgut bacterial community presented more *Acinetobacter* and less *Enterococcus* when comparing to the midgut. The *S. frugiperda* strains shared the same bacterial composition with some slight differences in their abundance (Fig. 20). On the other hand, the microbiota found in corn leaves was basically composed by ASVs assigned to the *Proteobacteria Sphingomonas, Methylobacterium* and *Acinetobacter*. Interesting the midgut and hindgut of both *S. frugiperda* strains were dominated by the Enterococcus, while the artificial diet was composed predominantly by the *Proteobacteria Methylobacterium* and *Stenotrophomonas* (Fig. 20).

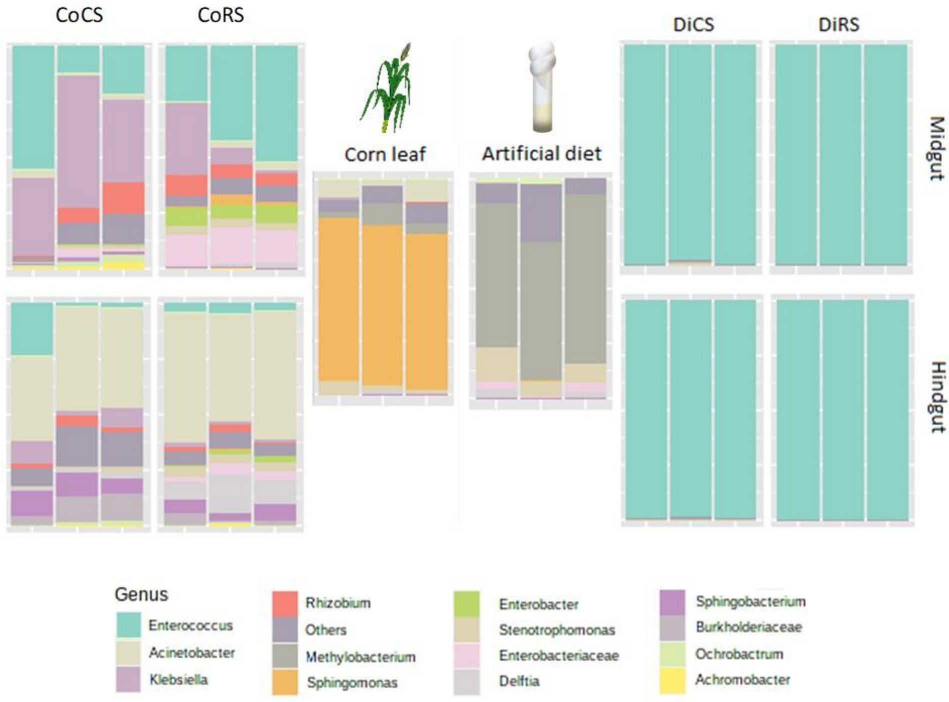


**Figure 20.** Taxonomic composition of midgut (A) and hindgut (B) microbial community of *Spodoptera frugiperda* corn (CR) and rice strains (RS) and at genus level using Stacked Bar plot.

Fluorescent *in situ* hybridization (FISH) using the Eubacteria probe designed to label 16S rRNA gene sequences revealed bacteria infecting oocytes and eggs at late stages of embryonic development distributed from the epithelium of the nurse cells to the oocyte (Fig 21). In early stage oocytes bacteria were visualized at the oocyte border close to follicular cells. We did not detect bacteria inside nurse cells or deeper in the oocyte cytoplasm. Bacteria were also visualized in late stage developing embryo (Fig. 22). As we expected the presence of bacteria in the *S. frugiperda* gut, we image it as a control (Fig 23).

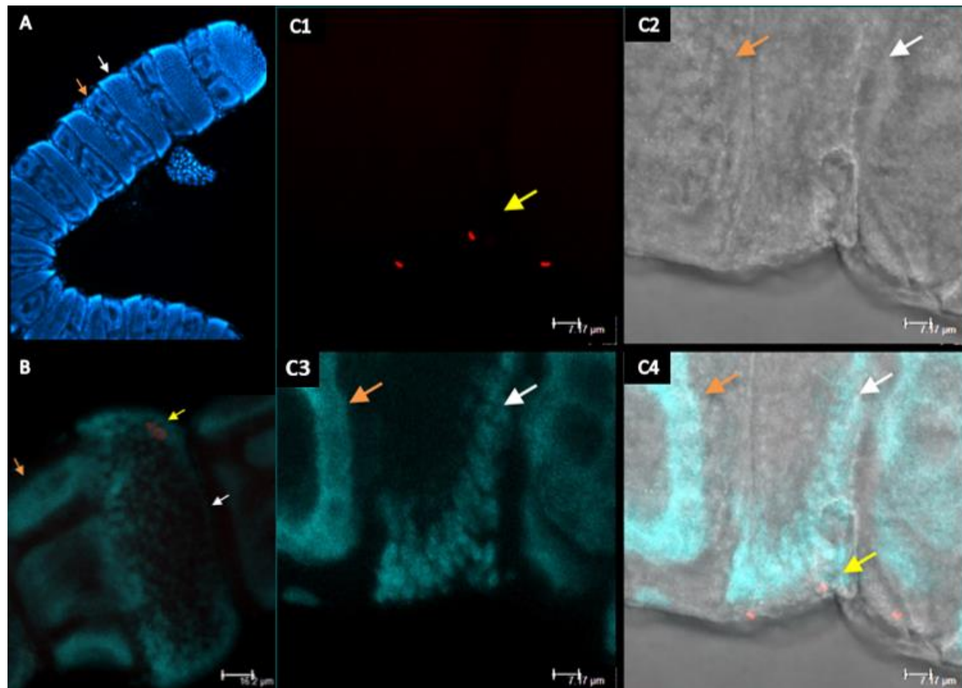


**Figure 21.** Ovariole of *Spodoptera frugiperda* (A) with DAPI staining showing the oocyte (white arrows) and nurse cells (orange arrows) nuclei. Scale bar equals 60 µm. (B) A small aggregate of bactetia in the oocity. Bacteria detected by universal probe (EUB) are highlighted with yeloow arrows Nurse cells are pointed by orange arrows, oocity is indicated by white arrow. (C1)Bacterial localization in the ovary of *S. frugiperda* with Fluorescent In Situ Hybridization. (C2) TIC images, (C3) DAPI staining.(blue) (C4) are merged images of TIC, EUB and DAPI.

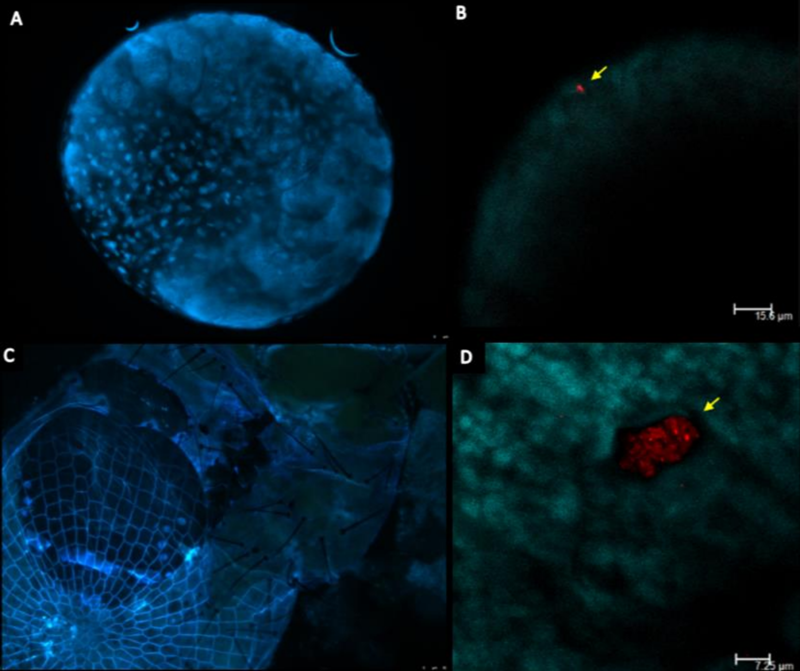


**Figure 22.** Eggs of *Spodoptera frugiperda* with DAPI staining showing a (A) earlier and (B) late embrio nuclei. Scale bar equals 50 µm. Bacterial localization in the embryo of *S. frugiperda* with Fluorescent In Situ Hybridization. Bacteria detected by universal probe (red). (D) Detection of bacteria In the late embryo in a clump. (B) Bacteria in the early embryo close to the egg edge Bacteria detected by universal probe are highlighted with yeloow arrows.

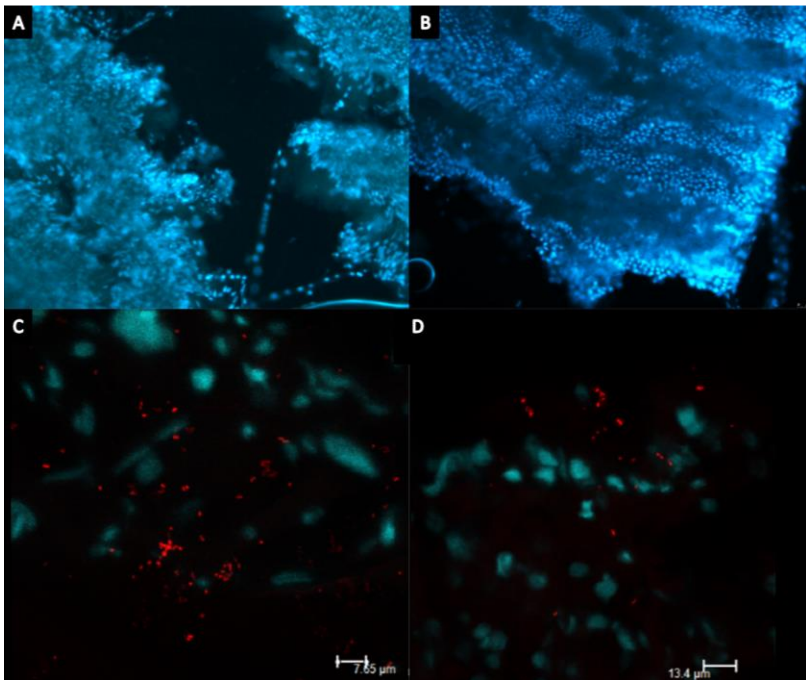


**Figure 23.** *Spodoptera frugiperda* midgut (A,B) with DAPI staining showing nuclei. Scale bar equals 50 µm. (C,D) Bacterial localization in the midgut of *Spodoptera frugiperda* with Fluorescent In Situ Hybridization. Bacteria detected by universal probe (red) and DAPI staining (blue).

## 4.4 DISCUSSION

The gut microbiota of *S. frugiperda* is strongly modulated by the diet consumed but the microbiota of the diet does not represent the gut microbiota. This finding along with the detection of bacteria in the reproductive tissues of females and in the eggs of *S. frugiperda* demonstrates the occurrence of horizontal transmission of bacteria in this species. This suggests the occurrence of close associations of *S. frugiperda* with bacterial symbionts, indicating associated bacteria can contribute to host fitness attributes. The presence of bacteria in the ovaries and eggs of a close related species *S. littoralis* has also been previously demonstrated (Chen*, et al.*, 2016, Teh, et al., 2016), as well as their survival and proliferation in the intestinal tract of the host at all life stages, safely passing through metamorphosis and benefiting the adult host (Johnston and Rolff, 2015); (Teh, et al., 2016). The mechanisms for survival of these bacteria belonging to *Enterococcus* involve upregulation of pathways for tolerating alkaline stress, forming biofilms and two-component signaling systems, resisting oxidative stress and quorum sensing (Mazumdar*, et al.*, 2021).

*Enterococcus* was also the most abundant bacteria found in our samples, especially in the larvae fed on artificial diet. This observation is similar to other reports (Chen, et al., 2016, Gichuhi*, et al.*, 2020, Jones*, et al.*, 2019). *Enterococcus* has been pointed out as an important symbiont for the *Spodoptera* genus. In addition to the high prevalence in both field and laboratory samples, it has been demonstrated to be metabolically active in the larval lifespan playing a defensive role (Rozadilla*, et al.*, 2020, Shao*, et al.*, 2014). *Stenotrophomonas, Sphingobacterium, Enterococcus* and *Delftia* here identified as part of the gut microbiota of *S. frugiperda* were also present in adult and larval stages of *S. frugiperda* from maize fields (Gichuhi, et al., 2020), which suggests that gut bacterial community members are transmitted across developmental stages. *Klebsiella, Enterobacter, Ochrobactrum* and *Enterococcus* were found in the regurgitant of fall armyworm collected in Puerto Rico and Pennsylvania (Jones, et al., 2019). *Klebisiella* and *Enterobacter*

were identified playing a role in mediating fall armyworm–plant interactions by upregulating the expression of the herbivore-induced maize proteinase inhibitor (*mpi*) gene in maize and in tomato. *Klebsiella* also downregulated the activity of peroxidase while upregulating the trypsin protease inhibitor, enhancing larval performance on tomato (Acevedo*, et al.*, 2016).

The fact that the gut bacterial community of strains are similar to each other and modulated by the food is also seen in humans for example, where host genetics play a less relevant role in determining microbial composition than environmental factors such as diet (Rothschild*, et al.*, 2018). Diet has also been shown to affect microbial colonization and proliferation (Mason, et al., 2020). Therefore, it is possible that some bacterial groups that are not present in the food source may have a maternal origin that varies in its abundance and prevalence in the gut, depending on the diet consumed by the host and the host strain examined. In our artificial diet, we did not use antibiotics, and this was also not autoclaved, which could explain the bacterial diversity in this food source. On the other hand, the low diversity of the bacterial community in the gut of *S. frugiperda* can suggest that the artificial diet when compared to plant food does not favor the microbial diversity in the intestine of caterpillars (Mason, et al., 2020). Moreover, *Enterococcus* was demonstrated to produce the antimicrobial mundticin, which strongly inhibits some of the competing and potentially pathogenic microorganisms inhabiting the gut of *S. littoralis* larvae (Shao, et al., 2017). Possibly the artificial diet favors the establishment of this taxa and this in turn inhibits the growth of other Bacteria.

We also observed differences in the midgut and hindgut microbial composition of the *S. frugiperda* strains. The midgut is involved with food digestion and nutrient assimilation, while the posterior region of the gut is involved with excretion and water and nutrient resorption. The different roles played by each gut region lead to environments differing in ion concentration, pH and redox potention which affect their microbial composition (Dillon and Dillon, 2004, Egert*, et al.*, 2003, Smith*, et al.*, 2017). The hindgut is known to be more favorable to bacteria function and growth due to the lack of digestive enzymes and the presence of ions and metabolites delivered to this gut region in the filtrate from the Malpighian tubules (Douglas, 2015). In most insects, the hindgut harbors the largest microbial populations when compared to other gut regions (Douglas, 2015). Therefore, the greatest differences between the strains in the hindgut may be due to greater bacterial diversity and the greater likelihood of finding symbionts more closely associated with the strains due to the better colonization conditions of the hindgut.

Our results with the larval gut microbiota of host-adapted strains of *S. frugiperda* do not support the claim that the larvae of lepidopterans do not carry true bacterial associates in their gut and that the gut community is simply a reflection of the bacteria associated with the food source larvae exploit. We also demonstrated bacteria is vertically transmitted in *S. frugiperda*, but future studies are still require to demonstrate these bacteria are indeed the prevalent bacteria inhabiting the larval gut lumen. Our results also highlight that when using the artificial diet in experiments, it is important to consider the high simplification of the caterpillar gut microbial diversity, which does not reflect the condition of this community when the host feeds on a natural diet such as maize. The function of these gut microbes in *S. frugiperda* strains need to be evaluated, as well as the ecological implications of this variability to *S. frugiperda* larval development.

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# 5. METATRANSCRIPTOME OF THE FALL ARMYWORM HOST STRAINS REVEALED DIFFERENCES IN MIDGUT BUT NOT IN GUT BACTERIAL TRANSCRIPTIONAL ACTIVITY

## ABSTRACT

The challenges herbivores face in plant feeding may be attenuated by herbivore-associated microorganisms that aid in the process of host plant adaptation. We assessed the gene expression responses of two host-adapted strains of *Spodoptera frugiperda*, the corn and the rice strains, to different natural and artificial food sources. Assessment of differential gene expression was based on mRNA-based Illumina metatranscriptome sequencing and analysis of the larva midgut and associated gut microbiota. Our analyses revealed the transcriptional activity associated with the gut epithelium of *S. frugiperda* differ between strains in all food sources investigated, with most of the differential expressed genes detected belonging to processes of detoxification, nutrient assimilation and immune response. However, few differences in transcriptional activity of the gut bacteria were detected between the rice and the corn strain to most of the food sources investigated. The only exception in the transcriptional activity of the gut microbiota of both races was the bean-based artificial diet. The gut microbiota of the host-adapted races of *S. frugiperda* responded very differently when larvae fed on the artificial diet. We conclude that the *S. frugiperda* strains have different adaptations to deal with the ingested food, while the associated microbiota plays basically the same role in both strains regardless the natural food sources tested. We also point out that the artificial diet generates a considerable difference in the functioning of the gut microbiota of the strains, mainly on *Enterococcus mundtii*

KEYWORDS: *Spodoptera frugiperda*, symbiosis, gut bacteria, host plant adaptation

## 5.1 INTRODUCTION

Plant feeding by herbivores represents a tough challenge. Plants utilize a myriad of structural and biochemical defense mechanisms to prevent their attack (Chen 2008, Strauss and Agrawal 1999). Therefore, insects that succeed in herbivory require a series of adaptations to overcome plant defenses and to acquire the nutritional components necessary for their survival and proper development. These adaptations include enzymatic detoxification, followed by excretion or sequestration, physiological tolerance, and/or behavioral avoidance (Zunjarrao et al. 2020). New mechanisms of detoxification have also been achieved through gene recruitment, neofunctionalization, and horizontal gene transfer (Zunjarrao et al. 2020, Wybouw et al. 2014, Heidel-Fischer et al. 2019, Heidel-Fischer and Vogel 2015).

Moreover, many insects benefit from the contribution of associated microorganisms to be successful in the process of adaptation to host plants (Acevedo et al. 2016, Hammer and Bowers 2015). In this context, gut bacteria can play an important role in this process of adaptation because they are present in the organ where food is initially processed and, therefore can respond relatively quickly to environmental changes, such as to plant defense molecules (Hammer and Bowers 2015). It has been reported that gut bacteria can improve the fitness of lepidopterans by protecting their host against pathogens (Shao et al. 2017) and by processing waste nitrogen, particularly ammonia (Rozadilla et al. 2020). The gut microbiota of lepidopterans has also been shown to facilitate digestion and nutrient acquisition (Indiragandhi et al. 2008, Xia et al. 2017), producing enzymes and amino acids, but also aiding in the detoxification of plant-derived (Ceja-Navarro et al. 2015, Zhang et al. 2020) and synthetic (Gomes, Omoto and Cônsoli 2020) xenobiotics.

The Fall armyworm (FAW), *Spodoptera frugiperda* (Lepidoptera: Noctuidae) is currently one of the most devastating agricultural pests in the western hemisphere that has recently spread to West and Central Africa, Asia and

Oceania (Li et al. 2020, Goergen et al. 2016, Zarkani, Wibowo and Sipriyadi 2020). The FAW is highly adaptable and polyphagous, feeding on many plants of agricultural importance (Deshmukh et al. 2021, Ashley et al. 1989, Casmuz et al. 2017, Montezano et al. 2018). Additionally, it has been reported to evolve resistance to many classes of pesticides (Carvalho et al. 2013, Gutiérrez-Moreno et al. 2019) and *Bacillus thuringiensis* (Bt)-crops (Chandrasena et al. 2018, Farias et al. 2014, Storer et al. 2010).

The FAW is a species composed of two morphologically identical, but genetically distinct host-adapted strains (Nagoshi and Meagher 2008). They are asymmetrically distributed among host plants in the field. The "corn strain" (CS) infests corn, sorghum, and cotton plants more commonly, while the "rice strain" (RS) is more often collected on rice and pasture grasses (Pashley 1989, Prowell 1998, Silva-Brandão et al. 2018, Juárez et al. 2014). Several differential effects of the host plants on the viability and development of the two strains have been described (Pashley, Hardy and Hammond 1995, Veenstra, Pashley and Ottea 1995, Meagher, Nagoshi and Stuhl 2011, Juárez et al. 2014, Pashley 1986, Meagher et al. 2004, Silva-Brandão et al. 2017). Additionally, the host-adapted strains also differ in their transcriptional activity, particularly in the expression of genes related to xenobiotic metabolization and mitochondrial genes, pointing to a role in driving strain divergence (Orsucci et al. 2020, Silva-Brandão et al. 2017). Moreover, the RS and CS also differ in their susceptibility to organic pesticides (Veenstra et al. 1995, Adamczyk Jr et al. 1997, Ríos-Díez and Saldamando-Benjumea 2011, Yu 1991) and Bt-plants (Adamczyk Jr et al. 1997, Ingber, Mason and Flexner 2018).

There are still few studies that address the functional role of the gut microbiota when compared to studies describing the taxonomic profile of the microbiota in lepidopterans, especially in the FAW host-adapted strains (McCarthy, Cabrera and Virla 2015, Rozadilla et al. 2020). The analysis of the composition of the microbiota is not sufficient to elucidate the real role of the microbiota of any particular host. The similarity in the taxonomic composition may reflect in different contributions to the host when different food substrates are available in the environment, which may alter the production of molecules by the microbiota (Graf et al. 2015). Moreover, some species that are very abundant in the gut community may have poor metabolic activity, and vice-versa (Chen et al. 2016, Shao et al. 2014).

Therefore, considering the limited current knowledge on the functionality of the gut microbiota of lepidopteran larvae (Paniagua Voirol et al. 2018) and the great potential that the gut microbiota has to be used in developing insect pest control methods, it is of paramount importance to deepen the knowledge on the ecology and functional activity of the gut microbial community of FAW. In this study we report the metatranscriptome profiling of the larval midgut of the CS and RS of FAW in order to elucidate the differential activity of the gut epithelium and the microbiota of both host-adapted strains feeding on different food substrates. An important and novel aspect of this study is that we not only identified the metabolically active gut microbiota, but also gained insight into what genes were being expressed.

## 5.2 MATERIAL AND METHODS

### 5.2.1 Insect rearing and strains identification

Colonies of the RS and CS of *S. frugiperda* were established in controlled laboratory conditions from field sampled specimens. The *RS* colony was obtained specimens collected in rice fields in Santa Maria, *RS,* Brazil (29°68'68"S, 53°81'49"W), and the *CS* colony from a maize field in Piracicaba, SP, Brazil (22°43'30"S, 47°38'56"W).

Field-collected larvae were reared individualized into plastic coffee cups containing an artificial diet based on wheat germ, beans and brewer's yeast (Greene, Leppla and Dickerson 1976). Individual larval exuviae were collected for each larva for strain identification (see below). Once pupation occurred, pupae were transferred to clean cups lined with filter paper for adult emergence. Newly emerged adults from strain-identified larvae were transferred to PVC tubes lined with paper for mating and egg laying. Egg masses were collected, surface sterilized, and transferred to artificial diet for later larval development (Parra 1999).

*Spodoptera frugiperda* strain identification followed Levy, Garcia-Maruniak and Maruniak (2002). The collected larval exuviae was subjected to tissue lysis for DNA extraction in 750 µL digestion buffer (60 mM Tris pH 8.0, 100 mM EDTA, 0.5% SDS) and proteinase K at a final concentration of 500 µg/mL. Samples were macerated using a plastic pestle, mixed well by inversion and incubated overnight at 55°C. Afterwards, 750 µL of phenol:chloroform (1:1) was added and rapidly inverted for 2 min. Samples were centrifuged at high speed for 10 minutes. The aqueous phase was collected and phenol:chloroform extraction was repeated twice before a final extraction with chloroform. The aqueous phase was collected, added 0.1 volume of 3M sodium acetate (pH 5.2) and an equal volume of 95% ethanol. Samples were then mixed by inversion, incubated for 40 min at -80°C, and centrifuged (27,238 g x 30 min x 4°C). The pellet obtained was washed twice with 1 mL of 85% ice-cold ethanol, centrifuged for 10 min after each wash and dried at 60°C during 5-10 min in a SpeedVac. Finally, the pellet was resuspended in nuclease-free water. DNA concentration and quality were estimated by spectrophotometry and agarose gel electrophoresis.

The genomic DNA (gDNA) obtained was subjected to polymerase chain reaction (PCR) amplification of a fragment of the mitochondrial cytochrome oxidase I (COI) gene using the primers set JM76 (GAGCTGAATTAGGRACTCCAGG) and JM77 (ATCACCTCCWCCTGCAGGATC) (Levy, Garcia-Maruniak and Maruniak 2002) to produce a 569 base-pair (bp) amplicon. The PCR mixture contained 100-150 ng of gDNA, 1.5 mM of $MgCl_2$, 1x PCR buffer, 0.2 mM of each dNTP, 0.32 µM of each primer, and 0.5U of GoTaq® DNA Polymerase (Promega) in a total volume of 25 µL. The thermocycling program was 94°C x 1 min (1x) followed by 33 cycles at 92°C x 45 s, 56°C x 45 s, 72°C x 1 min, with a final extension at 72°C x 3 min (1x). Ten-µL of the PCR reaction mixture was then subjected to restriction using the *Msp*I (HpaII) and overnight incubation at 37°C. The restriction pattern was verified using 1.5% agarose gel electrophoresis. The restriction pattern of the amplified COI fragment of the CS produces two fragments (497pb and 72pb), whereas that of the RS yields an unrestricted fragment.

### 5.2.2 Testing the diet effect on the larval gut metatranscriptome of CS and RS of *S. frugiperda*

Seeds of maize (*Zea mays*, Poaceae) var. "Conventional impact" and cotton (*Gossypium hirsutum*, Malvaceae) var. IAC FC2 were seeded in 500 mL plastic pots filled with a commercial soil conditioner, while rice seeds (*Oriza sativa,* Poaceae) var. BRS Esmeralda were seeded in 1 L plastic pots. All plants were maintained in a greenhouse. Leaves were sampled from cotton and maize plants at the v3-v4 stage, while rice leaves were sampled from plants at the v11-v13 stage. Leaves were collected and immediately immersed in a container with distilled water for 30 minutes to maintain turgidity, and offered *ad libitum* to newly emerged larvae of *RS* and *CS* strains in 25 mL plastic cups on top of a moistened cotton pad. Leaves were replaced and supplied with fresh ones in order to have no leaves older

than two days and no shortage of food. Insects were kept under controlled laboratory conditions (25 ± 1°C; 60 ± 10%; 14-hour photophase).

The experimental groups were composed by RS and CS larvae feeding on leaves of rice (RiRS, RiCS), corn (CoRS, CoCS) and cotton (CtRS, CtCS), and on artificial diet (DiRS, DiCS). We used five replicates for each treatment, with each replicate corresponding to a pool of midguts collected from five larvae. Sixth-instar larvae were surface-sterilized in cooled 0.2% sodium hypochlorite in 70% ethanol, washed in cold sterile water, and transferred to autoclaved water for gut dissection under sterile conditions. Tissues were sampled in RNAlater and stored at -80 until RNA extraction.

### 5.2.3 RNA extraction and sequencing

Three pools of five midguts were pulverized in liquid nitrogen, and 20 mg of the pulverized material was used for RNA extraction using the QIAGEN AllPrep DNA/RNA/miRNA Universal Kit. We followed the manufacturer's protocol for RNA isolation from animal tissue, and RNA samples were stored at -80ºC. Library preparation (Illumina Stranded Total RNA Prep Ligation with Ribo-Zero Plus kit) and paired-end (2 x 100 bp) sequencing on Illumina HiSeq plataform was performed at the Animal Biology Laboratory (ESALQ/USP, Piracicaba, SP).

All sequencing data (paired-end reads) were used to assemble a single metatranscriptome consisting of all libraries obtained for both host-adapted strains RNA (midgut tissues + associated bacteria). *De novo* assembly was achieved using Trinity (v 2.8.5) (Grabherr et al. 2011; Haas et al. 2013), with its built-in command for "gentle trimming" (MacManes, 2014) using Trimmomatic set with *slidingwindow*:4:5, *leading*:5, *trailing*:5, *minlen*:25.

### 5.2.4 Sequences analyses

All sequencing data (paired-end reads) were used to assemble a metatranscriptome consisting of both host RNA (from midgut tissues), and gut content RNA, including gene expression from midgut microorganisms. *De novo* assembly was achieved using Trinity (v 2.8.5) (Grabherr et al. 2011; Haas et al. 2013), with its built-in command for "gentle trimming" (MacManes, 2014) using Trimmomatic and the following settings: Slidingwindow:4:5 Leading:5 Trailing:5 Minlen:25.

In order to separate transcripts belonging to *S. frugiperda* from those belonging to bacteria, contigs of the *de novo* assembly were first annotated against the NCBI *nt* database (downloaded March, 2021) using dc-megaBLAST (e-value threshold of 1.0E-5), and the *nr* database (downloaded March, 2021) using DIAMOND's implementation of the BLASTx engine (e-value threshold of 1.0E-5). BLAST results were imported to R (v. 4.0.5) where contigs were filtered either as belonging to the "host" or "bacteria", according to the following set of rules: a read was considered to belong to the host if its taxonomical ID was included in the phylum Arthropoda in both blast results or in at least one blast result if the other blast yielded unidentified hit. Contigs with conflicting taxonomy or unidentified in both blast searches were excluded. The same set of rules was applied when isolating contigs belonging to the gut microbiome, but using "Bacteria" as the taxonomical ID. With this criterium we created three separate fasta files: one for host-associated contigs, one for bacteria-associated contigs, and one for contigs that were excluded (e.g. contaminants or unidentified).

To gain further insight on the bacterial functional profile, Trinotate (https://trinotate.github.io) (v 3.0.1) was used for protein prediction and functional annotation using a range of databases, such as SwissProt, EggNOG and KEGG.

Only the longest isoform assembled for each gene by Trinity was used in downstream analyses. Salmon, within Trinity, was used to estimate counts for bacterial and host contigs present in each sample. With this data, PCA and heatmaps were generated using Trinity to evaluate how samples clustered according to gene expression. Differentially expressed genes (DEG) were identified using DESEq2 within Trinity, using fold change (FC) |2| or higher and *p* (FDR) < 0.05 as thresholds for DEG. Finally, we characterized similarity in expression profile based on DEG expression using 60% of the height of a tree built using hierarchical clustering for highly expressed DEG genes (FC ≥2, p<0.05).

## 5.3 RESULTS

### 5.3.1 Transcriptome assembly and annotation

*De novo* base pairs (bp) and an N50 length of 1208 bp. After the filter to separate contigs of bacteria from the host, the total of trinity transcripts for the host was 73804 transcripts belonging to Arthropoda and 2259 to bacteria. The median contig length and N50 of the selected transcripts was 325 bp and 809 bp for those belonging to Arthropoda, and 331 bp and 542 bp for those to bacteria.

### 5.3.2 Differential transcript expression

Principal component analysis (PCA) of all expressed genes of the gut microbiota of *S. frugiperda* show that gene expression in the gut bacterial communities is generally similar among samples, regardless of the *S. frugiperda* strains, or type of diet. One exception was for the gene expression in microbiota found in *RS* fed artificial diet, which clustered away from all other treatments, including from *CS* under the same condition (Fig. 24). As a contrast, the PCA for host-associated reads showed that within each strain the gene expression profile was generally similar among samples, except for samples of *CS* feeding on artificial diet (Fig. 24). On the other hand, the overall host gene expression of *S. frugiperda* gut was remarkably distinct between strains (Fig. 25). In addition, hierarchical clustering analysis shows that both *CS* and *RS* strains fed cotton have a very distinct gene expression profile when compared to all other samples. Within the branch containing all other samples, we observe that samples from *RS* larva fed artificial diet tend to cluster more closely than larva fed grasses such as maize and rice; the same pattern is found in *CS* larva, even though these samples did not cluster together with *RS* larva fed the same diets (Fig.25)

**CoRS *vs* CoCS**

No transcripts were differentially expressed between the host-adapted strains of *S. frugiperda* when feeding on maize leaves. (Fig.26). On the other hand, a total of total of 1432 contigs were differentially expressed in the midgut of *RS* and *CS* on maize, of which 870 were down-regulated and 562 were up-regulated in the gut of *RS* when compared to *CS*. (Fig 27).

**RiRS *vs* RiCS**

Seventeen transcripts were differentially expressed between the gut microbiota of CS and RS larvae of *S. frugiperda* fed on rice leaves. Only one transcript of the gut microbiota of *RS* assigned to *Ralstonia solanacearum* was down-regulated in *RS* when compared to *CS*. The remaining 16 transcripts were up-regulated in the microbiota of RS larvae. One was assigned to *Ralstonia solanacearum*, two to *Staphylococcus aureus* and 13 to *Enterococcus mundtii*. All of the annotated transcripts were assigned to *Enterococcus mundtii* and were putatively identified as DNA-directed RNA polymerase subunit alpha, elongation factor G, 50S ribosomal protein L2, uncharacterized ABC transporter ATP-binding protein, accessory gene regulator protein A, formate acetyltransferase, intracellular maltogenic amylase, arginine deiminase, and aldehyde-alcohol dehydrogenase 2 (Fig.26).

On the host *S. frugiperda* midgut, a total of 1672 transcripts were differentially expressed (DE) in the gut of RS fed on rice leaves when compared to CS larvae. Most DE genes (1057) were down-regulated, but 615 were up-regulated. (Fig 27).

**CtRS *vs* CtCS**

Four genes were differentially expressed by the gut microbiota of the host-adapted strains of *S. frugiperda* fed on cotton. All of them were down-regulated in gut microbiota of *RS* larvae. Two transcripts were assigned to *Staphylococcus aureus* and two to *Enterococcus casseliflavus*. No annotation was obtained for these transcripts (Fig.26).

On the host midgut, cotton leaf was the diet that had the lowest number of differentially expressed genes between the *S. frugiperda* strains a total of 1115 genes. Moreover, this diet also provided an inverted pattern in the differential expressed gene between the strains comparing to the other diets: *RS* had a lower number of downregulated genes (493), and a higher number of up regulated genes (622), than the *CS* at the same condition(Fig 27).

**DiRS *vs* DiCS**

The artificial diet has the Three hundred and sixty-five (365) genes were differentially expressed by the gut microbiota of *S. frugiperda* strains when fed artificial diet. Five unannotated transcripts were down-regulated in the microbiota of *RS,* larvae, and were assigned to *Staphylococcus aureus* (3) and *Ralstonia solanacearum* (2), while 360 were up-regulated. All of the up-regulated transcripts were assigned to *Enterococcus mundtii,* except for one assigned to *Lactobacillus plantarum* and another to *Enterococcus faecium* (Fig.26).

The artificial diet was the food substrate that produced the highest number of DE transcripts on the midgut host when *RS* was compared to *CS*. We detected 1720 DE transcripts; 1016 were down-regulated and 704 were up-regulated in *RS* when compared to *CS*. (Fig 27).

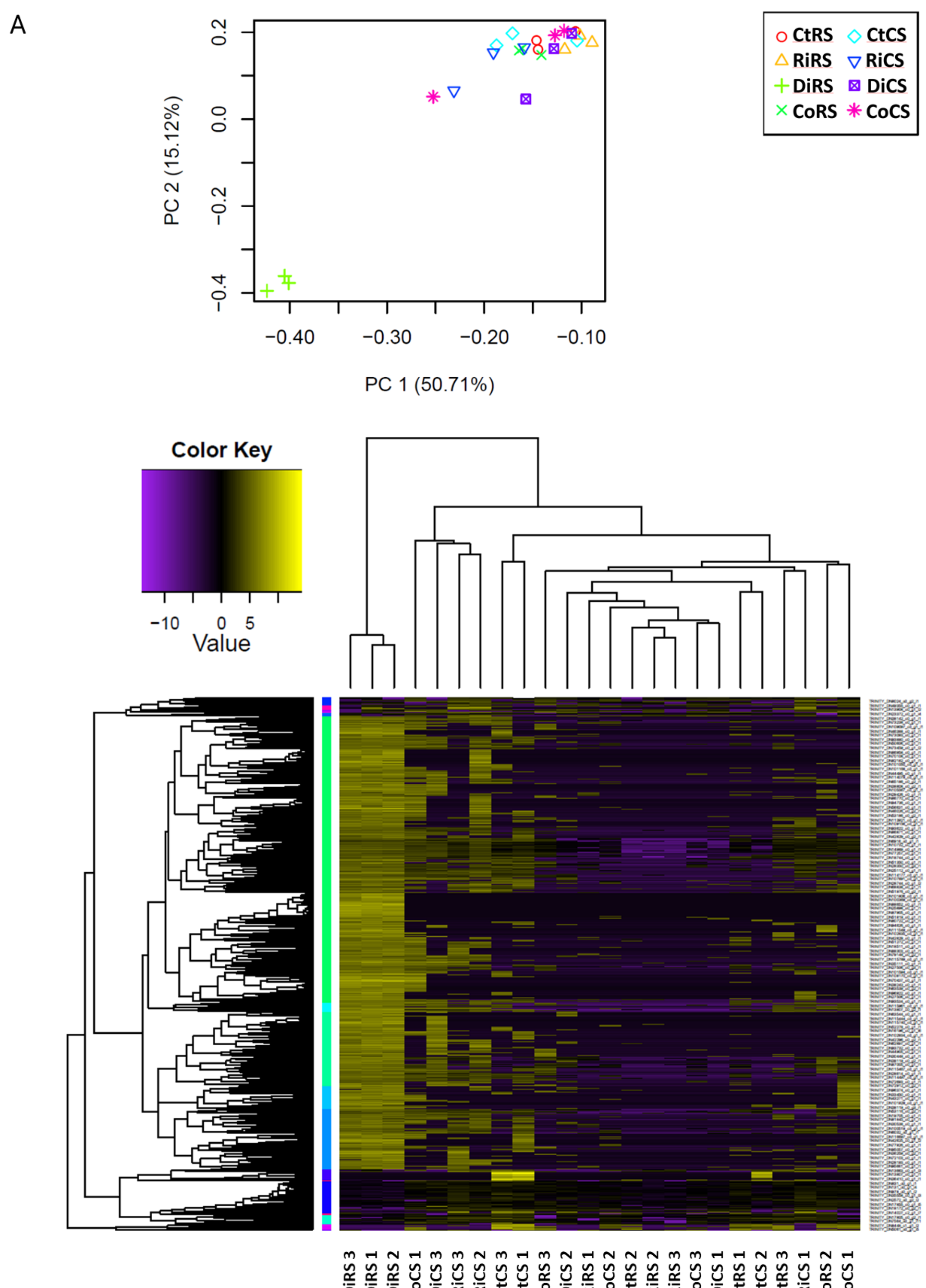


**Figure 24.** Principal components analysis (PCA) and hierarchical heat map cluster of all the genes analysed for DEseq2. (A) Principal Components Analysis of the DE from the transcripts of the gut microbiota *of Spodoptera frugiperda* corn (CR) and rice strains (RS) when fed on artificial diet (Di), cotton (Ct), rice (Ri) and Corn (Co). In brackets is indicated the percentage of variation explained by each of the components. (B) Hierarchical heat map cluster showing individual DE values for the treatments. Rows are standardized to have a mean of 0 and standard deviation of 1; purple indicates high and yellow indicates low DE values. Each rectangle represents individual DE values.

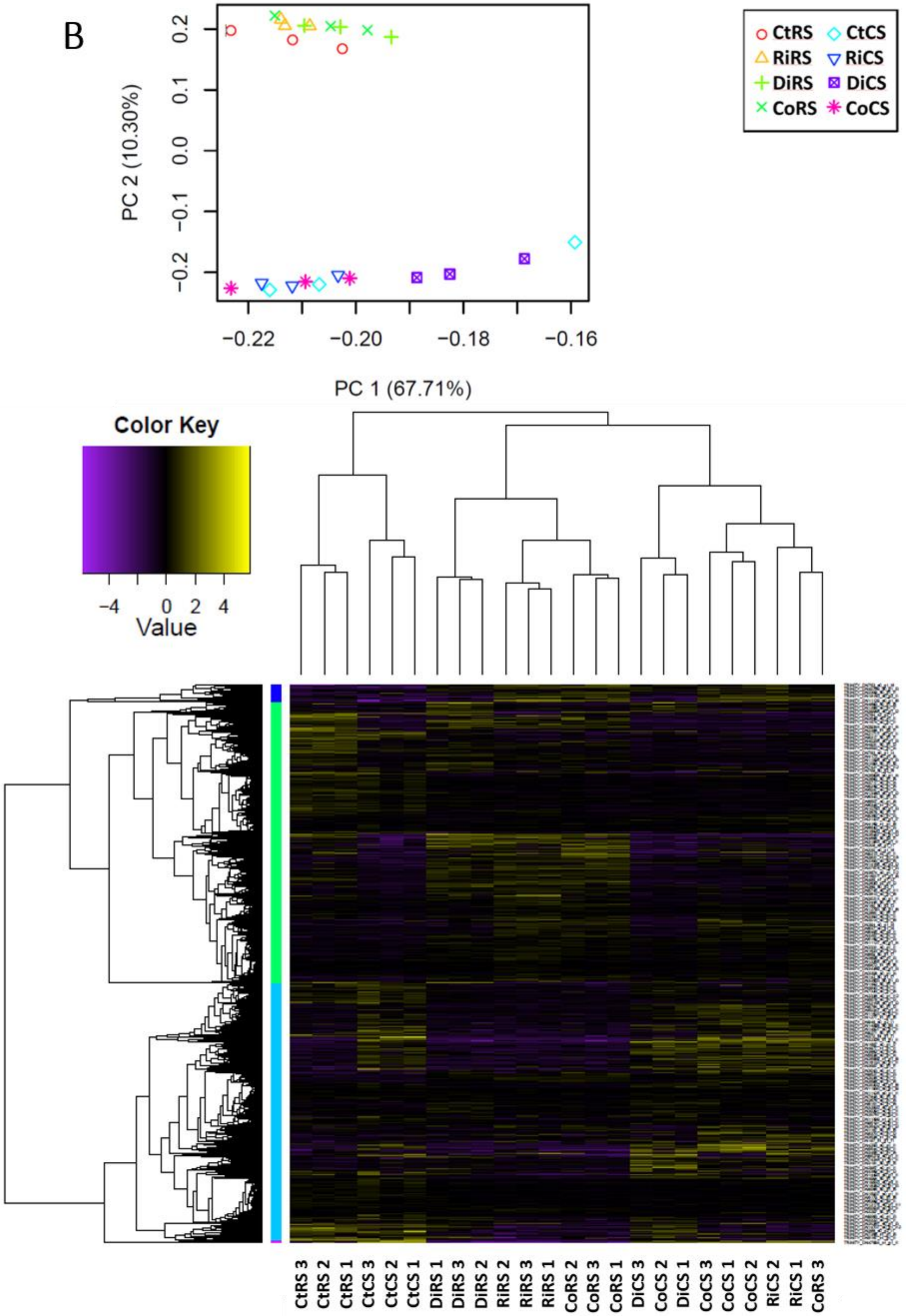


**Figure 25.** Principal components analysis (PCA) and hierarchical heat map cluster of all the genes analysed for DEseq2. (A) Principal Components Analysis of the DE from the transcripts of the *of Spodoptera frugiperda* corn (CR) and rice strains (RS) gut when fed on artificial diet (Di), cotton (Ct), rice (Ri) and Corn (Co). In brackets is indicated the percentage of variation explained by each of the components. (B) Hierarchical heat map cluster showing individual DE values for the treatments. Rows are standardized to have a mean of 0 and standard deviation of 1; purple indicates high and yellow indicates low DE values. Each rectangle represents individual DE values.

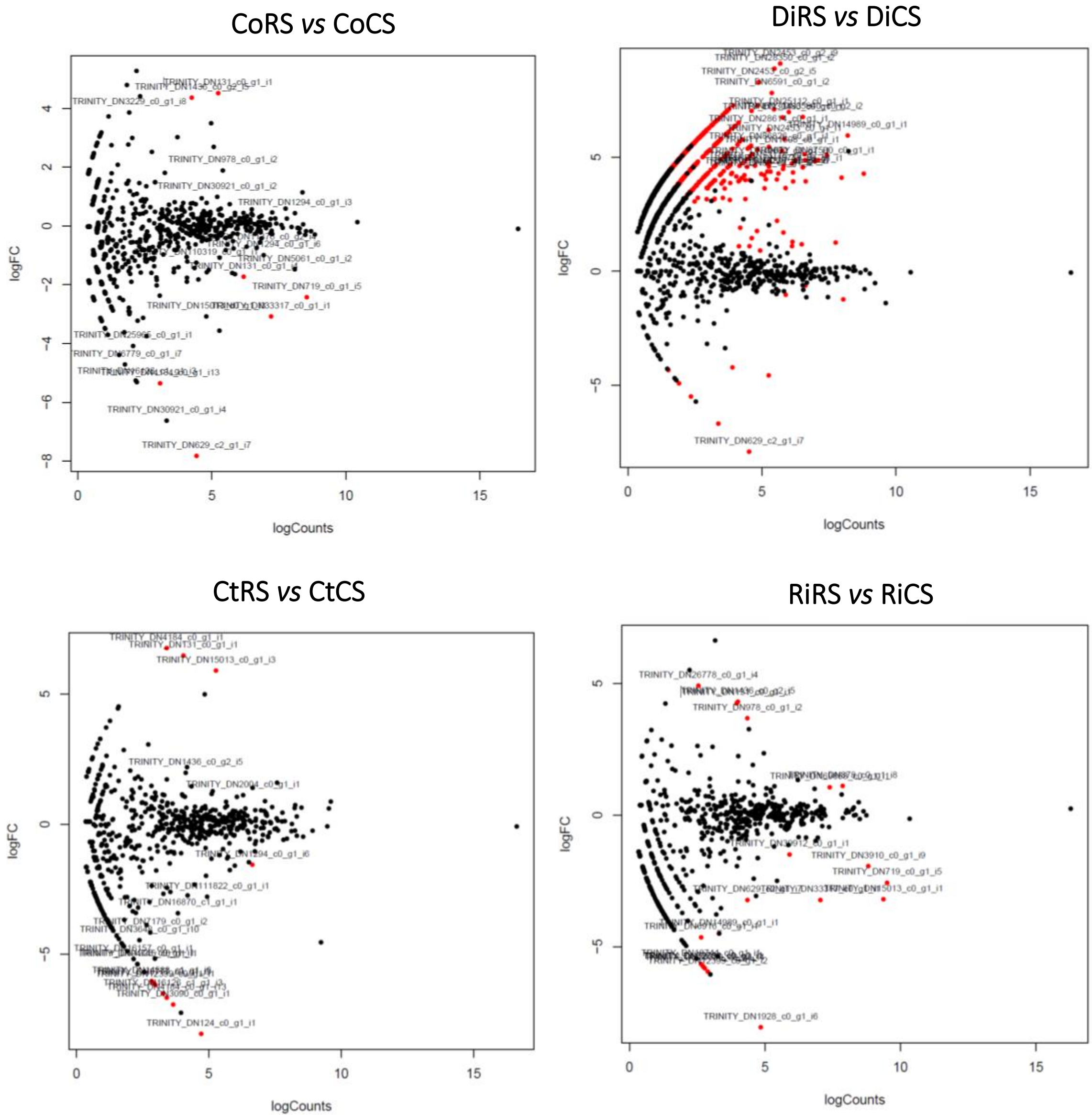


**Figure 26.** MA plot represents the differential expressed transcripts of the *of* gut microbiota *of Spodoptera frugiperda* corn (CR) and rice strains (RS) gut when fed on artificial diet (Di), cotton (Ct), rice (Ri) and Corn (Co). The red dot showed differentially expressed genes and the black dots were differentially not expressed genes

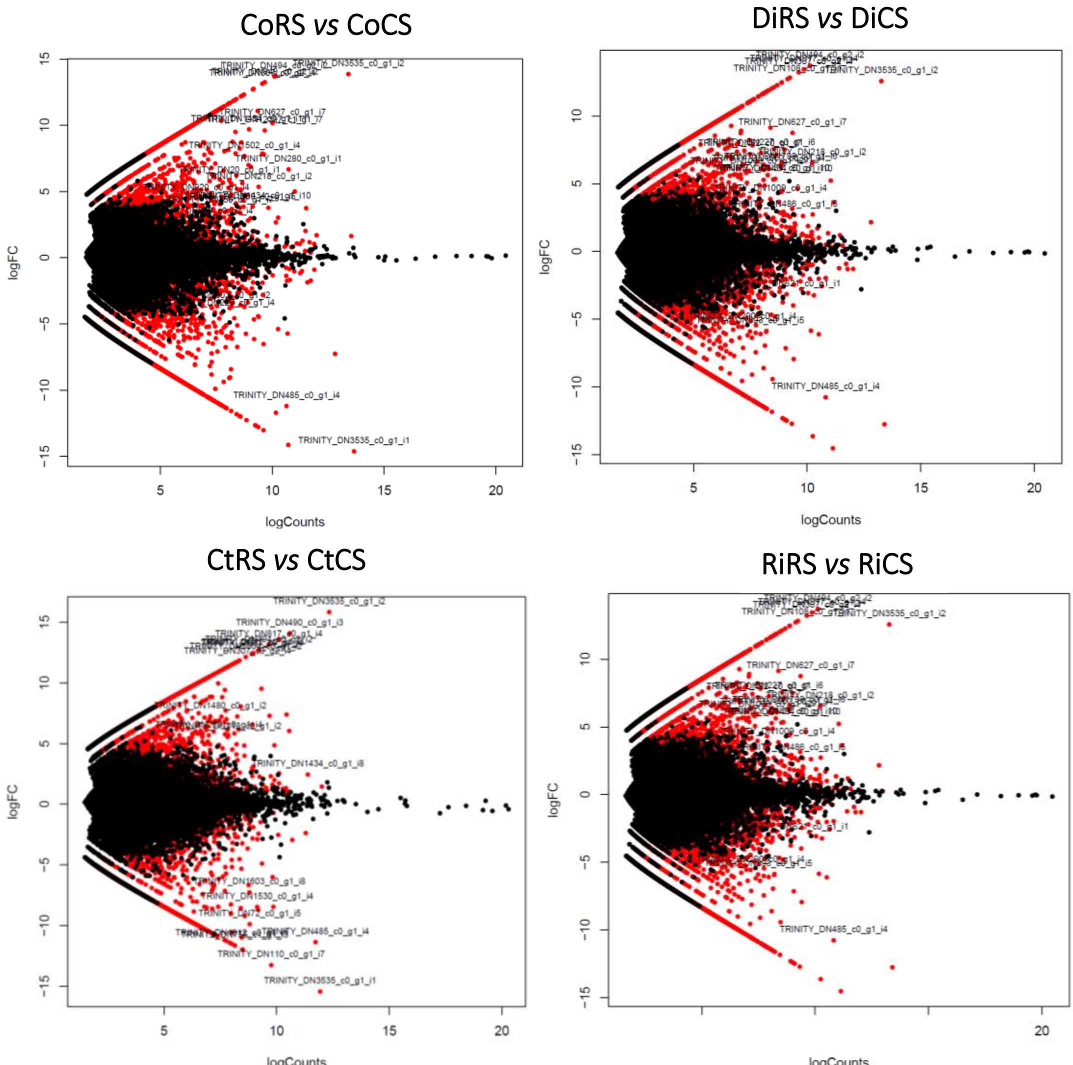


**Figure 27.** MA plot represents the differential expressed transcripts of the *of Spodoptera frugiperda* corn (CR) and rice strains (RS) gut microbiota when fed on artificial diet (Di), cotton (Ct), rice (Ri) and Corn (Co). The red dot showed differentially expressed genes and the black dots were differentially not expressed genes

## 5.4 DISCUSSION

*S. frugiperda* strains respond differently when feeding on the same diet by expressing distinct sets of genes and some of them can be associated to a better adaptation to the host plant, such as genes involved in digestion, detoxification and immunity processes. These differences are also consistent with the results using the whole caterpillar body of *S. frugiperda* strains, supporting the assertion that they respond differently to the same diet (Orsucci et al. 2020, Silva-Brandão et al. 2017).On the other hand the low number of differentially expressed genes among the microbiota of the strains may be explain by the similarity in the composition of the strains gut microbiota demonstrated previously (chapter 1,2 and 3). This suggests that the microbiota performs basically the same functions in the host strains of *S. frugiperda*. In general, we found that among the functions attributed to the microbiota, we found functions related to nutrition, such as carbohydrate metabolism, amino acids and energy supply, which could provide the nutritional complementation of the host in the different diets.

However, we still found differentially expressed genes in the gut microbiota of *S. frugiperda* host strains, especially when the caterpillars fed on artificial diet. And when we examined to which taxonomic groups these genes

were assigned, we noted that most of them were assigned to *Enterococcus mundtii*. *Enterococcus* has been described as an important member of the microbiota found in the genus *Spodoptera*(Paniagua Voirol et al. 2018). *E. mundtii* was also consistently found in the microbiota of field and laboratory *S. frugiperda* caterpillars in previous studies (chapter 1,2 and 3, (Gichuhi et al. 2020, Jones et al. 2019, Mason, Hoover and Felton 2021) It has also been demonstrated to colonize and proliferate in the gut of *S. litoralis* using strategies such as upregulated pathways for tolerating alkaline stress, forming biofilms and two-component signaling systems, resisting oxidative stress and quorum sensing(Mazumdar et al. 2021). Furthermore there are strong indications of vertical transmission of *E. munditi* in *S. litorallis* (Chen et al. 2016, Teh et al. 2016, Johnston and Rolff 2015). All together indicate that *E. munditi* may be a symbiont closely related to *S. frugiperda*.and since their strains have only recently diverged, it is possible that *E. munditi* found in each host strain may also represent different lineages of the same species.

The large number of genes differentially expressed by the gut microbiota when the caterpillar fed on the artificial diet suggests that this food substrate affects the function of the microbiota differently among strains. Most of the genes upregulated in RS were related to pathways associated with colonization and pathogenicity of *E. mundtii.* Here, the gene ontology term used for "pathogenesis" (GO:0009405), is defined as part of the initiation of a pathogenic interaction or a more neutral interaction, depending on the context. On the other hand, the gut half of the host RS, exhibited a dysregulation of biological process such immune responses and microorganism control when compared to the CS. Therefore, the host-microbiota interactions is context dependent, and will affect their host gene expression depending on the host strain and the type of diet being consumed. In this case, the artificial diet appears to favor a process of proliferation (pathogenicity) in the RS gut, which also involves downregulation of host defenses. The cause of this phenomenon, as well as whether this process is harmful or beneficial to the host, are questions that deserve further investigation in future studies.

In conclusion, the gut microbiota of *S. frugiperda* is metabolically active and its functioning is similar between rice and corn strains when feeding on the same food, except when larvae fed on artificial diet, where the rice strain showed a large number of upregulated genes when compared to the corn strain. On the other hand, the gut transcriptome of the host *S. frugiperda* was shown to be quite distinct between the strains when feeding on the same diet. Further studied are needed to explore more in details the complex interplay between the host and its microbiota functioning and our analysis provides a suitable framework for the development of further experiments.

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# 6. NON-TARGETED METABOLICS REVEAL DIFFERENCES IN THE METABOLIC PROFILE OF THE FALL ARMYWORM STRAINS WHEN FEEDING DIFFERENT FOOD SOURCES


## ABSTRACT

The moth *Spodoptera frugiperda,* the fall armyworm (FAW), is an important polyphagous agricultural pest feeding on nearly 350 host plants. *Spodoptera frugiperda* is undergoing incipient speciation with two well-characterized host-adapted strains, the "corn" (*CS*) and "rice" (*RS*) strains, which are morphologically identical but carry several genes under positive selection for host adaptation. We used a non-target metabolomic approach based on gas chromatography/mass spectrometry to identify differences in metabolite profiles of the larval gut of *CS* and *RS* feeding on different host plants. Larvae were fed on artificial diet, maize, rice, or cotton leaves from eclosion to the sixth-instar, when they had their midgut dissected for analysis. This study revealed that the midgut metabolomic profile of FAW differs on the each host plant depending on the host-adapted strain. Additionally, we identified several candidate metabolites that may be involved in the adaptation of *CS* and *RS* strains to their host plants. Our results shed light on our understanding of gut metabolic activities of the FAW, which associates the metabolomics of FAW with the metabolomics of the associated microbiota.


KEY WORDS: Metabolite, herbivore, gut microbiota, insect-plant interaction, nutritional ecology.

## 6.1 INTRODUCTION

*Spodoptera frugiperda,* the fall armyworm (FAW) (Lepidoptera, Noctuidae) has a remarkable number of host plants, feeding on approximately 350 different vegetal species from 76 families (Montezano et al., 2018). Despite this wide range of host plants, FAW is best known as one of the most important agricultural pests of grasses (maize, millet, rice and sorghum) and some cultivated dicots such as cotton (Barros, Torres, Ruberson, & Oliveira, 2010). The FAW is native from the New World, but in the last few years has invaded Africa and further spread to Asia and Oceania (Goergen, Kumar, Sankung, Togola, & Tamò, 2016; S. J. Johnson, 1987; Otim et al., 2018; Padhee & Prasanna, 2019; Piggott, Tadle, Patel, Gomez, & Thistleton, 2021). Therefore, FAW is currently considered of a global concern due its polyphagy and capacity for rapid evolution of resistance to pesticides and Bt-crops(F. Huang, 2020; Jakka et al., 2016), representing an imminent threat to food security and a source of significant economic losses.

The FAW is the only species of *Spodoptera* that usually feeds on grasses without having adapted, suitable mandibles. Larvae that feed on grasses typically have specialized mandibles with chisel-like edges adapted to the consumption of silica rich leaves, which causes adverse effects to larval mandibles (Brown & Dewhurst, 1975; Djamin & Pathak, 1967; Pogue, 2002; Smith, 2005). Mandibles of the *FAW* have serrate-like processes adapted to the consumption of dicots or monocots that do not accumulate silica (Pogue, 2002). FAW is primitively a polyphagous, but because of the mandible-type it is thought to have started exploiting cultivated grasses as host plants only recently (Kergoat et al., 2021; Kergoat et al., 2012).

Another interesting aspect of the FAW is the identification of two distinct strains known as the rice (*RS*) and corn (CS) strains (Gouin et al., 2017; Pashley, 1986). There are indications that this divergence occurred about 2 Myr ago (Kergoat et al., 2021; Kergoat et al., 2012). These strains differ in their performance and preference for host plants, and the correct classification of these two strains of the FAW is still controversial. Some authors refer to them as “sibling species” (Drès & Mallet, 2002; Dumas et al., 2015), “host strains” (Pashley, 1986; Prowell,

McMichael, & Silvain, 2004), and "host form" (Juárez et al., 2014). The lack of consensus is due to the fact these strains co-exist in sympatry and still hybridize, but also due to inconsistencies in the associations with the named host plants. At the adult stage, both corn and rice strains showed weak evidence of preference for their expected host-plant in choice and non-choice laboratory experiments (Robert L. Meagher, Nagoshi, & Stuhl, 2011; Orsucci et al., 2020). Despite the fact that the corn strain is often associated with maize, sorghum and cotton, while the rice strain with rice and pasture grasses, some reports show the rice strain larvae developed better on corn and sorghum than the corn strain larvae (R. L. Meagher, Nagoshi, Stuhl, & Mitchell, 2004). Moreover, both strains poorly performed when feeding on rice (Silva-Brandão et al., 2017). Therefore, further studies are still needed to understand how the process of host plant adaptation is taking place in FAW.

Every novel acquisition of host plants by herbivores constitutes a new niche adaptation program that opens several evolutionary possibilities, but not without associated costs. In order to exploit a novel host, insects have to become adapted to deal with new defensive secondary metabolites, such as phenolics and terpenoids, and the nutritional quality of the new host plant (Singer, 2008). However, the mechanisms behind the best performance of a given host-adapted strain on a given plant are poorly understood so far. Different approaches can be used to address this question. One alternative is to access the insect metabolome, the set of all low molecular weight metabolites that are produced during cell metabolism (Sun & Hu, 2016). Ultimately, the metabolome is a product of genomic, transcriptomic, and/or proteomic perturbations (C. H. Johnson & Gonzalez, 2012). Among the approaches that can be used, non-targeted metabolomics provide a holistic view of the insect's metabolic profile. It makes no assumptions about which metabolites are important in distinguishing sample types (Sévin, Kuehne, Zamboni, & Sauer, 2015). This approach provides a direct functional measurement of cellular activity and physiological state, reflecting environmental changes such as new host plants as well as aspects related to their genome, as different host-adapted strains (Sun & Hu, 2016). Therefore, the non-targeted study of metabolomes is a good tool to point chemical candidates involved in insect-plant interactions (Maag, Erb, & Glauser, 2015). Particularly, the assessment of the insect midgut may be useful, bearing in mind that it is a permeable, metabolically active tissue, in which most digestion and almost all nutrient absorption takes place (Dow, 1987). However, approaches focused on the assessment of the gut metabolomics of insect herbivores are not common, and little is known on how host plants interfere with the profile of primary metabolites of the gut of herbivores.

The gut microbiota is also a key player in the metabolic processes of their hosts. Gut microbes can play important roles in several metabolic functions, including vitamin production(Chen et al., 2016; Salem et al., 2014), amino acid synthesis(Ayayee et al., 2016; X. Xia et al., 2017), and detoxification of secondary plant compounds and synthetic insecticides (Almeida, Moraes, Trigo, Omoto, & Cônsoli, 2017; Ceja-Navarro et al., 2015), for example. Among the numerous factors that influence the gut microbiota(Dillon & Dillon, 2004; Yun et al., 2014), diet has received considerable attention due to its strong effect on the composition of the microbial community(Mason et al., 2020; Wongsiri & Randolph, 1962; Yun et al., 2014). Diet provides the substrates to produce a plethora of small molecules that can be converted by the gut microbiota and which are not produced by the host(Krishnan, Alden, & Lee, 2015; S. Wang et al., 2020). Therefore, the gut microbiota may also facilitate adaptation to new host plants by regulating or participating in the host's metabolic processes(Hammer & Bowers, 2015; Zhang et al., 2020). Microbial contribution will depend on substrate availability and on microbial gene diversity and activity(Wu et al., 2016). Thus, taxonomic or metagenomic information of the gut microbiota is limited in predicting the metabolome of a microbial community, as it may under or overestimate the functional contribution of associated gut microbiota depending on the nutritional conditions the host is exposed to (Wu et al., 2016).

The FAW is a good model to study adaptation of phytophagous insects to agricultural plants. Moreover, the metabolic processes underlying host shifts or differentiation in this species are not well understood. In terms of metabolome, we would expect different metabolic profiles to reflect new adaptations. This knowledge can help in understanding the processes lepidopteran larvae employ to face challenges as dietary nutritional deficiencies and host plant switches, considering the insect as a unit with their microbes associated. The aim of the present research is to investigate if the gut metabolome of FAW is determined by the diet and/or by host genotype. Highlighting the metabolic differences in the midgut of the FAW strains has the potential to indicate: 1) how different host plants affect insect nutritional metabolism and 2) how larvae of the two host strains differ in their utilization of diverse host plant nutrients.

## 6.2 MATERIAL AND METHODS

### 6.2.1 Insect rearing and strains identification

Colonies of FAW were initiated in the laboratory from field-collected populations. The *RS* was originally obtained from rice fields in Santa Maria, RS, Brazil (29°68'68"S, 53°81'49"W) and the *CS* from a maize field in Piracicaba, SP, Brazil (22°43'30"S, 47°38'56"O). Field-collected larvae were individualized into plastic cups containing an artificial diet based on wheat germ, beans and brewer's yeast (Burton & Perkins, 1972; Kasten Jr, Precetti, & Parra, 1978), brought to the laboratory and reared under controlled conditions (25 ± 1°C; 70 ± 10% RH; 14 h photophase) until pupation. Pupae were transferred to clean plastic cups lined with filter paper for adult emergence. The produced exuviae were used for DNA extraction for strain identification as described below. After strain identification, all newly emerged adults belonging to the same strain were transferred to PVC tubes lined with paper as a substrate for egg laying. Egg masses were collected and transferred to artificial diet for later larval development.

Strain identification followed Levy et al. (2002) (Levy, Garcia-Maruniak, & Maruniak, 2002). Briefly, DNA was extracted from individual pupal exuviae using the genomic DNA preparation protocol from RNAlater™ preserved tissues with some modifications. The exuviae were individually placed in 750 µL digestion buffer (60 mM Tris pH 8.0, 100 mM EDTA, 0.5% SDS) containing proteinase K at a final concentration of 500 µg/mL. Samples were macerated using plastic pestles and mixed well by inversion. Samples were incubated overnight at 55°C. Afterwards, 750 µL of phenol: chloroform (1:1) was added and samples were rapidly inverted for 2 min before centrifugation at a tabletop centrifuge at maximum speed (10 min). The aqueous layer was recovered and subjected to re-extraction twice before a final extraction with chloroform. The aqueous layer was collected and added to 0.1 volume of 3 M sodium acetate (pH 5.2) and an equal volume of 95% ethanol. Samples were then mixed by inversion, incubated for 40 min at -80°C, and centrifuged (27,238 g x 30 min x 4°C). The pellet obtained was washed twice with 1 mL of 85% ice-cold ethanol, centrifuged for 10 min after each wash and dried at 60°C during 5-10 min in a SpeedVac. Finally, the pellet obtained was resuspended in nuclease-free water. DNA concentration and quality were estimated by spectrophotometry and agarose gel electrophoresis (Sambrook, 2001).

Polymerase chain reaction (PCR) amplification of the mitochondrial COI gene was conducted using the primers set JM76 (5'-GAGCTGAATTAGGRACTCCAGG-3') and JM77 (5'-ATCACCTCCWCCTGCAGGATC-3') to produce an amplicon of 569 base pairs (bp)(Levy et al., 2002). The PCR mixture contained 100-150 ng of gDNA, 1.5 mM of $MgCl_2$, 1 x PCR buffer, 0.2 mM of each dNTP, 0.32 µM of each primer and 0.5U of GoTaq® DNA

Polymerase (Promega) in a total volume of 25 µL. The thermocycling conditions were one cycle at 94°C x 1 min followed by 33 cycles at 92°C x 45 s, 56°C x 45 s, 72°C x 1 min, with a final extension at 72°C x 3 min (1x). Amplicons were then subjected to endonuclease restriction analysis using *Msp*I (HpaII) to produce two fragments (497pb and 72pb) for amplicons of the *CS*, while no digestion is observed for *RS* amplicons. After the amplification, 10 µL of the PCR reaction mixture was subjected to digestion with 10 U of *Msp*I following the manufacture guidelines (product number ER0541®, Thermo Scientific). Samples were gently mixed, centrifuged for a few seconds and incubated overnight at 37°C. Subsequently, digestion efficiency and the resulting products were verified using a 1.5% agarose gel electrophoresis.

### 6.2.2 Essay with natural diet and gut dissection

Maize (*Zea mays,* family: Poaceae) var. "Conventional impact" and cotton (*Gossypium hirsutum,* family: Malvaceae) var. IAC FC2 seeds were seeded in 500 mL plastic pots filled with soil conditioner, while rice seeds (*Oriza sativa,* family: Poaceae) var. BRS Esmeralda were seeded in 1 L plastic pots. All plants were maintained in a greenhouse.

The leaves were cut and immersed in a container with distilled water for 30 minutes to maintain turgidity. Newly emerged larvae of *RS* and *CS* strains were placed in 25 mL plastic cups containing a 3-cm piece of the host plant leaf (cotton and maize - leaves from v3-v4 stages; rice - v11-13). The leaves were replaced with fresh ones according to the needs of each instar, and were replaced every other day or earlier to avoid food shortages. Insects were kept under controlled laboratory conditions throughout the experiments (25 ± 1°C; 60 ± 10%; 14-hour photophase).

The experimental groups were represented by larvae of rice (RS) and corn (CS) strains reared on the following substrates: rice (RiRS; RiCS), corn (CoRS; CoCS), cotton (CtRS; CtCS), and artificial diet (DiRS; DiCS). We used five replicates for each treatment, with each replicate corresponding to a pool of midgut collected from five larvae.

The gut was collected from sixth-instars after larvae were surface-sterilized in cooled 0.2% sodium hypochlorite in 70% ethanol and washed in cold sterile water. Surface-sterilized larvae were dissected in sterile water under asseptic conditions. Tissues were flash-frozen in liquid nitrogen and stored at -80 until metabolite extraction.

### 6.2.3 Metabolite extraction

Samples were subjected to metabolite extraction and analysis according to Hoffman et al. (2010), with some modifications. A pool of midguts from five larvae were macerated in liquid nitrogen, and 25 mg of the macerate was homogenized in TissueLyser II (QIAGEN ) at the highest speed for 1 min using 5 mm tungsten beads in 500 µL of methanol-chloroform-water solution (3:1:1). Then the sample was sonicated (60 $Hz.s^{-1}$ x 30 min) in an ice bath (4ºC) and centrifuged (16,000 g x 10 min x 4ºC). The supernatant was collected and filtered through a luer-lock 0.22 µM filter (Millex®, JBR6 103 03) directly into amber glass vials.

Aliquots (50 µL) of each sample were freeze-dried in a Terrone model LS 3000 lyophilizer, and subjected to derivatization with 30 µL methoxyamine-HCl (20 $mg.mL^{-1}$) in pyridine for 16 h at room temperature. Trimethylsilylation was accomplished with the addition of 1% trimethylchlorosilane (TMCS) in 30 µL of *n*-methyl-*n*-

(trimethylsilyl)-trifluoroacetamide (MSTFA) to the samples, followed by incubation for 1 h at room temperature. After silylation, 30 µL of heptane was added to samples, which were immediately analyzed in a random order in a 7890A Agilent Gas Chromatograph coupled to a Pegasus HT TOF Mass Spectrometer (LECO, St. Joseph, MI, USA) (GC-TOF/MS) (Technologies). Samples were injected together with mix of *n*-alkanes standards ($C_{12}$ - $C_{40}$) for the correct calculation of the retention times. Derivatized samples (1 µL) were injected in splitless mode using an automatic sampler-CTC Combi Pal Xt Duo (CTC Analytics AG, Switzerland) coupled to the GC-MS system equipped with two silica columns in line. The first was a DB 5 column (20 m long x 0.18 mm internal diameter x 0.18 µm thick) (Agilent J & W Scientific, Folsom, CA, USA), and the second a RXT-17 column (0.84 m long x 0.1 mm internal diameter x 0.1 µm thick) (ResteK Corporation, Bellefonte, PA, USA). The injector was set at 280°C, the septum bleed rate was 20 $mL.min^{-1}$ and began after 250s from the start of data acquisition (Budzinski, de Moraes, Cataldi, Franceschini, & Labate, 2019). The gas flow was 1 $mL.min^{-1}$. The temperature of the first column was maintained at 80°C for 2 min and increased at 15°$C.min^{-1}$ to 305°C, with a 10 min hold. The temperature of the second column was maintained at 85°C for 2 min, and then raised to 310°C at 15°$C.min^{-1}$, with a 10 min hold. The column effluent was introduced into the ionization source of the Pegasus HT TOF MS. The transfer line and ionization source temperatures were held at 280 and 250°C, respectively. The ions were generated by an electron source (70-eV) at an ionization current of 2.0 mA, and 20 $spectros.s^{-1}$ were acquired in a mass range of 45-800 $m/z$, with the detector voltage set to 1500 V.

### 6.2.4 GC-TOF/MS data processing

The processing of GC-TOF/MS data was performed in two steps. Initially the generated chromatograms were exported to the ChromaTOF program, version 4.32 Software (LECO, St. Joseph, MI, USA), in which base line correction, deconvolution of the spectra, retention rate correction (RI), retention time correction (RT), peak identification, and alignment and identification of metabolites were processed using the NIST library, version 11. Only metabolites with three or more characteristic masses and a score of 700 or higher were considered valid. Isomers were manually checked and merged metabolite intensities were normalized using total ion chromatogram (TIC).

MetaboAnalyst 5.0 was used to perform all the downstream analyses (Chong, Wishart, & Xia, 2019). Data was *log* transformed and scaled using Pareto. Hierarchical clustering was also performed with the *hclust* function in package stat (R v3.5.1) using Ward as a clustering algorithm and Euclidean distances as measures. Sample clustering was presented as a dendrogram. Type 1 two-way ANOVA was used to examine the effects of strain and diet, and their interaction on metabolite abundance. False discovery rate was applied to adjust the *p*-values (0.05). Heatmap was built based only on the significant features from ANOVA. The distance measure used was the Euclidean, and Ward was used for clustering algorithm (J. Xia, Sinelnikov, & Wishart, 2011). In order to identify the features that were potentially significant in discriminating the strains on each host plant, paired analysis was performed using volcano plot, which combine fold change ($FC \geq |2.0|$) and *t*-test analysis, that can control the false discovery rate ($p \leq 0.05$).

## 6.3 RESULTS

The host plants FAW larvae feed interfered with the midgut metabolome. The midgut metabolomes of RS and CS larvae also differed when feeding on the same diet. However, the food source had a greater impact shaping the gut metabolome of FAW than the host strain. Metabolomic analyses led to the identification of two major clusters of metabolites, allowing the clear separation of larvae fed on artificial diet when compared to those fed on natural diets (Fig. 28). The metabolomic profile obtained clearly separated FAW larvae fed on monocots (corn and rice) from those feds on the dicot cotton. Additionally, the profile of metabolites obtained for each FAW race on each food source also led to their clear separation (Fig.28).

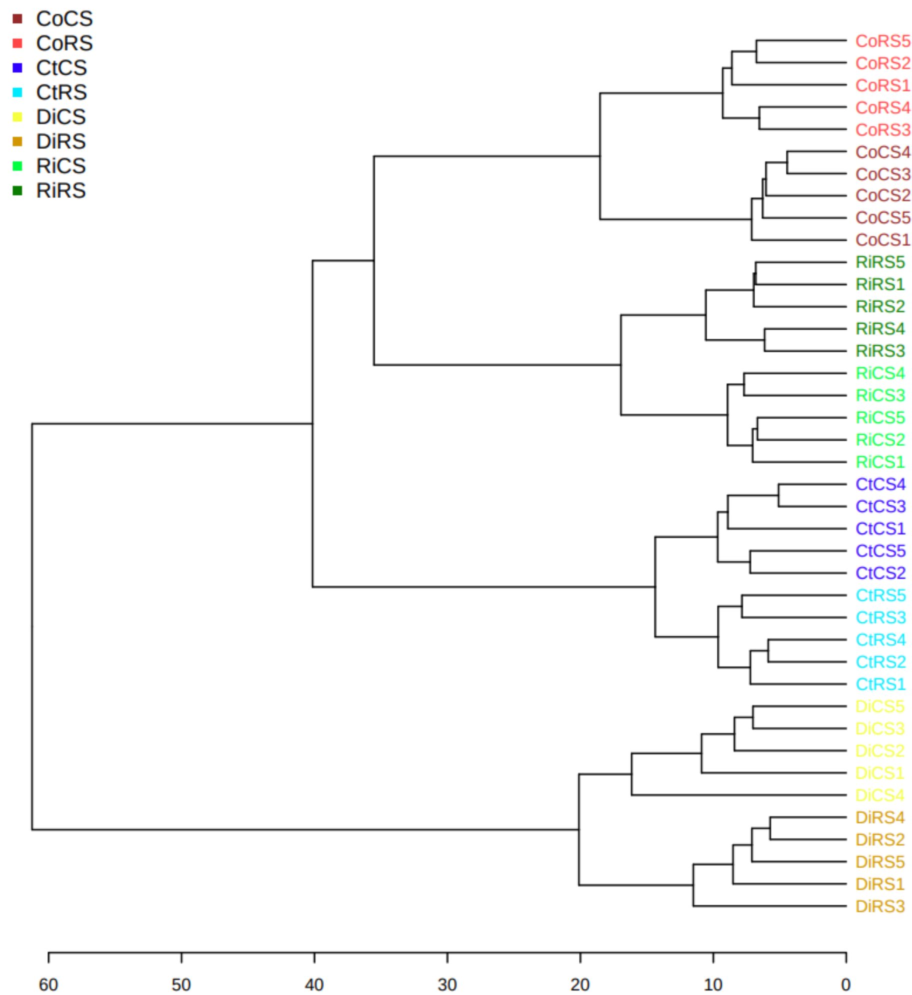


**Figure 28.** Clustering result shown as dendrogram with distance measure using Euclidean and clustering algorithm using ward.D of the midgut metabolite profiles of *S. frugiperda* larvae. The experimental groups were rice strain on rice (RiRS), corn (CoRS), cotton (CtRS) and artificial diet (DiRS) and corn strain on the same diets (RiCS, CoCS, CtCS and DiCS).

Among the 340 peaks identified in the gut samples of FAW larvae, 122 metabolites passed the filter criteria for analysis, with the abundance of 107 them being affected by the diet, 13 by the host-adapted race 13, and 50 by the interaction of both factors (Anexo E). The abundance of 12 metabolites was simultaneously affected by

diet, race, and their interactions (Fig.29). The compounds were predominantly classified as amino acids, sugars, fatty acids, and organic acids.

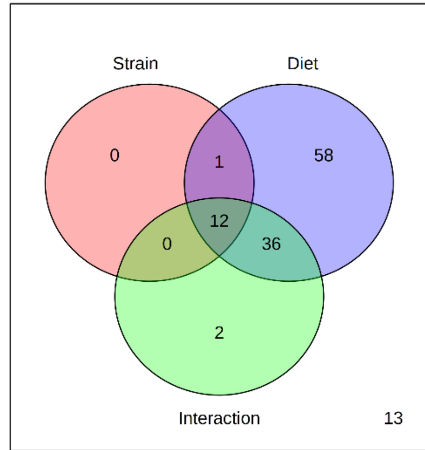


**Figure 29.** Venn diagram showing the important features of the midgut metabolome of *S. frugiperda* larvae selected by two-way ANOVA whose levels were affected by strain, diet or interaction of strain and diet.

Overall, there is a clear association of groups of metabolites with specific food resources. Furthermore, there is a clear distinction in the abundance of several metabolites between *CS* and *RS* samples, demonstrating a clear distinction in the metabolic profiles of each FAW race (Fig. 30). The first large cluster of metabolites is highly abundant in the gut of larvae fed on artificial diet. The second cluster presents metabolites abundant in the gut of larvae fed on all food sources, but not in the gut of those fed on corn. The third cluster characterizes the metabolic profile of *CS* on artificial diet. Next, the next clusters are followed by different metabolites abundant in the intestine of caterpillars that fed on plants. The fourth group is characterized by metabolites associated with the cotton plant, followed by the corn and rice groups. Finally, there is a cluster of metabolites common to all the natural diets (Fig. 30).

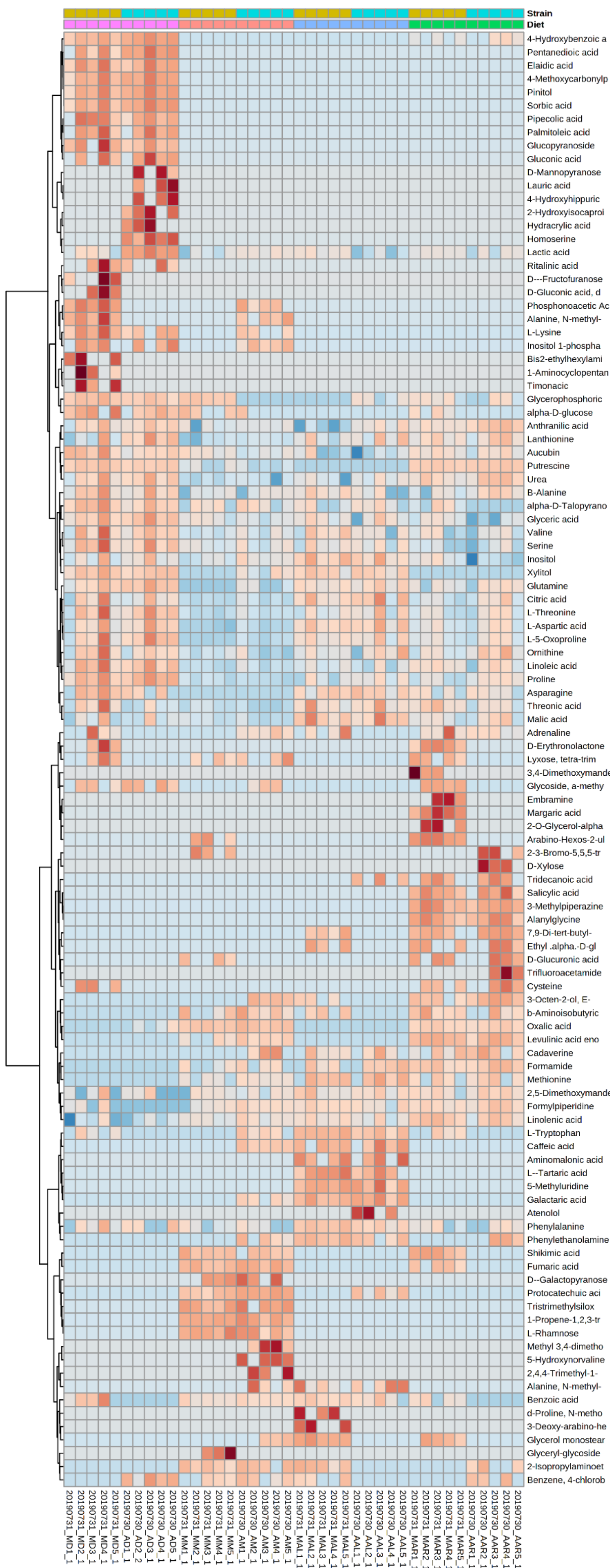


**Figure 30.** Heatmap of midgut metabolites from corn and rice strains of *Spodoptera frugiperda* larvae after feeding on artificial diet, corn, cotton, or rice. The experimental groups were rice strain on rice (RiRS), corn (CoRS), cotton (CtRS) and artificial diet (DiRS) and corn strain on the same diets (RiCS, CoCS, CtCS and DiCS).

Pairwise analyses of the gut metabolomes of *RS* and *CS* strains within each food source led to the identification of different sets of metabolites to differentiate the corn and rice strains (Table 5.2). *RS* feeding on corn had a higher abundance of caffeic acid, inositol 1-phosphate, adrenaline, protocatechuic acid and 5-hydroxynorvaline than the *CS* larvae, but the abundance of glycerophosphoric acid was higher in *CS* than in *RS* larvae (Tab. 5.2). In cotton, the abundance of glycerol monostearate and 2-isopropylaminoethanol was higher in *CS* than in *RS* larvae. In rice-fed larvae, the *CS* had higher levels of shikimic acid, 2-ketoglucose, D-erythronolactone and margaric acid, while the *RS* had higher levels of anthranilic acid. The midgut of larvae fed on artificial diet presented phosphonoacetic acid and alanine, N-methyl-N-ethoxycarbonyl-,dodecyl ester more abundant in *RS* , while homoserine lactic acid and 4-chlorobutyl-benzene were more abundant in the midgut of *CS* larvae (Table 6).

| | Metabolite | FC | log2(FC) | FDR | -log10(p) |
|---|---|---|---|---|---|
| Corn | Caffeic acid | 0.16099 | -2.635 | 1.59E-06 | 5.7988 |
| | Glycerophosphoric acid | 8.1756 | 3.0313 | 5.97E-05 | 4.224 |
| | Inositol 1-phosphate | 0.042813 | -4.5458 | 0.00238 | 2.6234 |
| | Adrenaline | 0.043827 | -4.512 | 0.027936 | 1.5538 |
| | Protocatechuic acid | 0.11284 | -3.1477 | 0.027936 | 1.5538 |
| Cotton | Glycerol monostearate | 6.349 | 2.6665 | 1.66E-05 | 4.7808 |
| | 2-Isopropylaminoethanol | 22.24 | 4.4751 | 0.00088159 | 3.0547 |
| Diet | Homoserine | 15.417 | 3.9465 | 0.0021244 | 2.6728 |
| | Lactic acid | 73.432 | 6.1983 | 0.011273 | 1.948 |
| | Phosphonoacetic Acid | 0.032468 | -4.9448 | 0.011273 | 1.948 |
| | Benzene, 4-chlorobutyl- | 680.08 | 9.4096 | 0.012464 | 1.9044 |
| | Alanine, N-methyl-N-ethoxycarbonyl-, dodecyl ester | 0.062969 | -3.9892 | 0.013014 | 1.8856 |
| Rice | Shikimic acid | 9.281 | 3.2143 | 0.00014586 | 3.8361 |
| | 2-ketoglucose | 8.6792 | 3.1176 | 0.00031354 | 3.5037 |
| | D-Erythronolactone | 23.033 | 4.5256 | 0.0023556 | 2.6279 |
| | Margaric acid | 20.9 | 4.3855 | 0.0023556 | 2.6279 |
| | Anthranilic acid | 0.28821 | -1.7948 | 0.025834 | 1.5878 |

**Table 6.** Significant features of midgut metabolome of *Spodoptera frugiperda* strains (*RS vs CS*) larvae after feeding on different food sources identified by Volcano plot with fold change threshold 2 and t-tests. False discovery rate correction was applied to adjust the p-values ($p \leq 0.05$).

## 6.4 DISCUSSION

The metabolic profile of the midgut of FAW larvae is largely influenced by the food source used, but the metabolome of the midgut of each strain is consistently different one from another in every single food source analyzed. Our data demonstrates the *RS* and *CS* interact differently with the substrate they are feeding on. Such interactions can be due to differential metabolization of nutrients they intake(Silva-Brandão et al., 2017). Molecular

differences at the genomic level are reported for these strains (Dumas et al., 2015), and particularly with the large variation they have in the number of copies of genes and gene sequences encoding for detoxification and digestive enzymes (Gouin et al., 2017). In addition, this is also consistent with the plethora of differences at the transcriptional level reported for the whole body of both strains when feeding on the same host plants. These strains were demonstrated to have differences in the expression levels of genes encoding for proteins with oxidoreductase activity, metal-ion binding, and hydrolase activity, which are also related to the metabolization of xenobiotics(Orsucci et al., 2020; Silva-Brandão et al., 2017).

The midgut metabolomes of FAW strains when feeding on different diets are not an exclusive product its own metabolism, but they are also composed of unmetabolized host plant components and compounds produced by the activity of the gut microbiota as well. It has been previously demonstrated that the gut microbiota plays an essential role in various aspects of host insect physiology (Dillon & Dillon, 2004; Engel & Moran, 2013). Particularly in aspects related to food adaptation, acting in the detoxification of xenobiotics, provision of nutrients and digestive enzymes. Furthermore, it is well know that the composition of the gut microbiota is also modulated by diet (Yun et al., 2014). Therefore, although we have not directly measured the effect of the microbial community on the gut metabolome, we consider the host and its microbiota as a unit, thus both work together to respond to environmental variations. Associations were found between *Plutella xylostella* gut metabolome, the host plant, and its gut microbiota composition. The metabolites correlated with gut bacteria belonged to the class of lipids and xenobiotics, indicating that bacteria may participate in detoxification metabolism and gut energy, possibly influencing the metabolism of the host insect to adapt to a new host plant(Yang et al., 2020).

Some compounds reported here in the midgut of *S. frugiperda* have a defensive function in plants against insects, such as shikimic acid. This compound has been shown to reduce intestinal proteolytic activity in insects by acidifying the intestinal lumen. However, some specialist insects such as *Gilpinia hercyniae* (Hymenoptera) are able to metabolize and neutralize the effect of this compound, through their gut bacteria (Schopf, 1986). Therefore, the higher abundance of shikimic acid in the *CS* larval midgut comparing to *RS* feeding on rice indicates the *CS* has a lower capacity to process this metabolite. Additionally, the difference in shikimic acid metabolization may also be due to the differential activity of the gut microbiota of the strains, similar to the adaptation found in *G. hercyniae*(Jensen, 1991). Likewise, margaric acid, or heptadecanoic acid, was shown to accumulate in *CS* when feeding on rice as well. This compound, also present in azalea (*Rhododendron sp.*), was negatively correlated with oviposition, eclosion, and nymphal survival of *Stephanitis pyrioides* (Hemiptera: Tingidae), but positively correlated with duration of development indicating an arrestment of the developmental period (Y. Wang, Braman, Robacker, Latimer, & Espelie, 1999). The same compound is also found in rice plants (Jones et al., 2011) so it is likely that margaric acid could help to explain the low performance and survival of *CS* on rice plants(Silva-Brandão et al., 2017) .

The similar pattern of response observed for strains feeding on corn, a host plant that resulted in a higher abundance of the corn defensive metabolites 5-hydroxynorvaline, caffeic acid and protocatechuic acid in *RS* larval midgut than in *CS*, raises a similar discussion. The accumulation of 5-hydroxynorvaline in maize leaves has been demonstrated after the feeding of *Spodoptera exigua* and the aphid, *Rhopalosiphum maidi*(Yan, Lipka, Schmelz, Buckler, & Jander, 2015), suggesting that this metabolite can provide protection against herbivores. When this compound was added to the artificial diet, it reduced aphid growth and reproduction, but no significant effect was found on *S. exigua* larval growth. However, 5-hydroxynorvaline plays also a defensive role by replacing amino acids in protein synthesis or by inhibiting the biosynthetic pathways of many microorganisms (Guirard, 1958; Heremans & Jacobs, 1994; T. Huang, Jander, & de Vos, 2011; Kurtin, Bishop, & Himoe, 1971; Washtien, Cooper, & Abeles, 1977). Thus, it is

possible 5-hydroxynorvaline negatively impact the gut bacteria and impairs its contribution to the host. The flavonoids caffeic and protocatechuic acids are referred as potential insecticides due to their toxic effects (War et al., 2013). *Helicoverpa armigera* larvae fed on caffeic and protocatechuic acids displayed reduced digestive and detoxification activity due to a reduction in serine protease, trypsin, and esterase activity. The larvae also showed greater reduction in larval weight and higher mortality when compared to the larvae fed on untreated control diet (War et al., 2013). Moreover, caffeic acid also increases the oxidative stress in the gut of insect herbivores due to the elevation of protein oxidation, lipid peroxidation products and release of free ions (Summers & Felton, 1994). The accumulation of these flavanoids in the *RS* larval midgut could explain why *RS* does not perform as well as the *CS* when feeding on maize(Orsucci et al., 2020; Silva-Brandão et al., 2017).

Therefore, the lower levels of defensive plant compounds in the midgut of the strains when they were feeding on their preferred host plants (*RS* on and rice *CS* on maize) and their higher abundancies in the midgut when larvae were feeding on the non-preferred host plants (Fig. S1), suggest either differential metabolization of the food source as discussed above, reflecting a metabolic machinery not yet adapted to the chemistry of those plants, and/or differential elicitation of metabolic response in the host plant. Additionally, FAW strains were demonstrated inducing different defense responses in maize and Bermuda grass via specific differences in their saliva composition(F. E. Acevedo et al., 2018). And the gut-associated microbes in their oral secretions also play a role mediating the insect-plant interaction by regulating plant defenses upon their secretion through insect oral secretions (Flor E. Acevedo et al., 2016).

Our findings also suggest that the strains of FAW metabolize the artificial diet differently. The diet has been widely used for several Lepidoptera, including FAW, demonstrating good performance(Gardner, Phillips, & Smith, 1984; Perkins, 1979; Silva, Baronio, Galzer, Garcia, & Botton, 2019), However, most experiments were performed using the corn strain. There is only one study as far as we know showing that *CS* larvae were significantly heavier than *RS* larvae when they fed the artificial diet (Silva-Brandão et al., 2018). Furthermore, artificial diets generally provide unrealistic amounts of soluble carbohydrates, proteins, and fats. Perhaps the reason for a greater accumulation of compounds in the larval gut when compared to other diets is due to the large amount of these compounds in the food, which does not allow their complete metabolization.

The fact that only glycerol monostearate and 2-Isopropylaminoethanol were differentially abundant between *CS* and *RS* strains, being both more abundant in the midgut of *CS* larvae suggest that the strains behave in a very similar way when feeding on this plant. Interesting, it is suggested that feeding on dicot is a primitive condition of the FAW complex and feeding on grasses is a more recent event (Kergoat et al., 2012). Additionally, studies also demonstrated that FAW presents low performance and low survival rate when feeding on cotton (Ali, Luttrell, & Pitre, 1990; Barros et al., 2010).

In conclusion, our study demonstrates the effects of host strains and dietary effects on the metabolome of the FAW midgut. we have provided us with an overview of these changes occurring in their metabolomes of the strains on different diets and identified a wide range of marker metabolites that may help us to better understand the mechanisms involved in the host adaptation process. Our results shed light on our understanding of metabolic activities in the FAW, being a unit composed of its own metabolome and the metabolome of the associated gut microbiota. Further analyses are essential to reveal the links between gut microbiota composition and host metabolic phenotype, thus providing a holistic understanding of the functionality and adaptability of strains to host plants.

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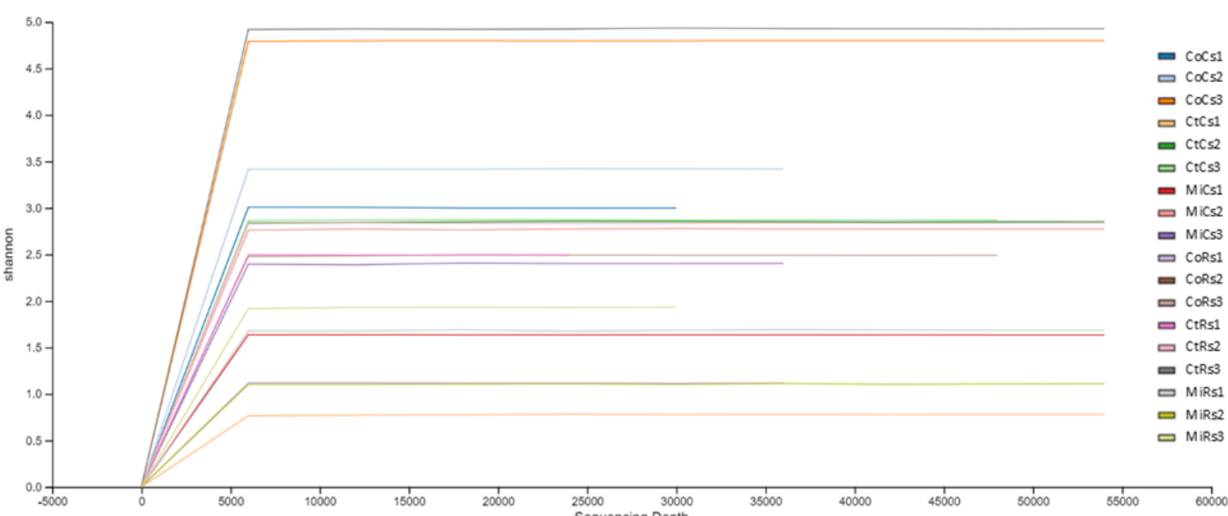
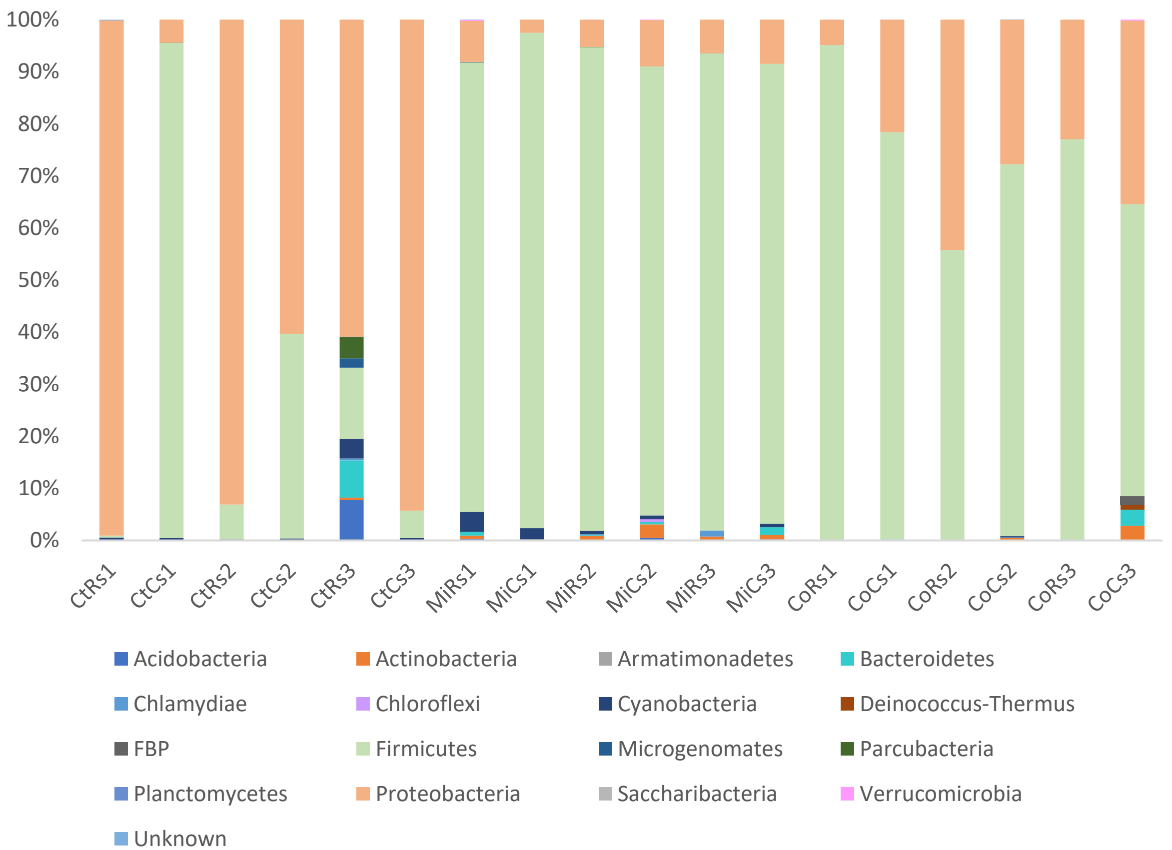
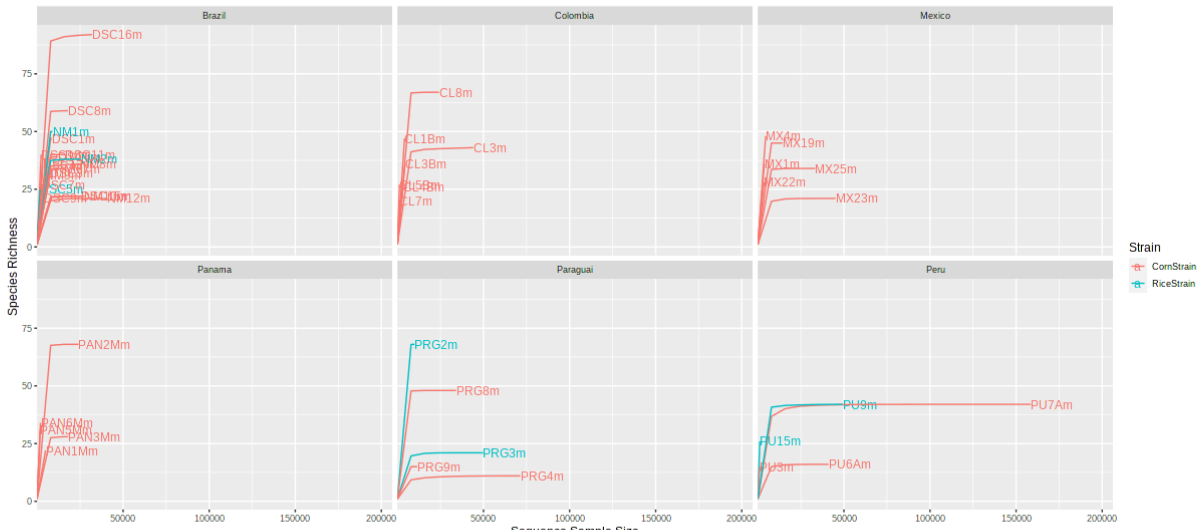
# ANNEXES



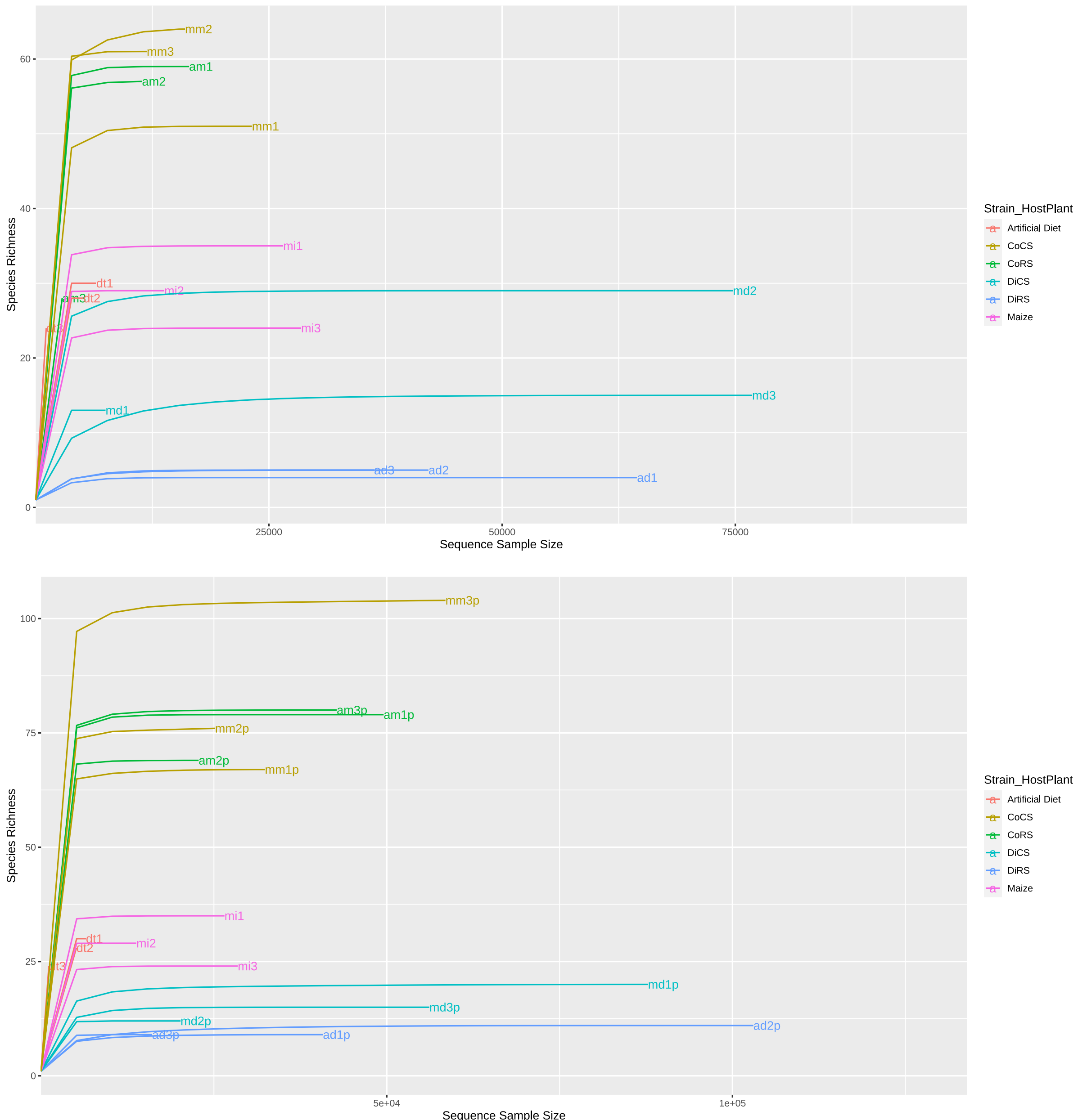

**ANEXO A.** Rarefaction curves based on Shannon diversity index for the number of sequences per sample of *S. frugiperda* corn (CS) and rice strains (RS) when feeding corn (Co), millet (Mi) and cotton (Ct). The level in each curve indicates the minimum number of sequences to capture the diversity. For all samples, curve tends to asymptote around 5000-7000 sequences.




**ANEXO B.** Taxa plot of bacterial genus of the microbiota associated with *S. frugiperda* corn strain (CS) and rice strain (RS) when feeding on corn (Co), millet (Mi) and cotton (Ct).




**ANEXO C.** Rarefaction curves showing the relationship between number of ASVs and number of sequences. The rarefaction curve for the midgut of *Spodoptera frugiperda* strains ( RS= red and CS=blue) fed on and maize collected in different countries.



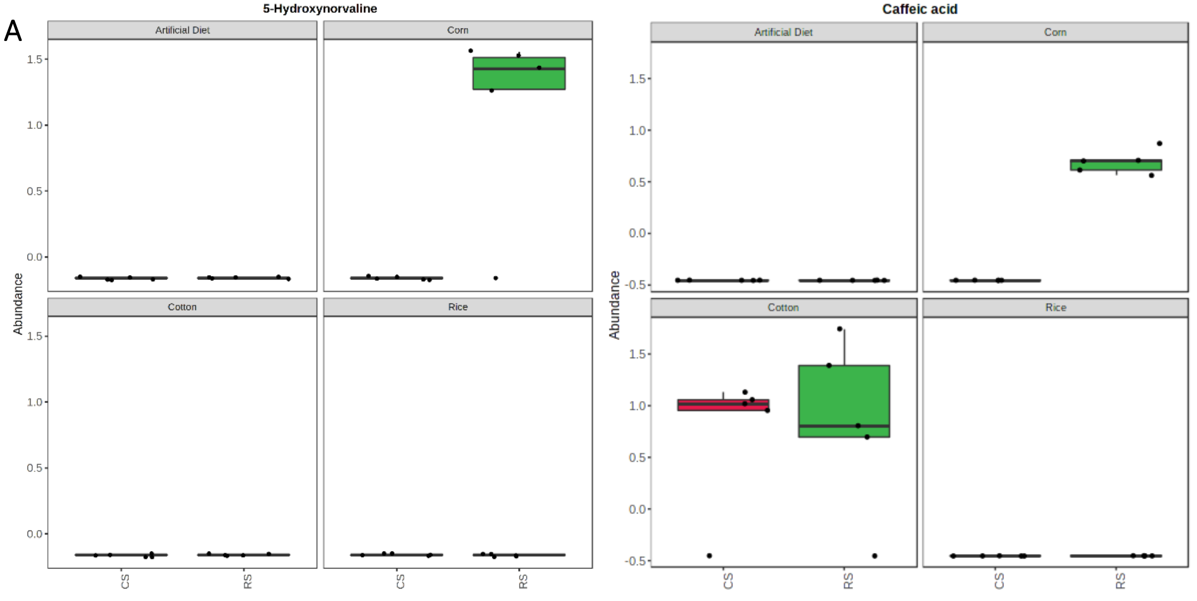

**ANEXO D.** Rarefaction curves based on Shannon diversity index for the number of sequences per sample of *S. frugiperda corn* (CS) and rice strains (RS) when feeding corn (Co) and artificial diet (Di). The level in each curve indicates the minimum number of sequences to capture the diversity.

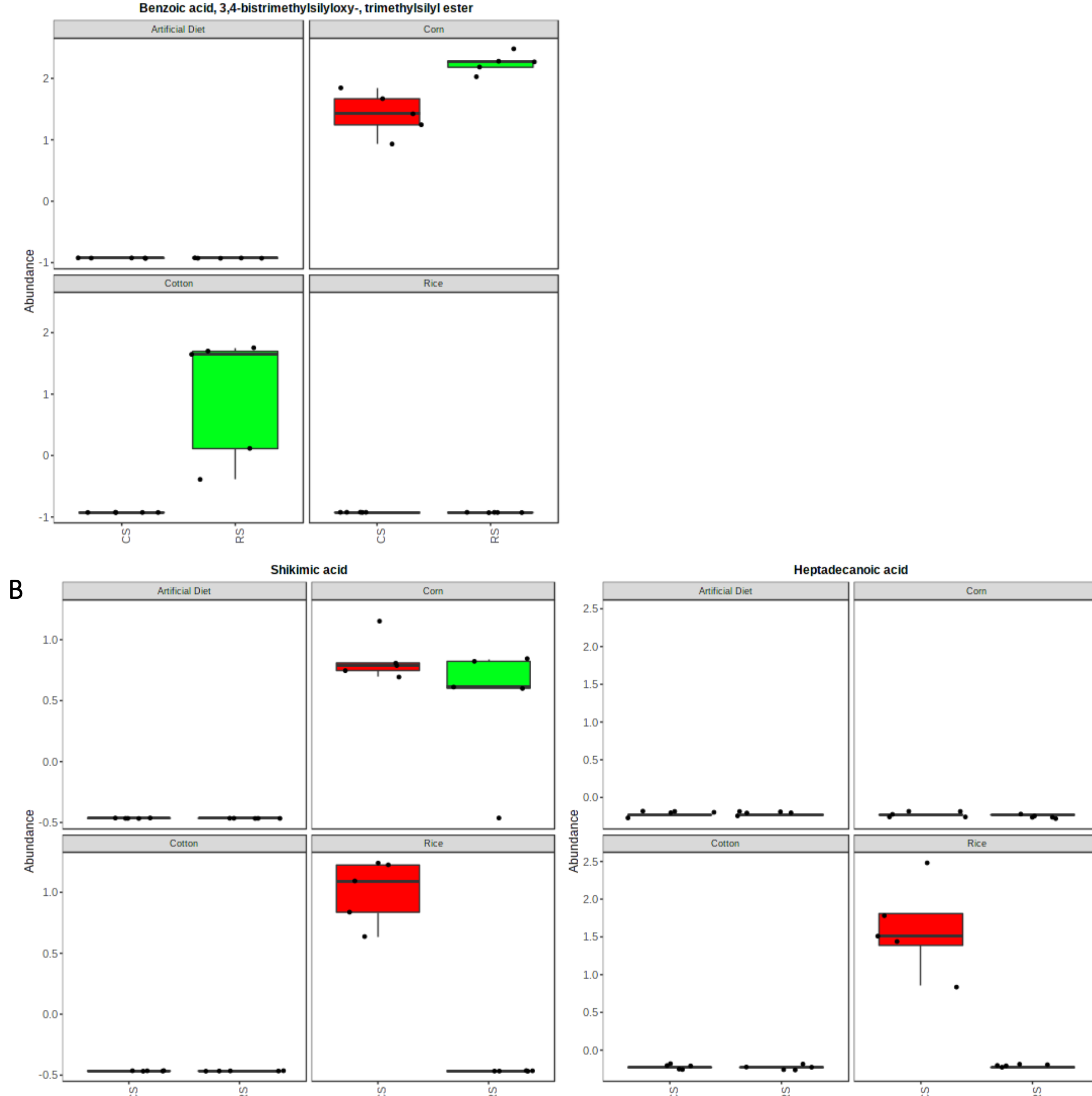
**ANEXO E.** Significant features identified by two-way ANOVA. FDR's correction was applied to adjust the p-values.

| Metabolite | Strain | | Diet | | Interaction | |
|---|---|---|---|---|---|---|
| | F.val | Adj.p | F.val | Adj.p | F.val | Adj.p |
| **Homoserine** | 72.91 | <0.01 | 72.91 | <0.01 | 72.91 | <0.01 |
| **Margaric acid** | 48.03 | <0.01 | 48.03 | <0.01 | 48.03 | <0.01 |
| **2-ketoglucose** | 46.17 | <0.01 | 19.54 | <0.01 | 19.54 | <0.01 |
| **Shikimic acid** | 42.12 | <0.01 | 63.49 | <0.01 | 24.54 | <0.01 |
| **Protocatechuic acid** | 30.70 | <0.01 | 112.80 | <0.01 | 13.37 | <0.01 |
| **Glycerol monostearate** | 22.08 | <0.01 | 10.12 | <0.01 | 24.41 | <0.01 |
| **D-Erythronolactone** | 20.78 | <0.01 | 7.28 | <0.01 | 7.28 | <0.01 |
| **Benzoic acid** | 18.96 | <0.01 | 7.16 | <0.01 | 4.89 | 0.02 |
| **Anthranilic acid** | 18.55 | <0.01 | 12.22 | <0.01 | 3.38 | 0.07 |
| **5-Hydroxynorvaline** | 15.64 | <0.01 | 15.64 | <0.01 | 15.64 | <0.01 |
| **Glycerophosphoric acid** | 11.30 | 0.02 | 23.96 | <0.01 | 20.50 | <0.01 |
| **Galactaric acid** | 10.50 | 0.03 | 115.80 | <0.01 | 6.30 | 0.01 |
| **2-Hydroxyisocaproic acid** | 10.33 | 0.03 | 10.33 | <0.01 | 10.33 | <0.01 |
| **Fumaric acid** | 8.47 | 0.05 | 94.81 | <0.01 | 13.43 | <0.01 |
| **Benzene, 4-chlorobutyl-** | 8.43 | 0.05 | 1.71 | 0.20 | 7.73 | <0.01 |
| **Lanthionine** | 7.45 | 0.07 | 6.31 | <0.01 | 1.42 | 0.41 |
| **Phosphonoacetic Acid** | 7.15 | 0.08 | 26.53 | <0.01 | 45.91 | <0.01 |
| **Lyxose, tetra-trimethylsilyl-ether** | 7.04 | 0.08 | 3.21 | 0.04 | 3.83 | 0.05 |
| **Methyl 3,4-dimethoxyphenylhydroxyacetate** | 6.74 | 0.09 | 6.74 | <0.01 | 6.74 | 0.01 |
| **Caffeic acid** | 6.58 | 0.09 | 23.93 | <0.01 | 5.34 | 0.02 |
| **Phenylethanolamine** | 6.49 | 0.09 | 13.26 | <0.01 | 6.12 | 0.01 |
| **Threonic acid** | 6.31 | 0.09 | 8.04 | <0.01 | 1.42 | 0.41 |
| **Glutamine** | 6.07 | 0.10 | 22.59 | <0.01 | 0.66 | 0.81 |
| **3-Deoxy-arabino-hexaric acid** | 5.64 | 0.11 | 5.64 | <0.01 | 5.64 | 0.02 |
| **Proline** | 5.64 | 0.11 | 31.28 | <0.01 | 1.69 | 0.33 |
| **4-Hydroxyhippuric acid** | 5.56 | 0.11 | 5.56 | 0.01 | 5.56 | 0.02 |
| **D-Xylose** | 5.49 | 0.11 | 5.49 | 0.01 | 5.49 | 0.02 |
| **2,4,4-Trimethyl-1-pentanol** | 5.34 | 0.11 | 5.34 | 0.01 | 5.34 | 0.02 |
| **d-Proline, N-methoxycarbonyl-, methyl ester** | 5.34 | 0.11 | 5.34 | 0.01 | 5.34 | 0.02 |
| **Bis2-ethylhexylamine** | 5.32 | 0.11 | 5.32 | 0.01 | 5.32 | 0.02 |
| **Atenolol** | 5.22 | 0.11 | 5.22 | 0.01 | 5.22 | 0.02 |
| **Embramine** | 5.14 | 0.11 | 5.14 | 0.01 | 5.14 | 0.02 |
| **2-O-Glycerol-alpha-d-galactopyranoside** | 5.10 | 0.11 | 5.10 | 0.01 | 5.10 | 0.02 |
| **4-Hydroxybenzoic acid** | 5.08 | 0.11 | 122.41 | <0.01 | 1.96 | 0.26 |
| **D-Gluconic acid, delta-lactone** | 4.98 | 0.11 | 4.98 | 0.01 | 4.98 | 0.02 |
| **Aucubin** | 4.91 | 0.11 | 12.16 | <0.01 | 0.22 | 1.00 |
| **Trifluoroacetamide** | 4.90 | 0.11 | 4.90 | 0.01 | 4.90 | 0.02 |
| **Hydracrylic acid** | 4.89 | 0.11 | 4.89 | 0.01 | 4.89 | 0.02 |
| **Timonacic** | 4.84 | 0.11 | 4.84 | 0.01 | 4.84 | 0.02 |
| **3-Octen-2-ol, E-** | 4.72 | 0.11 | 19.70 | <0.01 | 9.51 | <0.01 |
| **Lauric acid** | 4.63 | 0.11 | 4.63 | 0.01 | 4.63 | 0.02 |
| **Glyceryl-glycoside TMS ether** | 4.60 | 0.11 | 4.60 | 0.01 | 4.60 | 0.02 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Oxalic acid** | 4.45 | 0.12 | 169.11 | <0.01 | 3.32 | 0.07 |
| **D-Mannopyranose** | 4.24 | 0.13 | 4.24 | 0.02 | 4.24 | 0.03 |
| **alpha-D-glucose** | 4.11 | 0.14 | 10.21 | <0.01 | 0.49 | 0.86 |
| **Xylitol** | 4.07 | 0.14 | 60.84 | <0.01 | 4.43 | 0.03 |
| **Pentanedioic acid** | 3.98 | 0.14 | 59.34 | <0.01 | 3.98 | 0.04 |
| **3,4-Dimethoxymandelic acid** | 3.67 | 0.16 | 3.67 | 0.03 | 3.67 | 0.05 |
| **D---Fructofuranose, pentakistrimethylsilyl ether** | 3.61 | 0.16 | 3.61 | 0.03 | 3.61 | 0.06 |
| **Alanine, N-methyl-N-ethoxycarbonyl-, dodecyl ester** | 3.51 | 0.17 | 18.39 | <0.01 | 33.27 | <0.01 |
| **Alanine, N-methyl-N-methoxycarbonyl-, undecyl ester** | 3.30 | 0.18 | 7.29 | <0.01 | 1.29 | 0.46 |
| **Lactic acid** | 3.28 | 0.18 | 16.39 | <0.01 | 18.71 | <0.01 |
| **1-Aminocyclopentanecarboxylic acid** | 3.19 | 0.19 | 3.19 | 0.04 | 3.19 | 0.08 |
| **L-Aspartic acid** | 3.00 | 0.20 | 21.47 | <0.01 | 0.22 | 1.00 |
| **Levulinic acid enol** | 2.96 | 0.20 | 104.76 | <0.01 | 5.76 | 0.01 |
| **Inositol 1-phosphate** | 2.84 | 0.21 | 9.99 | <0.01 | 3.08 | 0.09 |
| **Cadaverine** | 2.84 | 0.21 | 5.00 | 0.01 | 1.73 | 0.33 |
| **Glyceric acid** | 2.62 | 0.24 | 10.33 | <0.01 | 1.96 | 0.26 |
| **Urea** | 2.41 | 0.26 | 14.40 | <0.01 | 0.49 | 0.86 |
| **L-5-Oxoproline** | 2.27 | 0.28 | 17.00 | <0.01 | 0.39 | 0.92 |
| **Sorbic acid** | 2.24 | 0.28 | 155.37 | <0.01 | 2.24 | 0.20 |
| **2-Isopropylaminoethanol** | 2.09 | 0.30 | 18.29 | <0.01 | 13.34 | <0.01 |
| **7,9-Di-tert-butyl-1-oxaspiro4,5deca-6,9-diene-2,8-dione** | 2.01 | 0.31 | 19.72 | <0.01 | 3.81 | 0.05 |
| **L--Tartaric acid** | 1.94 | 0.31 | 55.28 | <0.01 | 1.94 | 0.26 |
| **L-Threonine** | 1.93 | 0.31 | 9.18 | <0.01 | 0.03 | 1.00 |
| **Tridecanoic acid** | 1.83 | 0.33 | 7.29 | <0.01 | 1.52 | 0.39 |
| **b-Aminoisobutyric acid** | 1.80 | 0.33 | 9.42 | <0.01 | 3.19 | 0.08 |
| **4-Methoxycarbonylphenol** | 1.50 | 0.40 | 145.70 | <0.01 | 1.50 | 0.39 |
| **Pinitol** | 1.46 | 0.40 | 147.40 | <0.01 | 1.46 | 0.40 |
| **Formamide** | 1.41 | 0.41 | 15.23 | <0.01 | 0.36 | 0.93 |
| **Adrenaline** | 1.33 | 0.42 | 1.24 | 0.33 | 4.12 | 0.04 |
| **Citric acid** | 1.25 | 0.44 | 8.52 | <0.01 | 0.45 | 0.89 |
| **L-Tryptophan** | 1.23 | 0.44 | 25.95 | <0.01 | 9.77 | <0.01 |
| **Methionine** | 1.21 | 0.44 | 10.77 | <0.01 | 0.40 | 0.92 |
| **Ethyl .alpha.-D-glucopyranoside** | 1.12 | 0.46 | 7.08 | <0.01 | 1.73 | 0.33 |
| **alpha-D-Talopyranose** | 1.05 | 0.49 | 9.28 | <0.01 | 2.18 | 0.21 |
| **Cysteine** | 0.96 | 0.51 | 6.43 | <0.01 | 2.90 | 0.11 |
| **Inositol** | 0.93 | 0.51 | 11.02 | <0.01 | 4.47 | 0.03 |
| **Phenylalanine** | 0.79 | 0.55 | 4.86 | 0.01 | 0.04 | 1.00 |
| **D-Glucuronic acid** | 0.75 | 0.56 | 8.30 | <0.01 | 1.14 | 0.53 |
| **Formylpiperidine** | 0.74 | 0.56 | 18.18 | <0.01 | 5.28 | 0.02 |
| **Gluconic acid** | 0.67 | 0.58 | 15.41 | <0.01 | 0.67 | 0.81 |
| **Putrescine** | 0.67 | 0.58 | 164.10 | <0.01 | 0.79 | 0.72 |
| **L-Rhamnose** | 0.60 | 0.59 | 157.39 | <0.01 | 0.60 | 0.83 |
| **3-Methylpiperazine-2,5-dione** | 0.52 | 0.63 | 247.03 | <0.01 | 0.52 | 0.86 |
| **Linoleic acid** | 0.35 | 0.72 | 15.56 | <0.01 | 0.57 | 0.85 |
| **Valine** | 0.34 | 0.73 | 5.54 | 0.01 | 0.61 | 0.83 |
| **Ritalinic acid** | 0.29 | 0.76 | 7.75 | <0.01 | 0.29 | 0.98 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Asparagine** | 0.25 | 0.78 | 11.89 | <0.01 | 1.00 | 0.59 |
| **Glucopyranoside** | 0.23 | 0.78 | 28.63 | <0.01 | 0.23 | 1.00 |
| **2,5-Dimethoxymandelic acid** | 0.22 | 0.78 | 3.12 | 0.05 | 0.13 | 1.00 |
| **2-3-Bromo-5,5,5-trichloro-2,2-dimethylpentyl-1,3-dioxolane** | 0.21 | 0.78 | 3.45 | 0.03 | 6.69 | 0.01 |
| **D--Galactopyranose, pentakistrimethylsilyl ether** | 0.15 | 0.83 | 11.46 | <0.01 | 0.15 | 1.00 |
| **Salicylic acid** | 0.14 | 0.83 | 40.60 | <0.01 | 0.14 | 1.00 |
| **Pipecolic acid** | 0.09 | 0.89 | 35.56 | <0.01 | 0.09 | 1.00 |
| **Glycoside, a-methyl-trtrakis-O-trimethylsilyl-** | 0.08 | 0.89 | 6.91 | <0.01 | 8.00 | <0.01 |
| **Aminomalonic acid** | 0.08 | 0.89 | 16.51 | <0.01 | 0.08 | 1.00 |
| **Linolenic acid** | 0.07 | 0.89 | 13.44 | <0.01 | 1.05 | 0.56 |
| **Ornithine** | 0.04 | 0.93 | 4.81 | 0.01 | 1.52 | 0.39 |
| **5-Methyluridine** | 0.04 | 0.93 | 135.55 | <0.01 | 0.04 | 1.00 |
| **B-Alanine** | 0.03 | 0.95 | 3.81 | 0.02 | 2.80 | 0.11 |
| **Tristrimethylsiloxyethylene** | 0.02 | 0.97 | 55.79 | <0.01 | 0.02 | 1.00 |
| **Elaidic acid** | 0.01 | 0.98 | 93.77 | <0.01 | 0.01 | 1.00 |
| **Serine** | 0.01 | 0.98 | 10.86 | <0.01 | 0.05 | 1.00 |
| **1-Propene-1,2,3-tricarboxylic acid** | <0.01 | 1.00 | 562.08 | <0.01 | <0.01 | 1.00 |
| **Malic acid** | <0.01 | 1.00 | 11.09 | <0.01 | 0.23 | 1.00 |
| **Alanylglycine** | <0.01 | 1.00 | 127.33 | <0.01 | <0.01 | 1.00 |
| **L-Lysine** | <0.01 | 1.00 | 30.24 | <0.01 | 4.85 | 0.02 |
| **Palmitoleic acid** | <0.01 | 1.00 | 23.00 | <0.01 | <0.01 | 1.00 |






**ANEXO F.** Boxplot of significant features of midgut metabolome of *Spodoptera frugiperda* strains larvae after feeding on maize (A) and rice (B) identified by Volcano plot with fold change threshold 2 and t-tests threshold 0.05. False discovery rate correction was applied to adjust the p-values (0.05).