Beyond host regulation: Changes in gut microbiome of permissive and nonpermissive hosts following parasitization by the wasp *Cotesia flavipes*

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ABSTRACT

Koinobiont parasitoids regulate the physiology of their hosts, possibly interfering with the host gut microbiota and ultimately impacting parasitoid development. We used the parasitoid *Cotesia flavipes* to investigate if the regulation of the host would also affect the host gut microbiota. We also wondered if the effects of parasitization on the gut microbiota would depend on the host – parasitoid association by testing the permissive *Diatraea saccharalis* and the nonpermissive *Spodoptera frugiperda* hosts. We determined the structure and potential functional contribution of the gut microbiota of the fore-midgut and hindgut of the hosts at different stages of development of the immature parasitoid. The abundance and diversity of operational taxonomic units of the anteromedial gut and posterior region from larvae of the analyzed hosts were affected by parasitization. Changes in the gut microbiota induced by parasitization altered the potential functional contribution of the gut microbiota associated with both hosts. Our data also indicated that the mechanism by which *C. flavipes* interferes with the gut microbiota of the host does not require a host-parasitoid coevolutionary history. Changes observed in the potential contribution of the gut

microbiota of parasitized hosts impact the host's nutritional quality, and could favor host exploitation by *C. flavipes*.

Keywords: Bacteria, dysbiosis, functional contribution, host regulation, microbial ecology, symbiosis.

INTRODUCTION

Ecosystems are represented by complex interactions among living beings. These interactions and the interrelationships among organisms can directly or indirectly affect organisms linked at different levels in a chain of interactions. The term symbiosis (from the Greek *syn* "together" and *bios* "life") is used to describe any interaction between species alike (De Bary, 1879; Saffo, 1992) and the interactions among microorganisms and multicellular organisms are increasingly receiving attention due to the discovery of the role that microbial symbionts have in different ecological and multitrophic host relationships (Moran, 2006). Many of the issues that arise for a better understanding of host microbiota relationships and their variations are essentially ecological. This has led to the incorporation of models commonly used in ecology for these studies. However, given the particularities found in the host/microbiota relationships, the expansion of these models has been proposed, also favoring ecology in general (Miller *et al.*, 2018; Miller and Bohannan, 2019). Studies in this field benefited greatly from the advent of high throughput sequencing technologies (Bragg and Tyson, 2014). Such studies have extended our understanding of the diversity and complexity of associated microbial communities, thereby challenging the well-established concept of an individual (Gilbert *et al.*, 2012; Guerrero *et al.*, 2013). Thus, the behavior of the individual and its responses to the environment require a holistic investigation, which includes the relationship between the individual and its symbionts, leading to the definition of a single entity, the holobiont, which is evolving as a single unit of selection (Zilber-Rosenberg and Rosenberg, 2008; Gilbert *et al.*, 2012; Guerrero *et al.*, 2013). The term holobiont was first introduced by Mindell (Mindell, 1992) to describe the composite nature of species associated with primary symbionts, but has come to include all host-associated microorganisms (Margulis, 1993). There is an intense debate as to whether host - microbes are

or not a unit of selection (Bordenstein and Theis, 2015; Douglas and Werren, 2016; Skillings, 2016; Roughgarden *et al.*, 2018), regardless of the well-defined principles for holobiont – hologenome recognition (Bordenstein and Theis, 2015). Furthermore, even if the inheritable aspect of the holobiont is disregarded, the host microbial community can provide time for the host genome to evolve during periods of changes in the environment (Zilber-Rosenberg and Rosenberg, 2008).

Insects are widely used as models to study symbiosis because of the range of associations shared with microorganisms (from pathogenic to mutualistic), and the effects symbionts have on insect survival and fitness attributes (Bourtzis and Miller, 2003). One of such examples is the rich diversity of microorganisms associated with the gut of insects. Most of the gut-associated bacteria are free living, and are facultative associated with insects. Many are commensals or ordinary passengers, since they are acquired with the insect's food source (Dillon and Dillon, 2004; Engel and Moran, 2013). Nevertheless, there are several examples of obligate and facultative gut bacteria that contribute to host nutrition, food digestion, and nitrogen cycling, even in cases in which bacteria are acquired from the environment at every generation (Beard *et al.*, 2002; Kikuchi *et al.*, 2005). Thus, the gut microbiota of insects influences several physiological processes and interferes with the expression of the host phenotype, similarly to what occur with humans (Dillon and Dillon, 2004; Clemente *et al.*, 2012; Engel and Moran, 2013).

The gut microbiota of insects can assist with food digestion and utilization by producing enzymes that act on food digestion (Anand *et al.*, 2010; Krishnan *et al.*, 2014) and on the degradation of xenobiotics (Kikuchi *et al.*, 2012; Adams *et al.*, 2013). Additionally, gut microbes allow the exploitation of suboptimal food sources by the host through the synthesis and release of essential vitamins (Eichler and Schaub, 2002) and amino acids (Douglas, 2006; Nikoh *et al.*, 2011) and the recycling of nitrogen (French *et al.*, 1976; Ohkuma *et al.*, 1996; Hongoh *et al.*, 2008). Moreover, the gut microbiota affects the hosts' intra- and interspecific interactions, interfering with the process of speciation (Brucker and Bordenstein, 2013), mate choice (Sharon *et al.*, 2010; Sharon *et al.*, 2011), production of semiochemicals

(Dillon *et al.*, 2002; Leroy *et al.*, 2011) and protection against pathogens (Azambuja *et al.*, 2005; Cirimotich, Dong, *et al.*, 2011; Bahia *et al.*, 2014).

The successful association of insect hosts and their microbial symbionts also requires the maintenance of microbial populations within certain densities to avoid excessive costs or even damage to the host, at the same time microbial levels reach densities that enable them to provide the required contribution to the host (Ryu *et al.*, 2010; Charroux and Royet, 2012). Several factors (e.g., gut pH, redox potential, and food quality) are known to affect gut microbes (Dillon and Dillon, 2004; Engel and Moran, 2013). Moreover, molecules derived from the host immune system (lysozymes, reactive oxygen species, and antimicrobial peptides) are involved in keeping the gut microbiota under controlled levels (Dillon *et al.*, 2002; Azambuja *et al.*, 2005; Cirimotich, Dong, *et al.*, 2011; Bahia *et al.*, 2014).

Therefore, sources of stress that challenge the host immune system derange the process of regulation of the gut microbiota. Baculovirus suppresses the immune system of infected larvae of *Spodoptera exigua* (Huebner) (Lepidoptera: Noctuidae) which increases the load of gut bacteria, favoring the pathology of this viral infection (Jakubowska *et al.*, 2013). Alterations in the microbiota of *Drosophila melanogaster* were also shown to shape host resistance to the parasitoid *Asobara tabida* (Chaplinska et al., 2016). Even in nematodes, it is possible to find a greater diversity in the gut microbiota of infected than of uninfected nematodes (Vicente *et al.*, 2016). Insect parasitoids are also stressors of the host immune system, and parasitic wasps, particularly koinobionts, use a set of virulence factors to regulate the humoral and cellular immune responses of the host to allow host colonization by immature parasitoids. Koinobionts are parasitoids in which the hosts are not paralysed after parasitization, and parasitized hosts can actively move and feed during parasitoid development (Quicke, 2015). Parasitoids can regulate the host immune system by inactivating the IMD signaling pathway through inhibition of NF-κβ transcription factors (Bae and Kim, 2009; Bitra *et al.*, 2012), which also participate in the regulation of gut microbes (Charroux and Royet, 2012). Additionally, parasitoids alter host food preference (Smilanich *et al.*, 2011), food intake and utilization (Rossi *et al.*, 2014), nitrogen metabolism, and excretion (Kahn *et al.*, 1976). Moreover,

parasitoids also induce endocrine changes, affecting host molting and metamorphosis (Pfister-Wilhelm and Lanzrein, 1996; Mahmoud *et al.*, 2012). All these physiological changes are reported to interfere with the gut microbiota homeostasis (Dillon and Dillon, 2004; Broderick and Lemaitre, 2012; Hammer *et al.*, 2014; Yun *et al.*, 2014).

The successful development of parasitoids relies on the efficacy the host is regulated by the virulence factors produced by these wasps. The successful regulation and exploitation of hosts by parasitoids depends on the evolutionary history of the host-parasitoid interaction. During their evolutionary history, hosts developed defense mechanisms against natural enemies, while parasitoids improved their strategies to successfully exploit their hosts (Abrams, 2000; Pennacchio and Strand, 2006; Cônsoli *et al.*, 2012). Parasitoids can employ a range of molecules from their venom glands (Asgari and Rivers, 2011), calyx fluids (Tanaka and Vinson, 1991), larval secretions (Vinson and Iwantsch, 1980a) and teratocytes (Dahlman, 1991), besides proteins produced by the expression of genes from associated viral particles, polydnavirus, when infecting host tissues (Strand and Burke, 2013) to regulate their hosts physiology, growth and development (Beckage and Gelman, 2004; Cônsoli and Vinson, 2004). However, there are hosts that remain insensitive to the diversified tools parasitoids developed to guarantee a successful parasitization, particularly in host – parasitoid interactions that do not share an evolutionary history. Refractory hosts (nonpermissive hosts) rarely allow for the successful establishment and development of parasitoid progenies, contrarily with the highly successful rate of parasitization and effective parasitoid development in permissive hosts (Minchella, 1985; Bitra *et al.*, 2016).

Once the host and its associated gut microbiota function as a holobiont, the strategies that parasitoids employ to successfully parasitize and develop in their hosts would also be expected to result in the regulation of the host's gut microbiota. In order to test this hypothesis, we predicted host parasitization would induce quantitative and/or qualitative changes in the composition of the gut microbiota and would affect the functional contribution of the gut microbiota. We also predicted successful changes in the diversity of the gut microbiota and on its functional contribution would depend on the effective regulation

of the host by the natural enemy. To test our predictions, we investigated the changes induced in the diversity and potential functional contribution of the gut microbiota of the permissive *Diatraea saccharalis* (F.) (Lepidoptera: Crambidae) and the nonpermissive *Spodoptera frugiperda (*J. E. Smith) (Lepidoptera: Noctuidae) hosts when parasitized by the larval endoparasitoid *Cotesia flavipes* Cameron (Hymenoptera: Braconidae).

MATERIALS AND METHODS

Insects rearing

All insects used in our study were obtained from stock laboratory colonies maintained on artificial diets following standard procedures for insect rearing. Larvae of *D. saccharalis* were reared on an artificial diet based on soy flour and wheat germ (Parra and Mihsfeldt, 1992), while *S. frugiperda* larvae were reared on an artificial diet based on wheat germ, beans and brewer's yeast (Greene *et al.*, 1976), following standard rearing procedures (Parra and Panizzi, 1991). *Cotesia flavipes* was also obtained from a laboratory colony and reared using larvae of *D. saccharalis* as the host. Host larvae were individually offered to female wasps. Once stung, larvae were transferred to small plastic dishes containing a small piece of the artificial diet, in which they remained until parasitoid larval egression and pupation. The masses of cocoons were collected and transferred to a clean dish for wasp emergence, adult feeding in water-honey (1:1) solution and mating (Parra, 1999). Hosts and parasitoids were reared under controlled laboratory conditions (25 ± 1°C; 70 ± 10% RH; 14 h photophase).

Effects of parasitization by *Cotesia flavipes* on the gut microbiota of permissive and nonpermissive hosts

Once parasitized larvae grow at a different rate than the nonparasitized larvae, we opted to use larvae of *D. saccharalis* at the beginning of the last instar, allowing the sampling of the gut microbiota of control and parasitized larvae within the same instar. Larvae of the nonpermissive host *S. frugiperda* were subjected to parasitization by *C. flavipes* 12 h after the molt to the 3rd instar, once *C. flavipes* is unable to

attack older larvae of *S. frugiperda*. Thus, larvae of *D. saccharalis* at the premolt from 5th to 6th instar were selected, and only those molting within 12-h were later used for the experiments. Selected larvae were split into two distinct groups. In one group, larvae were individually offered to *C. flavipes* for parasitization; the other group was kept as control (non-parasitized). Larvae were individually placed into rearing containers under controlled conditions (25 ± 1°C; 70 ± 10% RH; 14-hour photophase) until dissection for the sterile collection of gut samples. Samples from control (C) and parasitized (P) larvae were collected at three time points relative to host parasitization, i.e., on the first (1DAP), fifth (5 DAP) and ninth day after parasitization (9 DAP). Seven larvae were dissected in each sampling period for each biological replicate in each treatment. Each sampling period had three biological replicates (1 replicate = 7 guts)/treatment.

Larvae were surface sterilized in cold 0.2% sodium hypochlorite in 70% ethanol solution, rinsed in cold sterile water and dissected in cold sterile saline solution (125 mM NaCl, 4°C) under aseptic conditions. The gut was removed, rinsed in sterile saline, and the foregut+midgut were separated from the hindgut. The foregut+midgut (anteromedial gut region – ANT) and the hindgut (posterior gut region – POS) were transferred to clean microtubes and immediately stored in absolute ethanol at -20°C until DNA extraction. The anteromedial region and the hindgut play different roles in the process of food digestion and assimilation. The anteromedial region of the gut is involved with food transport (foregut), food digestion and nutrient assimilation (midgut), while the posterior region of the gut is involved with excretion and water and nutrient resorption. The different roles each region of the gut play also lead to an environment that differs in ion concentration and pH, which may affect their microbial composition (Egert *et al.*, 2003; Dillon and Dillon, 2004; Ishak *et al.*, 2011; Tang *et al.*, 2012; Smith *et al.*, 2017). Thus, each one of these regions were sampled and analyzed separately.

DNA extraction, amplification and sequencing of the V4 region of the 16S ribosomal RNA gene

Each gut sample collected was macerated in liquid nitrogen and subjected to genomic DNA extraction (gDNA) using the commercially available product AxyPrep Bacterial Genomic DNA Kit (Axygen), following the manufacturer's recommendations. The gDNA samples obtained were checked for quality, integrity and purity by spectrophotometry and agarose gel electrophoresis, following standard procedures before storage at -20°C (Sambrook, 2001).

gDNA samples were used in PCR amplification of the V4 region of the 16S ribosomal RNA gene (16S rRNA) using the universal set of primers 16SV4F (5'AGTCAGTCAGCCGGACT ACHVGGGTWTCTAAT3') and 16SV4R (5'TATGGTAATTGTGTGCCAGCMGCCGCGG TAA3') (Kozich et al., 2013). Reactions were programmed at 98°C for 2 min (1 cycle), followed by 30 cycles at 98°C for 45 s, 56°C for 1 min and 72°C for 90 s, with a final extension (1 cycle) at 72°C for 10 min. Reactions were performed in a final volume of 50 µL, containing 100-150 ng gDNA, 1.5 mM $MgCl_2$, 1x PCR buffer, 0.2 mM of each dNTP, 0.32 µM of each primer and 2U GoTaq® DNA Polymerase (Promega). Amplification products were separated in 1.5% agarose gel electrophoresis containing 0.5 µg/mL of ethidium bromide in TAE buffer (40 mM Tris-acetate; 1 mM EDTA at pH 8.2) under constant voltage (100 V). PCR products were extracted from agarose gel and purified by centrifugation using an Ultrafree-DA spin column (Millipore). An aliquot of 50 ng of the purified amplicons was subsequently subjected to a nested-amplification for addition of tags and adapters required for paired-end sequencing (2x250 bp) of the different libraries in a MiSeq Illumina sequencing platform. The nested reaction was performed using the commercial product NEXTflex™ 16S V4 Amplicon-Seq Kit (Bioo Scientific) in a final volume of 50 µL, containing 50 ng of the amplicons obtained in the first amplification reaction, NEXTflexTM DNA PCR Master Mix, 0.2 µM of one of the 16SV4R NEXTflex™ antisense primer tagged with specific short sequences to identify each sample, and 0.2 µM of the 16SV4F NEXTflex™ sense primer, according to the manufacturer's guidelines. The reaction was programmed at 98°C for 2 min (1 cycle), followed by 30 cycles at 98°C for 45 s, 56°C for 1 min and 72°C for 90 s, with a final

extension (1 cycle) at 72°C for 10 min. The obtained amplicons were purified using the commercial system Agencourt® AMPure® XP (Beckman Coulter) and sent for sequencing at the Center for Functional Genomics, Luiz de Queiroz College of Agriculture, University of Sao Paulo in a MiSeq Illumina sequencing platform, using the paired-end strategy (2x – 250 bp).

Sequence analyses

The reads obtained were analyzed using the tools available as implemented in the software QIIME (Caporaso, Kuczynski, *et al.*, 2010). Reads were separated by their barcoding sequences (tags) and trimmed to remove residual primers. Reads were also subjected to quality filters to exclude sequences with quality values below 20 (Phred < Q20). Sequences obtained by combining sense and antisense reads were aligned against sequences available in the 13.5 GreenGenes database (Werner *et al.*, 2012) using the PyNAST method (Caporaso, Bittinger, *et al.*, 2010). Valid sequences were classified into operational taxonomic units (OTUs) with a 97% similarity threshold value being assumed using the "UCLUST" method (Edgar, 2010). OTUs were classified with the recommended open method of reference in which they were grouped according to the closest matched taxon, depending on similarity values, following the limits for their taxonomic designation: > 97% of similarity classified at species level; between 95%-97% classified at genus level; between 90%-95% at family level; between 85%-90% at order level; 80%-85% at class level; and 77%-80% at phylum level.

Data were rarefied using the samples with the smallest number of reads and then subjected to alpha-diversity analysis employing the Shannon index. UniFrac distance analysis, a β-diversity measure that uses phylogenetic information (Lozupone *et al.*, 2010) was subsequently used with each data set as a basis for hierarchical clustering with the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and Principal Coordinates Analysis (PCoA).

The Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) software (Langille *et al.*, 2013) was used to predict the potential functional contribution of the gut

microbiota. The GreenGenes database was used as a reference, but a different OUT-picking method was used to meet the requirements of PICRUSt. Therefore, in PICRUSt analysis we used the closed method in QIIME to classify the 16S rRNA sequences in OTUs. In PICRUSt, OTUs were normalized against the total number of copies of the 16S rDNA, while the metagenomes were predicted based on the Kyoto Encyclopedia of Genes and Genomes (KEGG). To evaluate changes induced by parasitism in the potential functional contribution of the gut microbiota of *D. saccharalis* and *S. frugiperda* larvae, the identified genes were grouped into four distinct categories: *enzymes*; *nutrition*; *defense*; *biodegradation*. This analysis used level 2 designations, namely "enzyme families" for *enzymes*; "amino acid metabolism, carbohydrate metabolism, cofactor and vitamin metabolism and lipid metabolism" for *nutrition*; "terpenoid and polyketide metabolism and biosynthesis of other secondary metabolites" for *defense*; and "xenobiotic metabolism and biodegradation" for *biodegradation*.

All statistical analyses were performed using the Statistical Analysis of Metagenomic Profiles (STAMP) software (Parks *et al.*, 2014). In one of the analysis only two experimental groups (parasitized x control) were considered, with the sampling period used as replicates. In this case, the White's non-parametric *t*-test was used for comparisons of the taxonomic and the functional groups, with bootstrap being used to calculate the confidence intervals. In the second analysis, the sampling periods were also considered as a factor of variation, and technical replicates were automatically generated by the software. Samples were then subjected to comparisons at the taxonomic level using the Fisher's exact test, and the Newcombe-Wilson method was used to calculate the confidence intervals (95% nominal coverage). The Benjamini-Hochberg test for false discovery rate (FDR) was used to indicate the percentage of false positives (reported by *q* values), which should be expected among all significant subsystems. The G + Fisher test, combined with the Newcombe-Wilson corrected by Bonferroni test were used for functional predictions.

RESULTS

16S rRNA sequences, and alpha and beta diversity indices

Illumina sequencing of the V4 region of the 16S rRNA gene obtained from the gut microbiota of *D. saccharalis* larvae generated a total of 4,599,312 valid reads, while 4,427,969 valid reads were obtained for the gut microbiota of *S. frugiperda* larvae after standard quality filtering. Filtering yielded a mean of 383,276 reads/sample, ranging from 256,911 to 459,908 reads/sample for the gut microbiota of *D. saccharalis*; for *S. frugiperda*, we obtained a mean of 368,997 reads/samples, ranging from 214,562 to 459,690 per sample. The mean length of the reads obtained for the microbiota of both species was 253 bp.

OTU rarefaction analyses from samples obtained for the gut microbiota associated with *D. saccharalis* and *S. frugiperda* were adequate. Analysis of the Shannon index indicated no significant gain in OTU diversity after sampling around 12,000 sequences of the gut microbiota for *D. saccharallis* and 8,000 for *S. frugiperda* (Figure S1). Alpha-diversity analysis of individual groups did indicate variations in the diversity of microbes in the gut among the samples analyzed (Figure S1). PCoA based on unweighted UniFrac analysis explained 63% of the variation observed in the microbiota associated with the anteromedial region of the gut of *D. saccharalis* under the different experimental conditions, while PCoA based on weighted UniFrac analysis explained 87% of such variation (Figures S2). Differences between the unweighted and weighted analyses suggest the analyzed microbiota is more strongly influenced by the relative abundance of their different components than by the number of OTUs. Differences in the gut microbiota associated with the hindgut between parasitized and control larvae were more conspicuous at the sampling times studied (Figure S2).

The distinct separation of the microbiota from the anteromedial gut region of *D. saccharalis* shown in the PCoA analysis gains a high level of bootstrap support in the UPGMA unweighted (50-100% bootstrap support) and weighted (75-100% bootstrap support) grouping analyses; albeit without the clustering of groups that are distinctly influenced by parasitism (Figure 1). The UPGMA analysis from

the weighted UniFrac also showed greater bootstrap support than the unweighted analysis, with supporting values between 75-100% for all groups, showing that differences in the abundance of the components from the community is a key factor in the separation of treatments (Figure 1). In both cases, there is a strong influence from the development stage of the host in determining the diversity of the microbiota associated with either the anteromedial region or hindgut. However, the only samples from parasitized larvae that resolved isolated from the control larvae in all analyses were those obtained at 5 DAP.

The microbiota of the anteromedial gut region of *S. frugiperda* also grouped in well-formed clusters depending on their sampling time and parasitization condition when using PCoA analysis (Figure S3). Seventy-three % of the total variability was explained by the unweighted UniFrac analyses and 97% by the weighted UniFrac analyses, also demonstrating the importance of the abundance of members of the gut microbiota in clustering the treatments. Similarly, 97% to 100% of the total variability of the microbiota of the foregut was explained by the unweighted and weighted analyses (Figure S3).

UPGMA analyses strongly supported the clades formed from both gut regions, especially when the abundance of the different components of the gut microbiota (weighted UniFrac) were taken into account (bootstrap values > 75%) (Figure 2). However, bootstrap values were below 50% when UPGMA analyses considered exclusively the phylogenetic relationships among members of the microbiota (unweighted UniFrac) (Figure 2). Analysis did not separate the parasitized and control larvae in specific clusters nor the different stages of development analyzed, except for those after one day of parasitization.

Effect of parasitism by *Cotesia flavipes* in the composition of the host gut microbiota

In general, the microbiota of the anteromedial gut region of *D. saccharalis* larvae is composed by *Proteobacteria,* followed by *Firmicutes* and *Actinobacteria* (Figure S4). Three classes of *Proteobacteria* were found. γ-*Proteobacteria* was the prevalent, and basically represented by unidentified OTUs of *Enterobacteriaceae*. *Rubrobacter* was the dominant representative of *Actinobacteria*, and *Firmicutes* was

represented by a dominant unidentified genus of *Planococcaceae*. Similarly, the hindgut of *D. saccharalis* larvae was predominantly inhabited by *Proteobacteria*, with *Actinobacteria* and *Firmicutes* representing larger relative proportions than those observed in the anteromedial gut region of *D. saccharalis* (Figure 3). *Proteobacteria* in the hindgut was mainly represented by an unidentified *Enterobacteriaceae*, while an unidentified *Planococcaceae* and *Streptomyces* were the prevailing representatives of *Firmicutes* and *Actinobacteria*, respectively (Figure 3).

No effects of parasitization were observed in the composition of the larval gut microbiota of *D. saccharalis* using only those genera with at least 5% relative abundance when sampling time was not considered (q-values > 0.05). However, significant differences in the diversity of the gut microbiota were detected when the sampling time was included in the analysis (Figure 4). A significant effect in the diversity of the gut microbiota of larvae of *D. saccharalis* was detected in parasitized larvae on day 1 after the parasitization (1DAP), mainly due to the decreased abundance of OTUs belonging to *Enterobacteriaceae* (q-value <1e-15). The decrease observed for *Enterobacteriaceae* was not followed by any detected beneficial effect on the abundance of the remaining bacteria of the anteromedial region of the gut of *D. saccharalis* larvae. *Enterobacteriaceae* abundance was recovered at day 5 after parasitization (q-value <1e-15). Recovery of the relative abundance of *Enterobacteriaceae* and the increased abundance of *Acinetobacter* in parasitized larvae as compared to the non-parasitized larvae were followed by the decreased abundance of all remaining bacteria (q-value <1e-15). The abundance of *Acinetobacter* continued to increase at expenses of the decreased abundance of the remaining microbiota in parasitized larvae at later stages of parasitoid development (9DAP) (q-value <1e-15) (Figure 4).

Parasitization negatively affected the abundance of *Streptomyces* and OTUs representing unidentified genera of *Enterobacteriaceae* and *Xantomonadaceae* in the hindgut of *D. saccharalis* larvae (q-values >0.05). On the other hand, all other OTUs increased in abundance 1 day after parasitization (Figure 4). The same pattern occurred 5 days after parasitization, except that the unidentified OTU of *Xanthomonadaceae* increased. Changes observed 9 DAP were less conspicuous, but *Shingobium*,

Streptomyces and the OTU representing the predominant unidentified *Enterobacteriaceae* increased. Abundance of the remaining OTUs decreased (q-value <1e-15) (Figure 4).

The gut microbiota of both regions of the gut of nonpermissive larvae *S. frugiperda* was comprised almost exclusively by two taxa of *Firmicutes, Enterococcus* and an unidentified genus of *Lactobacillales* (Figures S5 and 5). The effects of parasitization in the gut microbiota of the nonpermissive host were similar to that observed for the permissive host. Changes in the abundance of OTUs were observed at different sampling times (Figure 6). Abundance of *Enterococcus* slightly increased (q-value <1e-15) and that of *Lactobacillales* decreased in the anteromedial gut region after 1 DAP (q-value <1e-15), a pattern observed in the remaining sampling times (Figure 6). Changes in the composition of the hindgut of larvae of *S. frugiperda* parasitized by *C. flavipes* were less regular. Abundance of *Enterococcus* and *Lactobacillales* in the hindgut decreased 1 DAP (q-value <1e-15). *Enterococcus* abundance was recovered at 5 DAP, but decreased again at 9 DAP (q-value <1e-15) (Figure 6).

Effects of parasitism by *Cotesia flavipes* in the potential functional contribution of the host gut microbiota

Analysis of the potential functional contribution of the gut microbiota of larvae of *D. saccharalis* demonstrates metabolites produced by gut microbes are altered by parasitization by *C. flavipes*, which could impact the larval physiology (Figure 7). Such alterations in the potential functional contribution of the microbiota associated with the anteromedial gut region of *D. saccharalis* larvae after parasitization occurred regardless the sampling time (Figure 7). The changes induced in the composition of larvae gut microbiota of *D. saccharalis* parasitized by *C. flavipes* reduced the potential nutritional contribution of the gut microbiota to the host, mainly due to the decrease in the pyruvate metabolism of the gut microbiota (p-value <1e-15). A decrease in the enzymatic contribution of the gut microbiota was also observed but not in 5DAP, in which no differences were observed (p-value = 0.173). However, pathways related with the *degradation of xenobiotics* and *defense* mechanisms were increased (p-value

<1e-15). The potential contribution of the gut microbiota to host *nutrition* was decreased at 5 and 9 DAP, while the contribution to *degradation of xenobiotics* and *defense* against pathogens increased in parasitized larvae (p-value <1e-15) . Changes in the potential functional contribution of the gut microbiota of the hindgut of *D. saccharalis* were also detected (Figure 7). The contribution of the microbiota of the hindgut to *biodegradation* increased only at 1 DAP, followed by a decrease at 5 and 9 DAP. The functional contribution of microbes from the hindgut to *defense* was increased only at 5 DAP (p-value <1e-15), but significantly reduced at 9 DAP (p-value <1e-15) (Figure 7).

The potential functional contribution of the gut microbiota of the nonpermissive host *S. frugiperda* was also affected (Figure 8). Changes in the potential contribution of the microbiota associated with the anteromedial gut region at 1DAP were similar to that of the permissive host, *D. saccharalis*, with an increase in the potential contribution to *biodegradation*, a reduced contribution to *nutrition* (p-value <1e-15), and no effects on *enzymes* production (p-value =1.067). On 5 DAP, there were no differences in *enzymes* production (p-value =0.173). Only *nutrition* was negatively affected on 5 DAP, but effects were reversed on 9 DAP (p-value <1e-15) (Figure 8). The potential functional contribution of the microbiota associated with the hindgut of *S. frugiperda* larvae was also altered (Figure 8). The potential contribution to *biodegradation* was increased while the remaining groups observed decreased at 1 DAP; at 5 DAP, the potential contribution to *defense* was increased (p-value <1e-15) and followed by *biodegradation* (p-value <1e-15); only *biodegradation* remained high at 9 DAP (p-value <1e-15) (Figure 8).

DISCUSSION

Parasitization by *C. flavipes* altered the composition and functional contribution of the microbiota associated with the anteromedial and posterior regions of the gut of the permissive *D. saccharalis* and the nonpermissive *S. frugiperda* hosts. The effects of parasitization on the gut microbiota were also influenced by the time after parasitization. Such effects indicate the mechanisms parasitoids employ to regulate their hosts in order to promote a suitable environment for offspring establishment and

development also result in alterations in host gut microbiota. Host parasitization by *Cotesia flavipes* led to quantitative and qualitative changes in the community of bacteria associated with the anteromedial and posterior regions of the gut of the studied hosts. Such changes resulted in alterations in the potential functional contribution of the gut microbiota of their hosts. Our data suggests the resulting changes in the contribution of the microbiota could benefit the natural enemy as a part of the process of host regulation, enhancing parasitoid survival and development. Thus, the host gut microbiota may be one more factor for consideration when studying the nutritional ecology of parasitoids, as well as being an interesting source of new processes for parasitoids to directly and/or indirectly regulate the host.

Alterations in the gut microbiota of the permissive *D. saccharalis* and nonpermissive *S. frugiperda* hosts induced by the parasitism of *C. flavipes* characterize the process of dysbiosis, i.e., a process in which there is an alteration in the balance of the members of the microbe community without any specific changes in microbe diversity (Tamboli *et al.*, 2004). These results indicate that alterations in the host's gut microbiota are independent of the evolutionary history of the host-parasitoid interaction or the success of parasitoids to exploit preferable hosts. However, the alterations in the gut microbiota of the nonpermissive host seems to be less conspicuous than those in the permissive host, *D. saccharalis*. Such differences are thought to relate with changes resulting from the required regulation of the host to support the growth and development of the parasitoid that occur in one host (permissive) but not in the other (nonpermissive). Thus, factors related to the growth and development of parasitoids must influence direct or indirectly the community of microbes associated with the host gut. During the growth and development of *C. flavipes*, several virulence factors produced by teratocytes and parasitoid larvae affect the host physiology (Dahlman, 1991; Asgari and Rivers, 2011; Mahmoud *et al.*, 2011) and such virulence factors or the changes they induce in the host could individually or jointly influence the gut microbe community.

Parasitization by *C. flavipes* results in the immunosuppression of the cellular and humoral responses (Lavine and Beckage, 1995; Mahmoud *et al.*, 2011; Passos *et al.*, 2014) Immunosuppression is mainly obtained through the inhibition of phenoloxidase activity, production of reactive oxygen species (ROS)

and expression of NF-κβ transcription factors (Gillespie And *et al.*, 1997; Kanost and Gorman, 2008; Ryu *et al.*, 2008). Inhibition of NF-κβ transcription factors negatively affects the Toll and IMD signaling pathways (Bae and Kim, 2009; Bitra *et al.*, 2012). Disruption of these pathways lower the levels of antimicrobial peptides (AMPs) produced by the host and increase host susceptibility to gut microbes (Shrestha *et al.*, 2009). ROS and AMPs are key regulators of the gut microbial community, and alterations in their availability in the gut would certainly interfere with the maintenance of a desired microbial community (Charroux and Royet, 2012). But the immune response capacity of hosts parasitized by *Cotesia* species is partially recovered towards to the end of the parasitoid´s larvae development (Lavine and Beckage, 1996; Mahmoud *et al.*, 2011). The recovery of the host immune response coincides with the slightly taxonomic differences in the gut microbiota of parasitized and non-parasitized hosts, as observed at 9 DAP.

Host nutrition is extremely important in modulating the structure of the gut microbiota and is also targeted for regulation by parasitoids. Parasitoid development depends on the efficiency of food acquisition and utilization by the host (Harvey *et al.*, 1995; Cônsoli and Vinson, 2004; Pennacchio *et al.*, 2014). *Cotesia flavipes* reduces the food intake of parasitized larvae but increases the time the food remains in the gut by reducing gut motility (Rossi *et al.*, 2014). In this case, retention of food in the gut allows parasitized larvae to have similar nutritional indices than unparasitized larvae (Rossi *et al.*, 2014). The high efficiency in food utilization in parasitized larvae with reduced food intake has been argued to be a consequence of the prolonged exposure of the food to the host digestive enzymes, due to the reduction in the motility of the gut and the regulation digestive enzymes (Dillon and Dillon, 2004; Engel and Moran, 2013; Rossi *et al.*, 2014; Yun *et al.*, 2014). These changes contribute to modifications in the gut microbiota (Dillon and Dillon, 2004; Engel and Moran, 2013; Rossi *et al.*, 2014; Yun *et al.*, 2014), and could influence the microbiota of hosts parasitized by *C. flavipes*. Stress is another source of variation that regulates the gut microbiota in humans (Bailey *et al.*, 2011) and the changes parasitoids induce in their parasitized hosts, including significant alterations in the host hormonal balance (Pfister-Wilhelm and

Lanzrein, 1996; Lee and Kim, 2004), are sources of physiological stress. Moreover, bacteria are known to have a bidirectional relationship with the gut sensorimotor function, not only by responding to changes in the gut motor activity, but also severely influencing the gut sensorimotor function when the gut microbiota changes (Quigley, 2011). However, additional research would be required to investigate if the dysmotility of the gut of *D. saccharalis* parasitized larvae is a result of regulation of the host neuropeptides involved in gut motility, and the consequent alterations in the gut microbiota or if dysmotility is induced by the alterations of the gut microbiota induced by host parasitization.

Nevertheless, dysbiosis leads to drastic changes in host metabolism (Schilder and Marden, 2007; Sommer and Bäckhed, 2013). Dysbiosis can impair the contribution of the gut microbiota to the defense against pathogens (Azambuja *et al.*, 2004; Azambuja *et al.*, 2005; Cirimotich, Ramirez, *et al.*, 2011; Bahia *et al.*, 2014), metabolism of xenobiotics (Nikoh *et al.*, 2011; Adams *et al.*, 2013), food digestion (Kanost and Gorman, 2008; Anand *et al.*, 2010; Krishnan *et al.*, 2014), and nutritional supplementation through nitrogen recycling, production of vitamins, and other nutrients (Ohkuma *et al.*, 1996; Eichler and Schaub, 2002; Douglas, 2006; Hongoh *et al.*, 2008; Nikoh *et al.*, 2011). Dysbiosis induced in hosts parasitized by *C. flavipes* altered the potential functional contribution of the host gut microbiota and the consequent changes in the metabolism of the host may have direct and/or indirect beneficial roles in parasitoid development. Host-induced dysbiosis in hosts parasitized by *C. flavipes* could benefit the parasitoid through reduction of the host metabolic costs involved in food processing, mainly due the increased production of detoxifying enzymes that are reported to degrade anti-herbivory compounds (Hallahan and West, 1995; Thoss and Byers, 2006; Ibrahim *et al.*, 2008) or to act on a range of substrates (Feyereisen, 1999; Anzenbacher and Anzenbacherova, 2001). Additionally, the increased representation of polyketide synthesis pathways in the gut microbiota of parasitized larvae indicates a potential increase of biosynthesis of antibiotics (Bangera and Thomashow, 1999), would result in an environment better protected against pathogenic interactions and in a healthier environment for parasitoid development. Increased xenobiotic metabolization and degradation and antibiotic biosynthesis by the gut microbiota in

larvae of *D. saccharalis* parasitized by *C. flavipes* would aid the production of a more suitable environment for the natural enemy development, without an increased need of energy investment by the insect host, thereby favoring parasitoid development.

Differences in the functional contribution of the gut microbiota between the parasitized and unparasitized permissive host were detected even when no significant changes in the abundance of the taxonomic groups of the gut microbiota were observed. The gut microbiota of the larval anteromedial gut region of *D. saccharalis* parasitized by *C. flavipes* had a reduced contribution to *nutrition*, mainly due to the decrease in the metabolism of pyruvate of the gut microbes.

Nevertheless, the host suffers major stage-specific alterations when parasitized in addition to natural variations linked to their own development, which influence the host's gut microbiota. During the initial stages of parasitization, hosts are subjected to the maternal virulence factors, but upon egg eclosion virulence factors produced by teratocytes and the parasitoid larvae are also released, and affect the physiology of the host (Pinheiro *et al.*, 2010; Strand, 2014). Thus, the developing parasitoid requires an active nutritional environment to support its development as the nutritional requirements differs during the growth and development of the parasitoid (Vinson and Iwantsch, 1980b; Harvey and Malcicka, 2016). The molting process is an important factor to influence the gut microbiota of larvae of holometabolous insects (47). The parasitized nonpermissive host *S. frugiperda* does not stop molting during parasitoid development. And molting from one instar to another has been reported as a modulator of the gut microbiota (Hammer *et al.*, 2014). In the parasitized permissive host *D. saccharalis* no molt occurred as the host larvae were parasitized early in the last instar. Nevertheless, the physiological changes the larvae goes in preparation to the final molt to pupae are intense and induce changes in the gut microbiota (Johnston and Rolff, 2015).

The gut microbiota identified in association with *D. saccharalis* and *S. frugiperda* shared bacteria at the phylum-level with hundreds of species of insects belonging to 21 taxonomic orders, with

Proteobacteria and *Firmicutes* being the dominant representatives in the gut microbiota of insects (Yun *et al.*, 2014). The differences found between the gut microbiota in *D. saccharalis*, predominantly represented by *Proteobacteria*, and in *S. frugiperda*, predominantly represented by *Firmicutes*, may be due to differences related to their phylogeny, feeding habits and the diverse food substrates these insects feed on. These are the main factors reported to shape the composition of the gut microbiota of insects (Yun *et al.*, 2014). The observed differences in the prevalence of *Proteobacteria* and *Firmicutes* in the gut microbiota seem to occur naturally among the different representatives of Lepidoptera. *Proteobacteria* was the predominant group in most of the analyzed representatives of Lepidoptera, while *Firmicutes* prevailed in about 1/3 of the studied lepidopteran species (Yun *et al.*, 2014).

Little is known regarding the role of gut bacteria in most insects, including lepidopterans. In the case of Lepidoptera, *Enterococcus* has been reported as a common associate. *Enterococcus* is a facultative and dominant anaerobic genus in the gut microbiota of *S. frugiperda*, with more than half of its sequences identified in all the larval ages analyzed here. *Enterococcus* has also been reported as a dominant member of the gut microbiota of *Spodoptera littoralis*, and suggested to contribute to host protection against pathogens due the synthesis and release of antimicrobial peptides (Shao et al., 2014). Curiously, *Enterococcus* was also significantly favored in the gut of larvae of *S. frugiperda* parasitized by *C. flavipes*, particularly in the anteromedial region where this bacterial genus was highly abundant.

CONCLUSIONS

The gut microbiotas of the permissive *Diatraea saccharalis* and the nonpermissive *Spodoptera frugiperda* hosts are dominated by different bacterial groups. *Proteobacteria* predominates in the permissive host, and *Firmicutes* in the nonpermissive host. The permissive and the nonpermissive hosts suffer from dysbiosis when parasitized by *Cotesia flavipes.* Alterations in the balance of the gut microbial community in parasitized hosts affect the potential functional contribution of the gut microbiota. Changes in the potential functional contribution of the gut microbiota in parasitized larvae are suggested to benefit

parasitoid development, although differences the biological significance of the variations detected is yet to be test. The mechanisms inducing host dysbiosis remain unclear, although the reported dysmotility observed in the permissive host parasitized by *C. flavipes* may serve as a source of alteration of the gut microbiota.

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Figure 1 - UPGMA cluster analysis of unweighted (A, B) and weighted (C, D) Unifrac distances. The colors of the nodes of UPGMA analysis represent the bootstrap support: red: 75-100%; Yellow: 50-75% bootstrap support. The pie charts in "C" and "D" illustrate the relative abundance of the microbiota of the gut anteromedial region (ANT) (C) and hindgut (POS) (D) of *D. saccharalis* larvae at the phylum level, after different periods of development (1, 5 and 9 days) parasitized (P) or not (C) by *C. flavipes*.

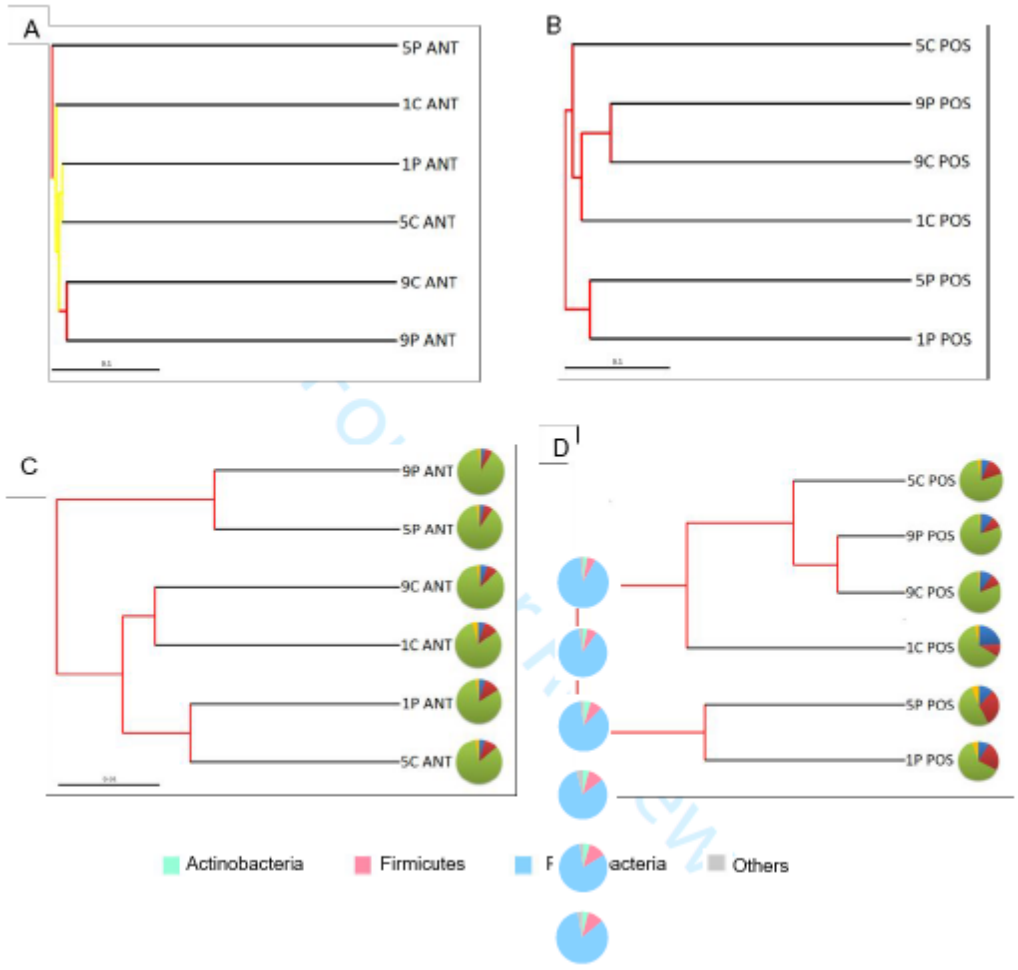

Figure 2 - UPGMA cluster analysis of unweighted (A, B) and weighted (C, D) Unifrac distances. The colors of the nodes of UPGMA analysis represent the bootstrap support: red: 75-100%; Yellow: 50-75% bootstrap support. The pie charts in “C” and “D” illustrate the relative abundance of the microbiota of the gut anteromedial region (ANT) (C) and hindgut (POS) (D) of *S. frugiperda* larvae at the phylum level, after different periods of development (1, 5 and 9 days) parasitized (P) or not (C) by *C. flavipes*.

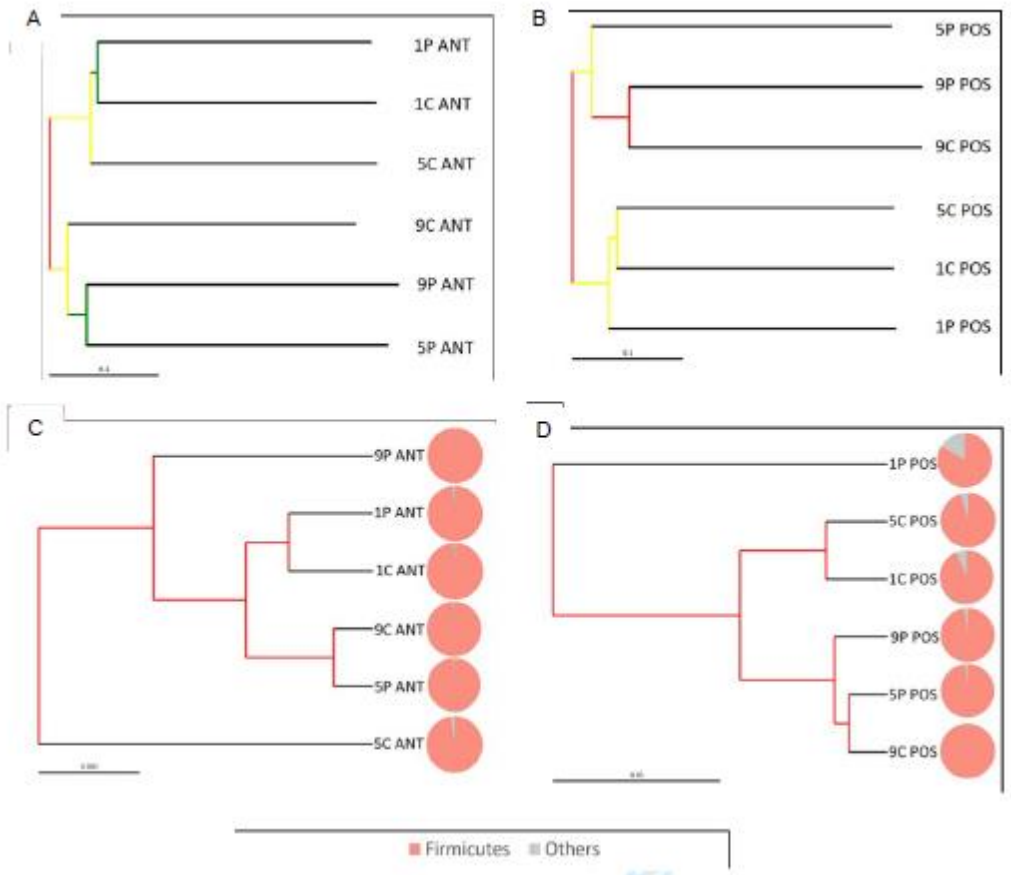

Figure 3 - Relative abundance (%) of bacterial genera of the microbiota of the gut anteromedial region (A, B) and the larval hindgut (C, D) of *D. saccharalis* parasitized (B, D) or not (A, C) by *C. flavipes*.

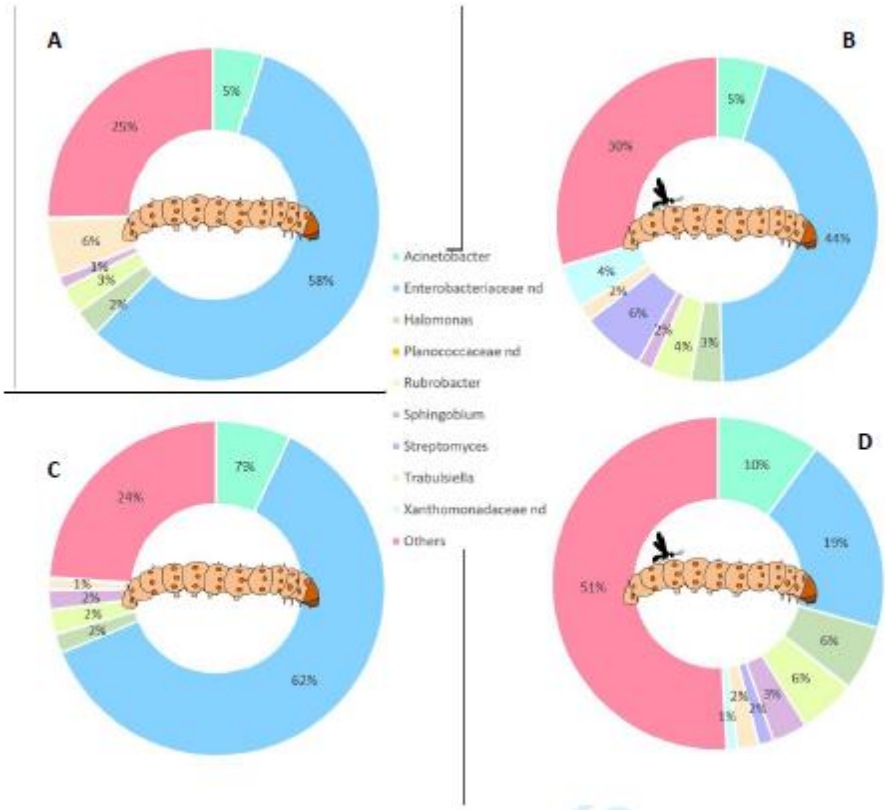

Figure 4 - Differences in relative proportion (%) of bacterial genera with 5% minimal abundance in the microbiota of the gut anteromedial region (ANT) (A) and the hindgut (POS) (B) of *D. saccharalis* larvae in different periods of development (1, 5 and 9 days), parasitized (P) or not (C) by *C. flavipes.*

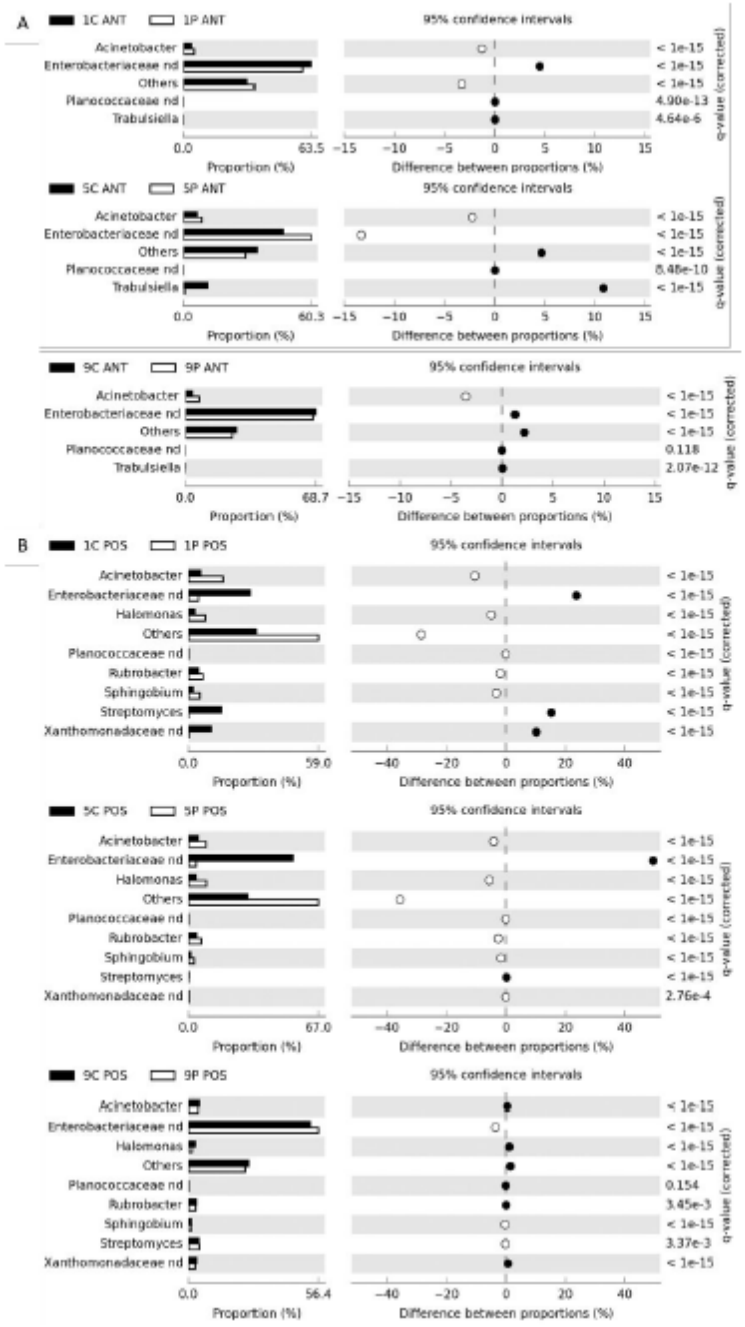

Figure 5 - Relative abundance (%) of bacterial genus of the microbiota of the gut anteromedial region (A, B) and the larval hindgut (C, D) of *S. frugiperda* parasitized (B, D) or not parasitized (A, C) by *C. flavipes.*

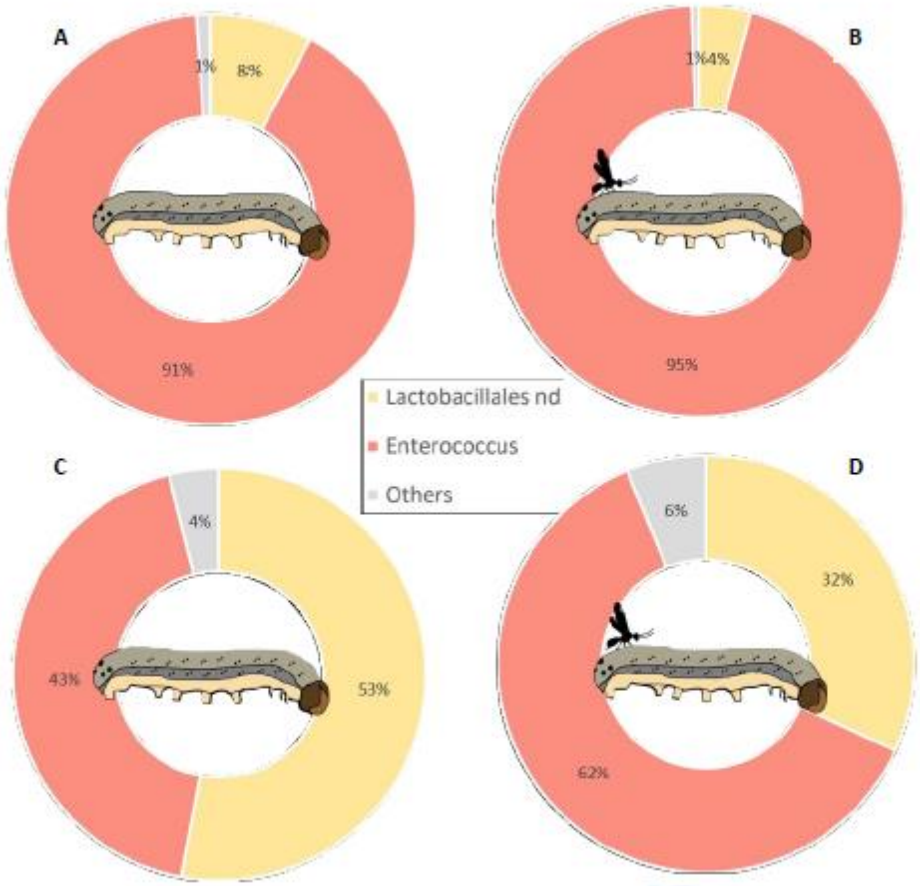

Figure 6 - Differences in relative proportion (%) of bacterial genera with 5% minimal abundance in the gut microbiota of anteromedial (ANT) (A) and hindgut (POS) (B) of *S. frugiperda* larvae in different periods of development (1, 5 and 9 days) after being parasitized (P) or not (C) by *C. flavipes*.

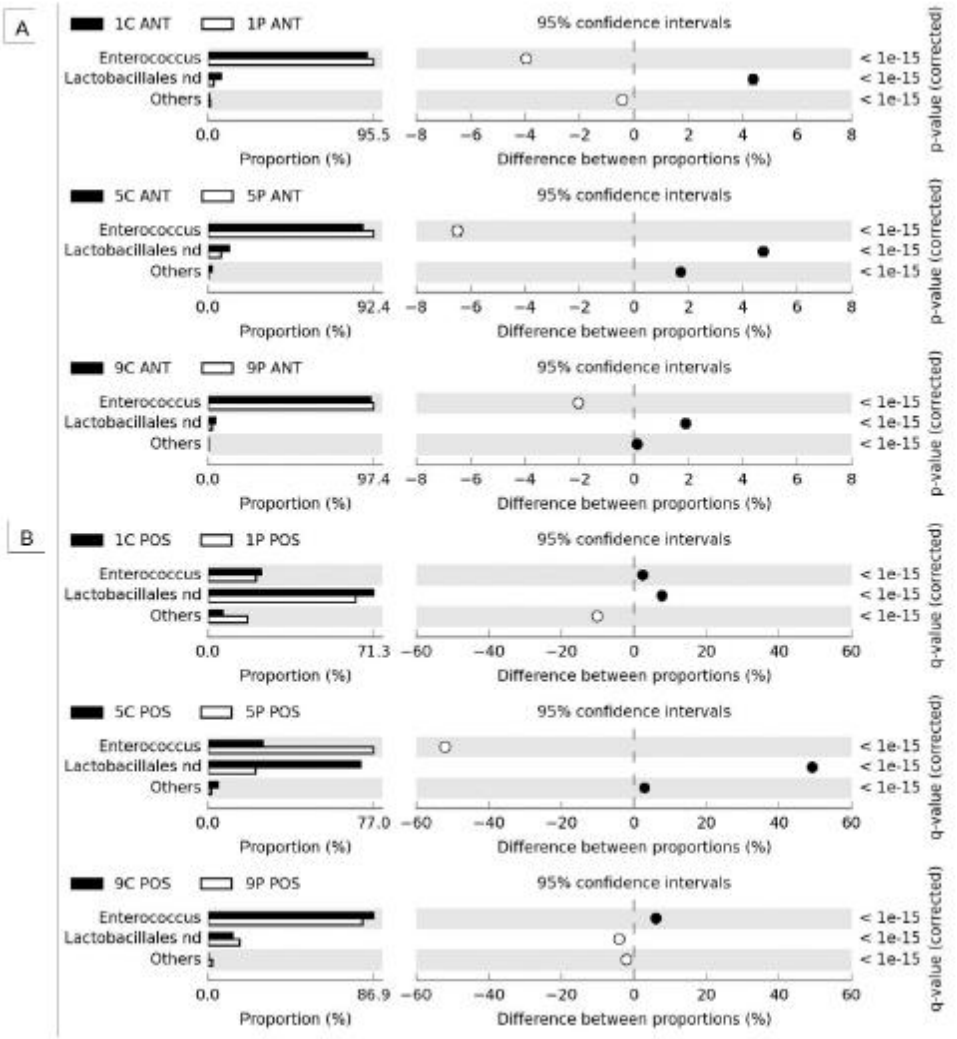

Figure 7 – Differences in the potential functional contribution (%) of the microbiota from the gut anteromedial region (A) and the hindgut (B) of *D. saccharalis* larvae in different periods of development (1, 5 and 9 days) after being parasitized (P) or not (C) by *C. flavipes* in relation to *biodegradation*, *defense*, *nutrition* and *production of enzymes*.

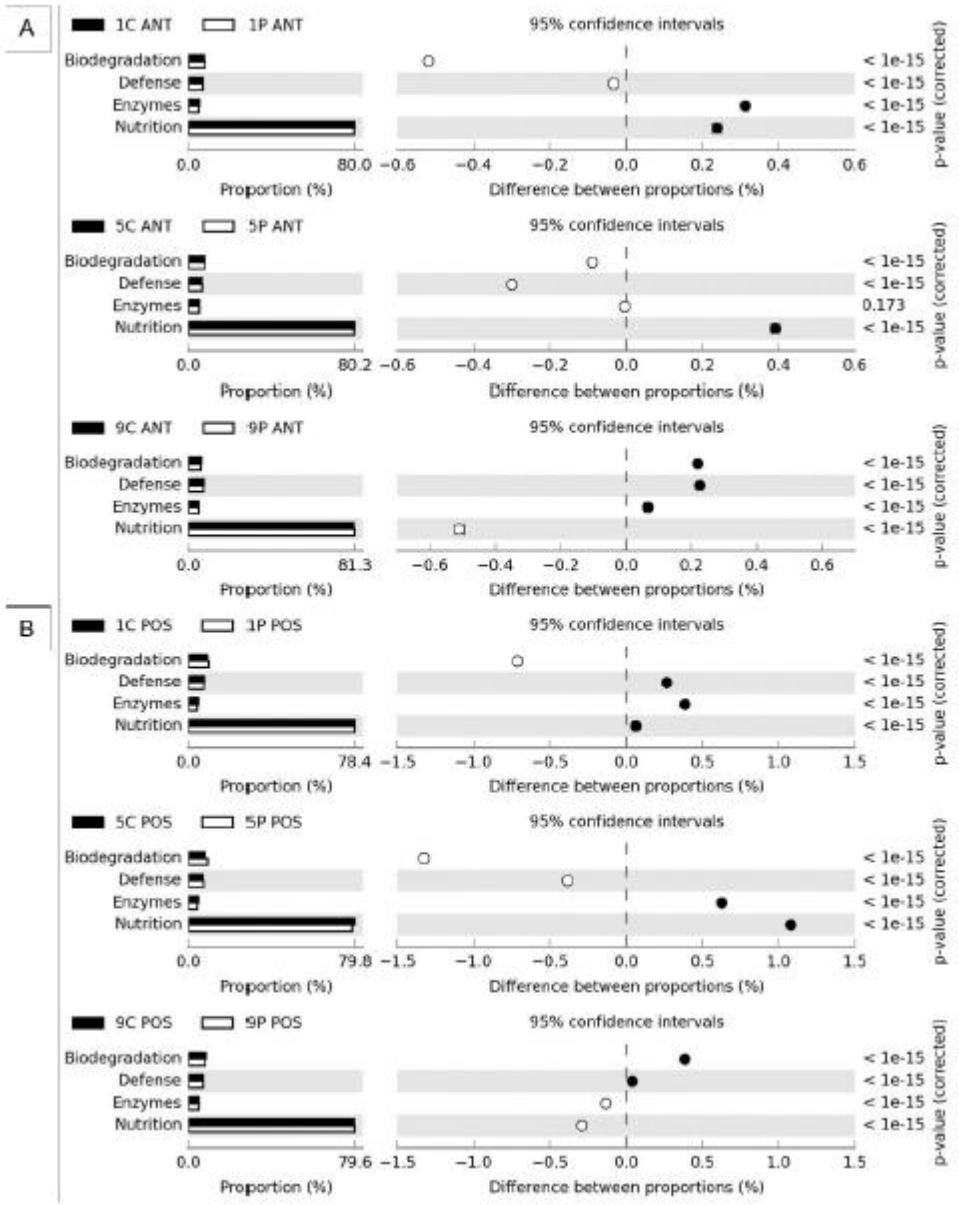

Figure 8 – Differences in potential functional contribution (%) of the microbiota from the gut anteromedial region (A) and the hindgut (B) of *S. frugiperda* larvae in different periods of development (1, 5 and 9 days) after being parasitized (P) or not (C) by *C. flavipes* in relation to *biodegradation*, *defense*, *nutrition* and *production of enzymes*.