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Comparative gut microbiota analysis in newly emerged adults of five economically important *Anastrepha* species (Diptera: Tephritidae)

Daniel Cerqueda-García and Martín Aluja

Red de Manejo Biorracional de Plagas y Vectores, Clúster Científico y Tecnológico BioMimic®, Instituto de Ecología, A.C. – INECOL, Xalapa, Veracruz, Mexico

Research Paper

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Corresponding author:
Daniel Cerqueda-García;
Email: daniel.cerqueda@inecol.mx
Martín Aluja;
Email: martin.aluja@inecol.mx

Abstract

This study investigates the gut microbiota of newly emerged adult females and males of five economically important *Anastrepha* species (Tephritidae) – *A. ludens, A. obliqua, A. serpentina, A. striata*, and *A. fraterculus* – by analyzing 16S rRNA amplicon sequences from 36 samples collected from ecologically relevant fruit hosts and locations in Mexico. We chose to concentrate only on newly emerged adults to identify bacteria that females could potentially transmit vertically to progeny via oviposition, a topic that remains poorly studied. Results revealed that Proteobacteria dominated the microbiota in all species, but substantial variation was observed in genus-level composition. Differentially abundant genera included *Enterobacter, Gluconobacter, Tatumella, Providencia, Ochrobactrum, Siccibacter, Sphingobacterium*, and *Sphingobium*. Significant differences in alpha diversity were observed between species, particularly between *A. obliqua* and *A. striata*, and between *A. obliqua* and *A. serpentina* based on the Shannon index. *Anastrepha ludens, A. obliqua*, and *A. striata* males exhibited higher species richness than females, although these differences were not statistically significant within individual species likely due to limited sample size. Interestingly, across all species, significant differences in microbiota composition were observed between males and females. Our findings suggest that morphological, physiological (i.e., metamorphosis) and ecological factors, such as possible gut structural differences and host fruit preferences, may influence the composition of the gut microbiota, potentially affecting the ecological adaptability and pest behavior of these flies.

Introduction

Tephritid fruit flies stand out as one of the most economically significant pests globally, infesting a wide variety of fruit and vegetable crops and triggering strict quarantine measures that severely restrict international trade (Dyck *et al.*, 2005; Clarke, 2019; Perez-Staples *et al.,* 2019). The genus *Anastrepha* (Diptera: Tephritidae) includes some of the most economically significant fruit pests in the Americas due to their ability to infest a wide variety of fruits, leading to substantial economic losses (Aluja, 1994). Among the more than 300 species within this genus (Norrbom *et al.*, 2018), *A. ludens, A. obliqua, A. serpentina, A. striata*, and *A. fraterculus* are particularly pestiferous. These species infest economically important fruits such as mango, citrus, guava and various species within the Sapotaceae (*A. serpentina*), causing direct damage through oviposition and larval feeding, which renders the fruit unmarketable. This damage also triggers strict quarantine restrictions, further exacerbating the economic impact by hindering international trade (Aluja *et al.*, 2014; Aluja and Mangan, 2008; Mello-Garcia, 2024).

From an evolutionary perspective, these species display varying degrees of specialization and adaptability to different host plants (Aluja and Mangan, 2008; Birke and Aluja, 2018). Phylogenetically, *A. serpentina* and *A. striata* are considered more ancestral within the *Anastrepha* genus (Mengual *et al.*, 2017; Norrbom, 2002), showing a closer relationship to host plants within restricted families. *Anastrepha striata* is classified as oligophagous because females primarily lay eggs into fruit within a single plant family (Aluja and Mangan, 2008). *Anastrepha striata* specializes in fruit within the Myrtaceae, particularly the genus *Psidium*, and *A. serpentina* within the Sapotaceae (Birke and Aluja, 2011). In contrast, *A. ludens* and *A. fraterculus* represent more derived species within the *fraterculus* group (Mengual *et al.*, 2017), which exhibit greater adaptability and polyphagy, allowing them to exploit a wider range of fruit hosts across different genera (Birke and Aluja, 2018). *Anastrepha obliqua* is also considered polyphagous but is more specialized compared to *A. ludens* and *A. fraterculus*, as it primarily infests fruits within

the Anacardiaceae family (Aluja and Mangan, 2008; Birke and Aluja, 2011; McPheron *et al.*, 1999). These evolutionary relationships highlight the complex host-plant interactions that have shaped the ecological niches of these species, influencing their capacity to adapt to new hosts and enhancing their pestiferous status.

Recent studies have highlighted the likely role of gut microbiota in these evolutionary processes. The microbiota associated with tephritids species not only supports nutrient assimilation but also plays a vital role in the flies' interactions with their host plants, and this symbiotic relationship is crucial for the flies' ability to colonize new fruits and expand their host range (Behar *et al.*, 2005; Cárdenas-Hernández *et al.*, 2024; Nikolouli *et al.*, 2020; Ochoa-Sánchez *et al.*, 2022). The concept of phylosymbiosis in which the structure of microbial communities reflects the phylogenetic relationships of their host species, has been observed in various insect groups. Phylosymbiosis suggests that as the genetic differences between host species increase, so do the differences in their associated microbiota (Brooks *et al.*, 2016). This pattern indicates that the microbiota evolves in tandem with the host, potentially providing adaptive advantages such as enhanced digestion and detoxification, which could facilitate the infestation of both native and exotic fruit hosts (Lim and Bordenstein, 2020; Mazel *et al.*, 2018; Raza *et al.*, 2020).

In a recent study, Ventura *et al.* (2018) concluded that the microbiota's composition in four species of *Anastrepha* (*A. ludens, A. obliqua, A. serpentina* and *A. striata*) is influenced by the host plant and environmental conditions. These authors indicated that at community level, bacterial diversity in adult flies was higher than the one found in larvae, which is surprising. Likely the technology used (pyrosequencing), and the fact that they used adult individuals captured in traps (not newly emerged) resulted in this finding. Aluja *et al.* (2021) went a step further performing a study on the gut microbiota of larvae and newly emerged adults in the Mexican fruit fly, *A. ludens* stemming from wild ancestral, commercially grown and marginal, toxic hosts. They concluded that the host plant greatly influenced larval gut microbiota, and importantly, that metamorphosis from larvae to adult, changed gut microbiota in adults, significantly reducing its diversity (contradicting the results by Ventura *et al.*, 2018). The pioneering studies by Petri (1909), Hagen (1966), Girolami (1973), and Lauzon *et al.* (2009), and many additional more recent studies (and references therein; e.g., Behar *et al.*, 2008; Ben-Yosef *et al.*, 2015; Majumder *et al.*, 2019; Mason *et al.*, 2023; Nikolouli *et al.*, 2020; Raza *et al.*, 2020) have flushed out the incredible complexity of the topic of bacterial-tephritid fly associations and their role in metabolic processes (among them degradation of toxic allelochemicals), overall fitness, and their potential control.

In this study, we explored the gut microbiota of newly emerged/teneral adults of five *Anastrepha* species (*A. ludens, A. obliqua, A. serpentina, A. striata*, and *A. fraterculus*) by amplicon sequencing of the 16S rRNA gene. We only concentrated on newly emerged adults and not the larvae, as Aluja *et al.* (2021) recently showed that during the process of metamorphosis, many bacteria present in the guts of larvae are lost, and consequently, the diversity of bacteria in the guts of adults is much reduced. This has important ecological connotations as females are known to vertically transmit bacteria to the progeny via de eggs (e.g., Aharon *et al.*, 2013; Hassan *et al.*, 2020; He *et al.*, 2022a; Lauzon *et al.*, 2009; Sacchetti *et al.*, 2019), and some of these bacteria partially inoculate the pulp in which the larvae will develop. The latter, on top of the many additional bacteria that are gathered by larvae from the host pulp (e.g., Aluja *et al.*, 2021; Ochoa-Sánchez *et al.*, 2022). The samples were collected from various fruit hosts across distant regions from Mexico, to provide a glimpse of the bacterial communities associated with these economically important pests. Our study represents only an initial step at better understanding a complex phenomenon in economically important species of genus *Anastrepha*, particularly from the perspective of newly emerged adults. This topic warrants further investigation due to its evolutionary, ecological, and pest management relevance. We hypothesized that given their close phylogenetic relationship, the gut microbiota of newly emerged *A. ludens, A. obliqua* and *A. fraterculus* adults, would be more similar that the one found in *A. serpentina* and *A. striata*, each one belonging to different phylogenetic groups within *Anastrepha*.

Materials and methods

Naturally infested fruit collection

Naturally infested fruits were collected in the field to obtain the five species of commercially important fruit flies in Mexico. Thirty-two kilograms (kg) of 'Ruby Red' grapefruit (*Citrus x paradisi* Macf., Sapindales: Rutaceae) were collected in Cuautla, Morelos for *A. ludens*; 38 kg of 'Criollo' mango (*Mangifera indica* L., Sapindales: Anacardiaceae) in Los Ídolos, Actopan, Veracruz for *A. obliqua*; 50 kg of guava (*Psidium guajava* L., Myrtales: Myrtaceae) in Cuajilote, Jamapa, Veracruz for *A. striata*; 5.6 kg of pear guava (*P. guajava*) in Loma Bonita, Ocosingo, Chiapas for *A. fraterculus*; and 68 kg of mamey sapote (*Pouteria sapota* Jacq., Ericales: Sapotaceae) in Izapa, Tuxtla Chico, Chiapas for *A. serpentina* (table 1 and fig. 1).

Sample processing

For adult sampling, fruits with signs of infestation were collected directly from the tree in the field, transported to the fruit processing laboratory of the Biorational Pest and Vector Management Network – INECOL and processed as described Aluja *et al.* (2021). Collected fruits were placed in plastic baskets over plastic trays (length: 35 cm, width: 30 cm, height: 13 cm) containing a thin layer of sterile vermiculite as pupation substrate. Every day, pupae were separated and placed in clean plastic containers (250 mL) with sterile vermiculite. Pupae were sprayed with sterile distilled water every third day until the emergence of adults. Guts (from the cardia to the anus) of newly emerged adults were dissected and preserved in RNA later at − 80 °C until DNA processing. Each sample consisted of two female and male guts, with five replicates per sex.

DNA isolation, 16S rRNA gen amplification and sequencing

DNA from each sample was isolated using QIAamp® DNA Mini Kit (Qiagen GmbH, Hilden, NRW, Germany). DNA (100 ng) was used for 16S rRNA gene amplification using primers based on Klindworth *et al.* (2013) containing Illumina adapter overhang nucleotide sequences (Illumina Inc.©): 16S Amplicon PCR Forward = 5′ TCGTCGGCAGCGTCAGAT GTGTATAAGAGACAGCCTACGGGNGGCWGCAG and 16S Amplicon PCR Reverse = 5′ GTCTCGTGGGCTCGGAGA TGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC.
PCR reaction (50 µL) consisted of Qiagen buffer 1X, Qiagen dNTPs 0.2 mM, 16S Amp F & R 0.2 µM, Qiagen Taq polymerase 2.5 U. PCR cycles used were: an initial 94 °C/2 min denaturation step; 25 cycles of 94 °C/15 sec, 55 °C/30 sec, and 72 °C/1 min;

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Table 1. Host fruit and details on collection sites in Mexico from where the five fruit fly species studied stemmed

Species	Host fruit	Collection site	Coordinates	Altitude (masl)
A. striata	*Psidium guajava*	Cuajilote, Jamapa, Veracruz	19° 2′35.70′′N 96°12′8.37′′W	12
A. ludens	*Citrus* x *paradisi*	Cuautla, Morelos	18°47′41.02′′N 98°57′15.95′′W	1307
A. fraterculus	*Psidium guajava*	Loma Bonita, Ocosingo, Chiapas	16° 4′59.69′′N 90°59′58.18′′W	196
A. obliqua	*Mangifera indica*	Los Idolos, Actopan, Veracruz	19°24′53.78′′N 96°31′6.36′′W	93
A. serpentina	*Pouteria sapota*	Izapa, Tuxtla Chico, Chiapas	14°49′48.59′′N 92°19′46.08′′W	53

masl: meters above sea level.

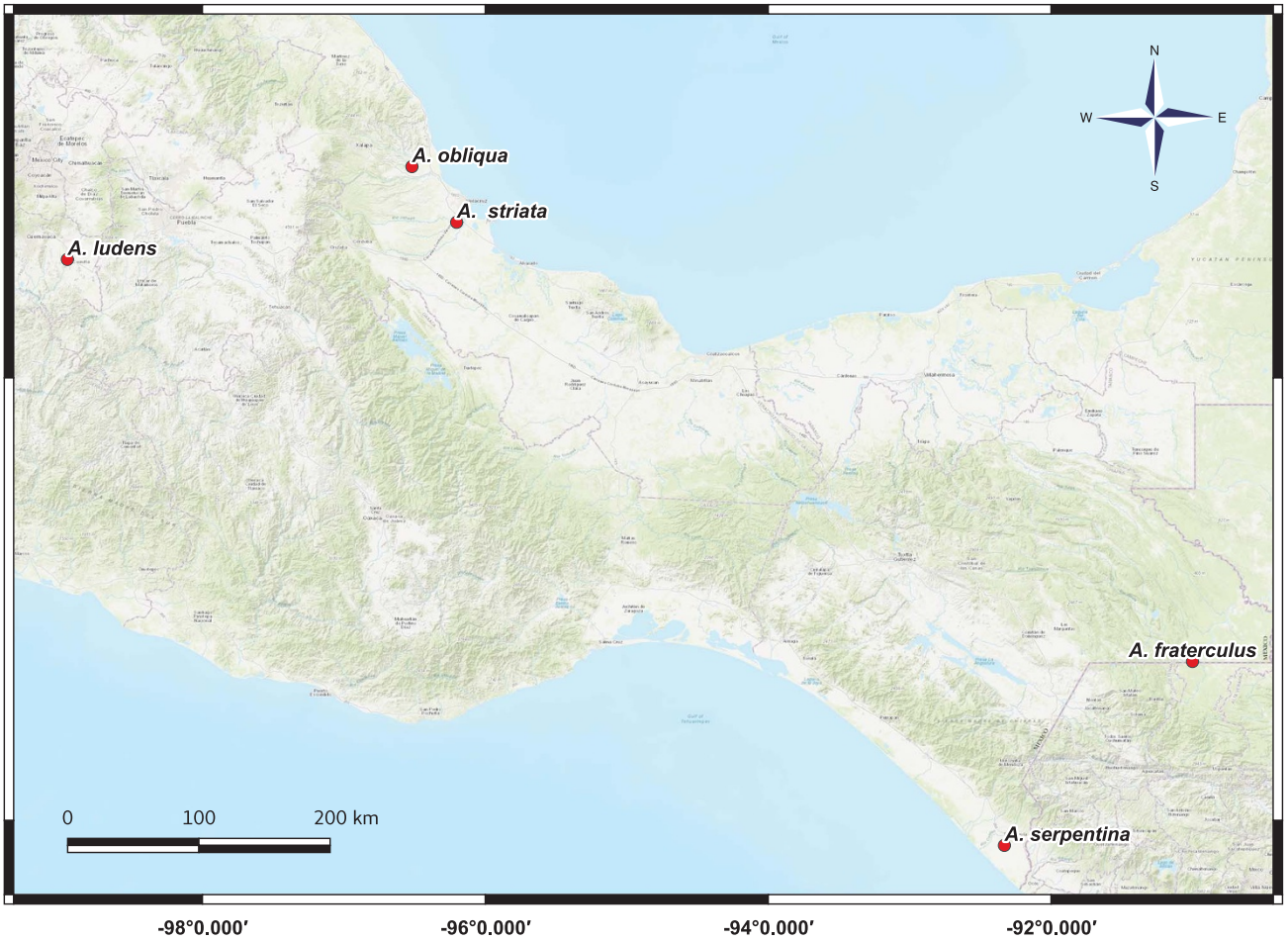

Figure 1. Map of sampled sites across Mexico to collect infested hosts of the five economically important *Anastrepha* species studied here. *Anastrepha ludens*: Cuautla, Morelos; *A. obliqua*: Los Ídolos, Actopan, Veracruz; *A. striata*: Cuajilote, Jamapa, Veracruz; *A. fraterculus*: Loma Bonita, Ocosingo, Chiapas; *A. serpentina*: Izapa, Tuxtla Chico, Chiapas. Note the great distance between collection sites which added ecological value to our sampling scheme and rendered our comparisons among species more robust.

and a final 72 °C/5 min elongation step. Amplicons were purified with the Wizard® SV gel and PCR clean-up system (Promega, Madison, WI, USA). Library preparation and sequencing were performed at the Sequencing Unit of INECOL by adding individual indexes (Nextera XT Index Kit v2 set A, Illumina Inc.) per sample using the NEB Q5 Hot Start High-Fidelity 2X Master Mix and polymerase (New England Biolabs, M0494). All libraries were immediately purified using 0.8X Ampure XP (Beckman coulter, A63881). Libraries were quantified afterwards using an Invitrogen Qubit 2.0 system with the DNA High-Sensitivity kit (Invitrogen, Q32853) and library presence and size was confirmed using a Tapestation 2100 system (Agilent, G2964AA) running a DNA HS screen tape (Agilent, 5067- 5584) with DNA HS reagents (Agilent, 5067- 5585). All libraries were normalized and pooled to an equimolar concentration before diluting to 12 pM following the standard normalization method and loaded in an Illumina

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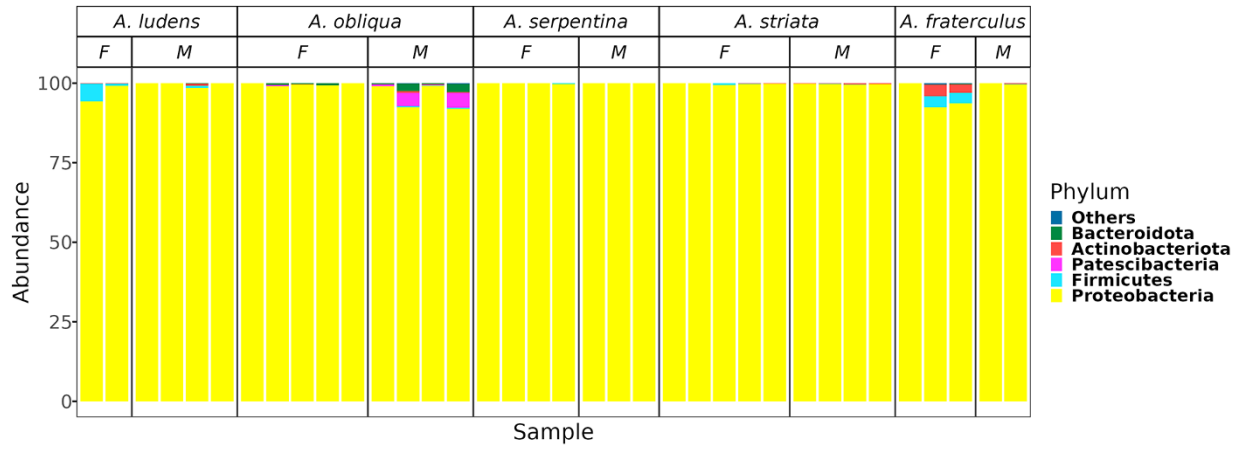

Figure 2. Relative bacterial abundance represented at phylum taxonomic level in the guts of newly emerged adults of five economically important *Anastrepha* species.

MiSeq sequencer using a MiSeq reagent kit v3 flow cell and reagents (Illumina, MS-102-3003) alongside a 20% PhiX control v3 (Illumina, FC-110-3001) spike to improve run performance.

Bioinformatic analyses

The raw paired end reads (2 × 300 bp) obtained from Illumina sequencing were processed using the QIIME2 platform (v.2020.6) (Bolyen *et al.*, 2019). The reads were denoised and trimmed with the DADA2 plugin (Callahan *et al.*, 2016) to resolve amplicon sequence variants (ASVs). Forward reads were trimmed at position 20 from the 5′ end and truncated to 270 bp, while reverse reads were trimmed at position 20 and truncated to 200 bp. Chimeric sequences were removed using the 'consensus' method, and other DADA2 parameters were kept at their default values.

The taxonomic classification of the ASVs was conducted using the classify-consensus-v-search plugin (Rognes *et al.*, 2016) against the SILVA v138 database (Quast *et al.*, 2013). A phylogenetic tree of the representative ASVs was constructed using the align-to-tree-mafft-fasttree plugin, which aligns sequences with MAFFT (Katoh, 2002) and constructs a tree using FastTree2 (Price *et al.*, 2010). The resulting ASV abundance table and phylogeny were exported to the R environment (v.4.1.2) for further analyses.

In R, the phyloseq package (McMurdie and Holmes, 2013) was used to assess alpha and beta diversity metrics. Importantly, plastid and mitochondrial ASVs were filtered out to focus on gut-specific microbiota. The dataset was normalized using rarefaction at a depth of 5000 reads per sample to account for differences in sequencing depth. The relative abundances of bacterial taxa at various levels were visualized with bar plots generated by the ggplot2 package (Wickham, 2011). Alpha diversity indices, including observed species and Shannon index, were calculated. Beta diversity was assessed using weighted and unweighted UniFrac distance matrices, and statistical differences were tested using PERMANOVA in the vegan package (Oksanen, 2015), with pairwise comparisons adjusted via the Benjamini-Hochberg method. Principal Coordinate Analysis (PCoA) plots were generated to visualize sample ordination. Differentially abundant genera between species were identified using LEfSe (Segata *et al.*, 2011), with statistical significance set at p-value < 0.05. All the raw data generated was deposited in the SRA platform under the BioProject PRJNA1292975.

Results

Overall bacterial taxonomic composition in the five Anastrepha species

We obtained a total of 36 valid samples, yielding 3,681,897 high-quality reads. The samples were normalized to a depth of 5,230 reads, resulting in a total of 648 ASVs. The phylum-level abundance was consistent and similar across the five fly species. Phyla with a relative abundance of at least one percent in any sample, were considered dominant. Using this criterion, we identified five dominant phyla. Considering their mean relative abundance across all samples, these were Proteobacteria (98.76%), Firmicutes (0.44%), Patescibacteria (0.28%), Actinobacteriota (0.26%), and Bacteroidota (0.02%) (fig. 2).

At the genus level, the variability within species and between sexes was much more pronounced (fig. 3). The most abundant genera, represented by their mean abundance, were as follows: In *A. ludens, Enterobacter* (47.88%) in females and *Ochrobactrum* (48.49%) in males. For *A. obliqua, Enterobacter* was the most abundant in both females (72.51%) and males (72.32%). In *A. serpentina, Providencia* was the predominant genus in both females (65.01%) and males (93.23%). In *A. striata*, the most abundant genera were *Klebsiella* (22.21%) in females and *Ochrobactrum* (62.24%) in males. Lastly, in *A. fraterculus, Klebsiella* (33.28%) in females and *Kosakonia* (49.88%) in males were the most abundant.

Differences in alpha and beta diversity among five tephritid species

Observed species (richness) was highest in *A. ludens*, with a decreasing trend noted in *A. obliqua, A. fraterculus, A. striata* and *A. serpentina* (fig. 4). Interestingly, *A. serpentina*, specializing in fruits within the Sapotacea exhibited the lowest diversity in both sexes. However, significant differences were only observed between *A. obliqua* and *A. serpentina* (table S1, Supplementary material). For the Shannon index, significant differences were noted between *A. obliqua* and *A. striata* (see fig. 4 and table S1 in Supplementary material). Differences in alpha diversity between sexes within each species were also observed, with a trend toward higher richness in males of *A. ludens, A. obliqua*, and *A. striata* compared to females. Despite this trend, there was no statistical significance within each

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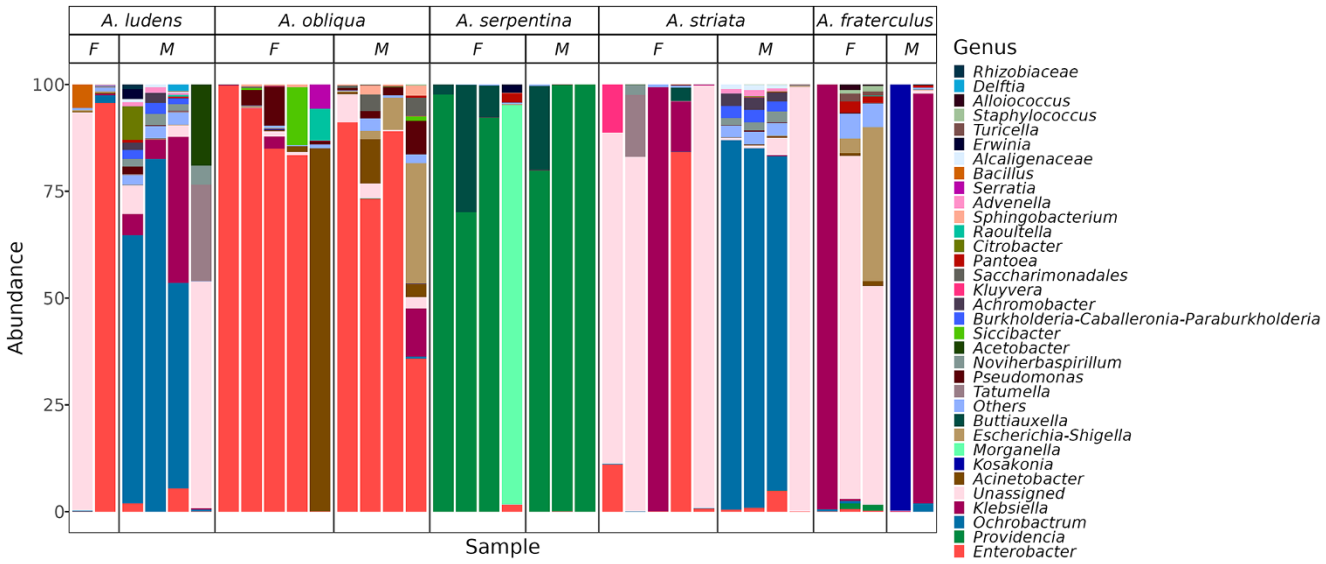

Figure 3. Relative bacterial abundance represented at genus taxonomic level found in the guts of newly emerged adults of five economically important *Anastrepha* species.

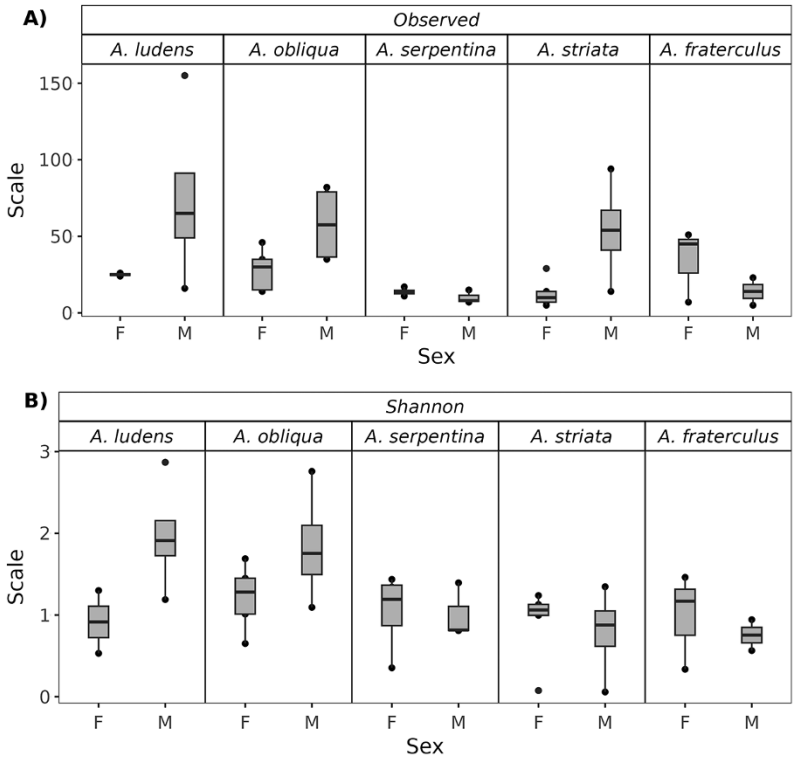

Figure 4. Boxplots of the alpha diversity indexes (Figure 4A Observed species and Figure 4B Shannon index) of bacteria found in the guts of newly emerged adults of across the five economically important *Anastrepha* species.

species, likely due to small sample size. However, a significant difference was found between males and females across all species combined (t-test p-value = 0.021). Alpha diversity among females across all species was more homogeneous, with no significant differences found in the Shannon index, but *A. ludens* exhibited significant differences in observed species compared to *A. serpentina* and *A. striata* (t-test p-value < 0.05). In males, *A. ludens, A. obliqua*, and *A. striata* exhibited greater observed number of species, but only the comparisons of *A. obliqua* with *A. serpentina* and *A. fraterculus* resulted significant (t-test p-value < 0.05). The Shannon index in males showed that *A. ludens* have significant differences with *A. striata* and *A. fraterculus* (t-test p-value $<$ 0.05).

PCoA (fig. 5) and PERMANOVA analyses suggested that *A. obliqua* adults harbor the most distinct microbiota composition, exhibiting significant differences when compared to all other species, followed by *A. serpentina*, which had significant differences when compared to three species (*A. striata, A. fraterculus*, and *A. ludens*). The species with the least differences in composition were *A. ludens, A. striata*, and *A. fraterculus*. Among sexes,

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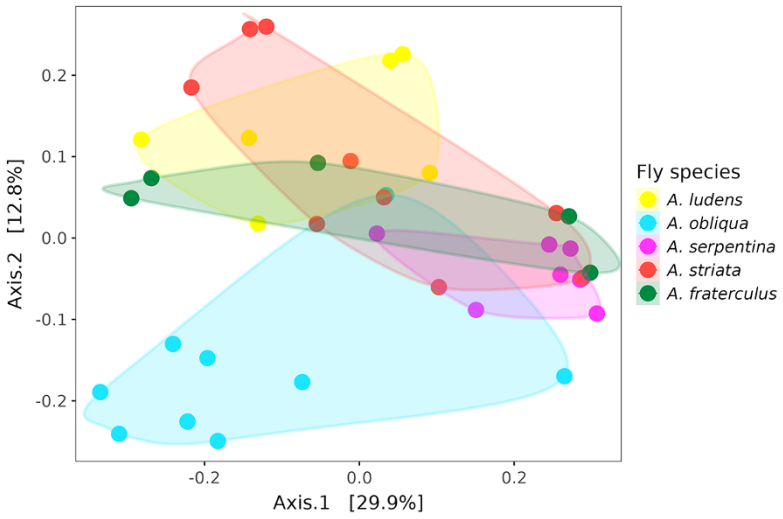

Figure 5. Principal Coordinate Analysis (PCoA) based on unweighted UniFrac distances illustrating gut microbiota composition among newly emerged adults of five *Anastrepha* species. Distinct clustering is observed, with *A. obliqua* exhibiting the most differentiated microbiota composition relative to all other species, followed by *A. serpentina*. In contrast, the microbiota of *A. ludens, A. fraterculus*, and *A. striata* exhibit considerable overlap.

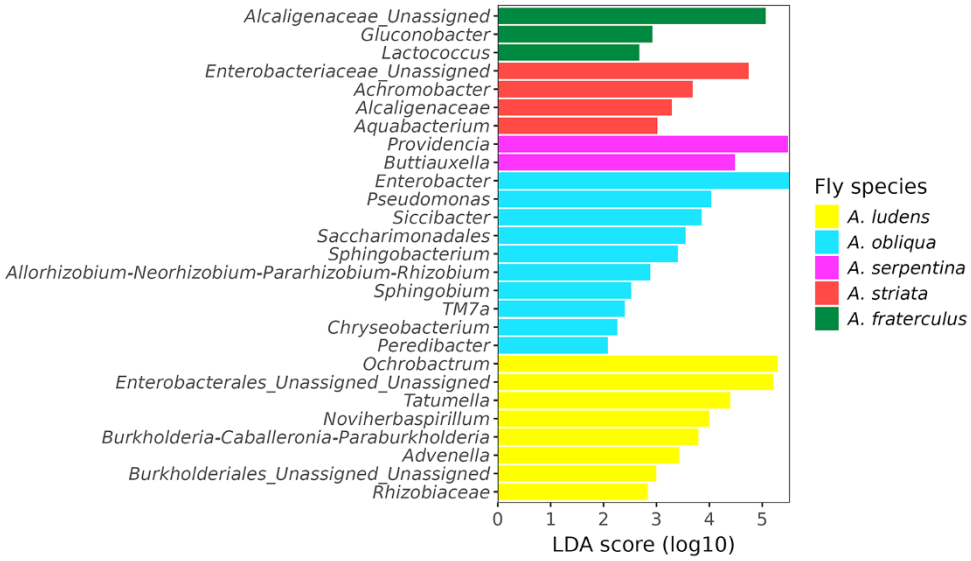

Figure 6. LEfSe analysis showing the differentially abundant bacterial genera found in the guts of newly emerged adults of five economically important *Anastrepha* species.

differences were observed between males and females in the cases of *A. obliqua* and *A. striata*.

Common and differentially abundant genera between the fly species

The LEfSe analysis identified twenty-seven genera with differential abundance between the five *Anastrepha* species studied (fig. 6). *Anastrepha obliqua* and *A. ludens* were the species with the most differentiated genera. In *A. obliqua*, the enriched genera included *Enterobacter, Pseudomonas, Siccibacter, Saccharimonadales, Sphingobacterium, Allorhizobium-Neorhizobium, Sphingobium, TM7a, Chryseobacterium*, and *Peredibacter*. In contrast, *A. ludens* was enriched with *Ochrobactrum, Tatumella, Noviherbaspirillum, Burkholderia-Caballeronia-Paraburkholderia, Advenella, Rhizobiaceae*, and two unassigned genera from the classes *Enterobacterales* and *Burkholderiales*, as well as one from the family *Alcaligenaceae*. In *A. striata*, the enriched genera were *Achromobacter, Alcaligenaceae, Aquabacterium*, and one unassigned genus from the *Enterobacteriaceae* family. *Anastrepha serpentina* and *A. fraterculus* had the fewest enriched genera, with *A. serpentina* being enriched in *Providencia* and *Buttiauxella*, and *A. fraterculus* in *Lactococcus, Gluconobacter*, and one unassigned genus from the *Alcaligenaceae* family.

On the other hand, ten genera were present in all fly species. Despite being enriched in *A. obliqua* and *A. ludens* respectively, *Enterobacter* and *Ochrobactrum* were also present in all fly species. The other eight genera present in all fly species were *Stenotrophomonas, Acinetobacter, Pseudomonas, Escherichia-Shigella, Pantoea, Enterococcus, Klebsiella*, and one unassigned genus from the *Alcaligenaceae* family.

Discussion

Although we worked with small sample sizes (albeit ones of significant ecological value), we were able to flush out very interesting patterns that add novel aspects to the fast-growing literature on the topic of tephritid fly microbiota. As noted in the introduction, working with newly emerged adults, that had no opportunity to feed in the sterile environment they were kept in

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until gut dissection, has been a neglected aspect of microbiota studies in tephritid flies. Most studies on adult fly microbiota so far, considered specimens captured in traps, which represents a totally different scenario and addresses very different questions than the ones we consider here (e.g., Goane *et al.*, 2022; Ravigné *et al.*, 2022). Knowledge on the bacterial diversity harbored in guts of newly emerged adults is crucial to our understanding of the system, particularly given the global economic impacts and ecological risks posed by invasive fruit flies, such as those extensively documented for the genus *Bactrocera* (Zhao *et al.*, 2024). Likely, some of these bacteria will end up being vertically transmitted by females when inserting eggs into ripening fruit where larvae will develop. In fact, recent research with *B. dorsalis* indicates that, although larval diet significantly shapes the gut microbiota composition, certain key bacteria remain stable across various fruit hosts, underscoring the potential importance of vertically transmitted microbiota (Kempraj *et al.*, 2024). But note too that adults are very mobile and need to seek protein sources upon emergence for sexual maturation (Birke and Aluja, 2018; Prokopy and Roitberg, 1984), so they will ingest throughout their lifetime a wealth of other bacteria (and additional microorganisms such as yeasts). So, much work lies ahead to find out if the vertically transmitted bacteria are those that females are 'born' with, or if it's a combination of the later with ones accrued during adult life. In this sense, our results, independent of species, indicate an overwhelming presence of bacteria in the guts of newly emerged adults within the Proteobacteria, a phylum containing over 460 genera and close to 2,000 species (Kersters *et al.*, 2006; Sharma *et al.*, 2022) among them the Enterobacteriaceae, which, in a previous study by us dominated the guts of both newly emerged female and male *A. ludens* adults originating from larvae infesting a wide array of hosts (Aluja *et al.*, 2021). Consistent with this, here Enterobacter dominated in the guts of newly emerged *A. ludens* females, but interestingly not males. We note that the overwhelming presence of Proteobacteria (97.7%) was recently also reported by Ravigné *et al.* (2022) working with other genera of economically important fruit flies (e.g., *Bactrocera, Ceratitis, Dacus, Neoceratitis* and *Zeugodacus*) in a study seeking to find out if fruit fly phylogeny could possibly imprint adult bacterial gut microbiota.

In broad terms, we can summarize our main findings as follows: (1) Sexes, independent of species, did not necessarily share the same types of bacteria (measured at the genus level), which is fascinating as both originated from larvae developing in the same host. This possibly means that during metamorphosis bacteria may be differentially retained or eliminated depending on sex. (2) *Anastrepha obliqua* and *A. ludens* were the species with the most differentiated genera, but interestingly, ten genera were present in the fly species stemming from three phylogenetic groups, among them *Enterobacter* and *Ochrobactrum*. This opens the door to the question of whether there is a shared, core microbiota among newly emerged adults of these species even though they infest very different hosts and, in our study, stemmed from geographically very distinct locations. But, despite the later, and except for *A. ludens* and *A. obliqua*, each species exhibited a clear abundance of certain bacteria 'unique' to them. (3) With respect to the observed species richness, *A. ludens* exhibited the highest values and *A. serpentina* the lowest, which could perhaps relate to their larval feeding habits (i.e., highly polyphagous in the case of *A. ludens* and much more specialized in the case of *A. serpentina*). But again, we need to bear in mind that during metamorphosis many bacteria present in the gut of larvae, are filtered out and don´t appear in adults (Aluja *et al.*, 2021). In what follows, we will discuss these main findings and outline future lines of research based on our results here.

Differential adult gut microbiota between the sexes

We measured a larger trend in alpha diversity in male *Anastrepha* species, particularly in *A. ludens, A. obliqua*, and *A. striata*, when compared to females of the same species (fig. 4). This is one of the most interesting and puzzling findings of our study as it likely implies as noted before, that, possibly, during metamorphosis bacteria are apparently differentially filtered out between the sexes. This hypothesis aligns with recent findings showing that differences in intestinal microbiota can significantly influence physiological traits related to invasiveness and adaptability, as observed in invasive populations of *Bactrocera dorsalis* (Wang *et al.*, 2023). If confirmed, this is fascinating and will help us gain further insights into the ecology and behavior of these insects. It also opens the challenge of discovering the mechanisms through which such a process could materialize. We will seek this question further by substantially increasing our sample size in future studies, expanding the geographic scope and host number in our sampling scheme. Also, by comparing the gut microbiota of newly emerged adults, and those captured in nature after they had the opportunity to feed on varied food sources, including adults kept under laboratory conditions and provided artificial sources of protein and sugar. It will be interesting to find out if the guts of these adults maintain the types of bacteria found in the guts of newly emerged ones, or if bacteria ingested via the food replace those adults are 'born' with. But it will be particularly interesting if our initial results here, indicating that the microbiota in the guts of newly emerged females and males differs, can be confirmed. In principle, this would make sense as females and males follow different paths during the process of sexual maturation and, importantly, as noted by Yuval (2017 and references therein), 'bacteria resident in the gut of *Drosophila* modify the fly's innate chemosensory responses to nutritional stimuli' and their subsequent foraging behavior. Females need to quickly seek protein sources for ovary development and maturation and then concentrate in finding suitable oviposition substrates for egg laying and progeny development. In contrast, males, on top of finding protein for gonad maturation, need to guarantee sexual pheromone quality and vigor for sexual displays (Aluja *et al.*, 2000; Benelli *et al.*, 2014a, 2014b). The ability to identify ideal lekking sites, territory defense within them, vigorous wing fanning, and high-quality sexual pheromone release require substantial energy reserves and appropriate muscle development. Could the 'initial load' of bacteria in newly emerged adults influence these processes? Another line of research that needs to be pursued to better understand the potential mechanisms driving the phenomenon being discussed here, is to investigate hormonal and structural differences in the gut that might influence bacterial colonization.

Related to the above, Guillén *et al.* (2019) reported that in *A. ludens*, males significantly regurgitate more than females, and that there were structural differences in the digestive tract between the sexes. The latter could possibly affect the bacterial transmission from larvae, via the pupae, to the adults during metamorphosis. Also, based on what Yuval (2017) reported, the bacterial load of newly emerged male adults, could possibly influence regurgitation behavior, as it has been speculated that through the regurgitated droplets, flies may accrue bacteria from the environment (Guillén *et al.*, 2019). So, in sum, our findings here indicating that there are

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differences in the gut microbiota between females and males, opens many fascinating questions we will pursue in future studies.

Is there a 'core microbiota' shared among newly emerged adults?

Our results also open the door to the question of whether there is a shared, core microbiota among these species despite the fact that they infest very different hosts and were collected in very distant locations. The presence of ten shared bacterial genera across the five *Anastrepha* species, despite their sampling from geographically distant locations in Mexico (Morelos, Veracruz, and Chiapas), may suggest the presence of a core gut microbiota maintained by biological factors rather than environmental influences. This retention across species highlights the importance of these bacteria in the flies' biology, suggesting they are likely transmitted vertically or retained due to selective pressures within the host, rather than being acquired from the environment (Yun *et al.*, 2014). *Enterobacter* and *Ochrobactrum* were enriched in *A. obliqua* and *A. ludens*, respectively, but were present across all species. *Enterobacter*'s metabolic versatility and *Ochrobactrum*'s role in insect guts suggest that they may contribute to the flies' adaptability (Jing *et al.*, 2020; Swings *et al.*, 2006). *Stenotrophomonas*, known for its role in biocontrol, as well as *Acinetobacter* and *Pseudomonas*, which aid organic matter breakdown, may indicate symbiotic relationships beneficial to the flies (Hagen, 1966; Kumar *et al.*, 2023; Wilson *et al.*, 2016). In the gut of newly emerged flies, *Enterococcus* species likely play a pivotal role in the initial colonization and stabilization of the gut microbiota, which is essential for maintaining overall health and immune function (Cox and Gilmore, 2007; Dillon and Dillon, 2004; Engel and Moran, 2013). Additionally, *Escherichia-Shigella* species could be implicated in the digestion of complex carbohydrates, providing vital nutrients that support the early developmental stages and energy requirements of adult flies (Dillon and Dillon, 2004; Zheng *et al.*, 2020), while *Pantoea*'s association with plants may enhance interactions with fruit hosts (Walterson and Stavrinides, 2015). The unassigned Alcaligenaceae genus further underscores the potential for these bacteria to be essential, conserved components of the insects' gut microbiota (Bextine *et al.*, 2004; Engel and Moran, 2013; Ramírez-Camejo *et al.*, 2017). *Klebsiella*, could participate in the gut microbiota regulation or nitrogen fixation processes within the gut, possibly aiding in the synthesis of amino acids that are crucial for the growth and maturation of *Anastrepha* flies (Bar-Shmuel *et al.*, 2020; Ben Ami *et al.*, 2010). Collectively, these bacteria establish a stable and functional gut environment, crucial for the survival and fitness of newly emerged adults.

Does phylogenetic placement count when comparing the gut microbiota composition of newly emerged adults?

The concept of phylosymbiosis suggests that the composition of an organism's microbiota is closely related to its phylogeny (Brooks *et al.*, 2016; Lim and Bordenstein, 2020). Here we studied five economically important *Anastrepha* species exhibiting varying degrees of phylogenetic relatedness (Mengual *et al.*, 2017). Our beta diversity results do not necessarily concur with Brooks *et al.* (2016) and more recent ones by Ravigné *et al.* (2022), working with other Tephritid fruit flies as indicated previously. As noted by us in an earlier study (Aluja *et al.*, 2021) comparing the gut microbiota of larvae and newly emerged *A. ludens* adults stemming from native ancestral and commercially grown hosts, and studies by Wong *et al.* (2013), and Chen *et al.* (2022) working with various drosophilid species, gut microbiota is shaped more by diet specialization and the last host plant in which the larvae developed. But much work lies ahead in this area as more enlightening comparisons are needed involving basal/primitive species, derived ones, and representatives of intermediate groups in the phylogeny of a particular fruit fly genus before we can reach definitive conclusions. This study represents only a first step in the direction needed.

Our PCoA and PERMANOVA analyses suggest that newly emerged *A. obliqua* adults, independent of sex, harbor the most distinct microbiota composition, exhibiting significant differences compared to all other species. This pattern is followed by *A. serpentina*, which exhibits significant differences when compared to *A. striata, A. fraterculus*, and *A. ludens*. Conversely, *A. ludens, A. striata*, and *A. fraterculus* show the least differences in microbiota composition. Thus, although *A. obliqua* and *A. ludens* are phylogenetically closer to *A. fraterculus*, their microbiota compositions are more distinct. This discrepancy suggests that there is not a clear differentiation of the gut microbiota within these tephritids within the *fraterculus* group, at least when comparing newly emerged adults. Thus, our hypothesis in the sense that, given their close phylogenetic relationship the gut microbiota of newly emerged *A. ludens, A. obliqua* and *A. fraterculus* adults would be more similar that the one found in *A. serpentina* and *A. striata*, each one belonging to different phylogenetic groups within *Anastrepha* (Mengual *et al.*, 2017), was only partially confirmed. We are currently working on a much more all-encompassing study that will hopefully shed additional light into this topic.

Initial insights into the differences in gut microbiota among the five Anastrepha species studied

Are there preliminary insights to be gained with respect to the gut microbiota of newly emerged adults in relation to the possible roles some of the bacteria found may have in toxic allelochemical degradation in the pulp of fruits the first instar larvae will encounter? Starting with the oligaphagous *A. striata*, the guts of newly emerged adults were enriched in two bacterial genera from the family Alcaligenaceae: *Achromobacter* and an unidentified genus from the Enterobacteriaceae family. The first two bacterial genera may have pectinolytic and cellulolytic activity, enabling them to break down complex carbohydrates and aromatic compounds such as polyphenols (Aiysha and Latif, 2022; Callegari *et al.*, 2020; Gladkov *et al.*, 2022; Mohamadpoor *et al.*, 2022). Additionally, newly emerged *A. striata* adults, exhibited an enrichment of bacteria within the genus *Aquabacterium*, which includes species capable of degrading aromatic compounds (Summers *et al.*, 2024; Wilson *et al.*, 2016). Based on Ochoa-Sánchez *et al.* (2022) who reported the conspicuous presence of bacteria within the *Komagataeibacter* genus, known for their capacity to degrade a wide spectrum of tannins and polyphenols, we expected this group of bacteria to show up in the guts of newly emerged adults. But as noted by Aluja and collaborators (Aluja *et al.*, 2024) working with *A. ludens* stemming from a single host (*Citrus* x *aurantium* along an 800 m longitudinal transect and an altitudinal transect spanning all the way from cero to 2000 masl, there is sometimes a shift in bacterial species, but not in the functional role they play (e.g., there was a trade-off between Acetobacteraceae and Rhizobiaceae from northern and southernmost samples). Here, we found two other groups of bacteria (pointedly *Achromobacter*) known to degrade the toxic compounds found in unripe guavas, the maturity stage at which *A. striata* females lay their eggs. So perhaps, newly emerged females

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indeed harbor the types of bacteria the larvae will later need to cope with a toxic environment.

The guts of newly emerged *A. fraterculus*, a polyphagous species when considering its entire distribution range (Mexico to Argentina), but locally behaving as a stenophagous species, was also enriched with an unassigned genus of Alcaligenaceae, with representatives exhibiting pectinolytic activity, and also contained the genus *Lactococcus*, which includes species with cellulolytic activity, such as *Lactococcus lactis* (Román Naranjo *et al.*, 2022). Importantly, from an ecological/functional perspective, *A. fraterculus* and *A. ludens* shared the characteristic of being enriched with genera that can utilize or occupy niches with simple carbohydrate sources and perform nitrogen recycling, such as *Gluconobacter, Tatumella, Novihervaspirillum, Advenella*, and an unassigned genus of the family Rhizobiaceae (Deppenmeier *et al.*, 2002; Fahde *et al.*, 2023; He *et al.*, 2022b; Ishii *et al.*, 2017; Jakob *et al.*, 2019; Kuzmina *et al.*, 2022; Liu *et al.*, 2022; Papalexandratou *et al.*, 2019; Swings *et al.*, 2006). All the above supports the hypothesis that the microbiota composition in these three species is partially related by their host plant associations.

In contrast, the microbiotas of *A. serpentina* and *A. obliqua* are significantly different from each other and from the three species mentioned above. *Anastrepha serpentina* is phylogenetically distant and stenophagous, and its microbiota was characterized by the presence of genera such as *Providencia* and *Buttiauxella. Providencia* is known for its adaptability to diverse environments and for harboring species with pathogenic potential. Notably, *P. alcalifaciens* and *P. rettgeri* have been identified as pathogenic to *A. ludens*, negatively impacting mass-rearing efficiency by reducing larval and pupal yields. A study by Salas *et al.* (2023) demonstrated that different isolates of *P. alcalifaciens/P. rustigianii* and *P. rettgeri/P. vermicola* were pathogenic to *A. ludens*, causing significant reductions in the conversion of eggs to pupae and overall fly production. Further research is needed to find out if *Providencia* could be involved in degrading the latex typically found in fruits within the Sapotaceae, the preferred hosts of this fly species. As the latex is released by the fruit immediately after the female stings the skin, eggs will encounter a very challenging environment. Besides, *A. serpentina* newly emerged adults, were enriched *Buttiauxella*, part of the Enterobacteriaceae family, which is known to produce phytases. These phytases are crucial for breaking down phytates, complex molecules commonly found in grains, seeds and fruit peels, into bioavailable phosphorus (Dersjant-Li and Dusel, 2019; Dersjant-Li *et al.*, 2017).

Among the five species examined, newly emerged *A. obliqua* adults exhibited the greatest number of differentiated bacterial genera, namely *Enterobacter, Pseudomonas, Siccibacter, Saccharimonadales, Sphingobacterium, Sphingobium,* and *TM7a*. Specifically, members of the genus *Enterobacter* are involved in nitrogen fixation and the degradation of toxic organic compounds, which could likely aid *A. obliqua* in detoxifying secondary plant metabolites such as tannins which are ubiquitous in some species of *Spondias* (Anacardiaceae), purportedly an ancestral host of this species (Da Silva *et al.*, 2012; Sevim *et al.*, 2016). Similarly, *Pseudomonas* species are well-documented for their capabilities in biodegradation, particularly of complex organic compounds and phenolic substances, which are common in many plant materials. These bacteria produce a variety of enzymes that allow them to break down recalcitrant compounds, rendering them essential for detoxifying plant secondary metabolites (Huerta-García and Álvarez-Cervantes, 2024; Jing *et al.*, 2020; Medić and Karadžić, 2022). On the other hand, *Siccibacter*, although less extensively studied, has been associated with cellulose degradation (Dhakal *et al.*, 2020). This function could be likely beneficial to *A. obliqua*, allowing larvae to efficiently utilize the carbohydrates present in various fruits, which may vary greatly in water content. Meanwhile, although there is limited information on *Saccharimonadales* in the gut microbiota of phytophagous insects, its potential role can be inferred from its activities in other ecosystems, such as soil and the rhizosphere. In these environments, *Saccharimonadales* have been associated with nutrient cycling, particularly in enhancing phosphorus availability through phosphatase activity and in the metabolism of complex carbohydrates. In the gut microbiota of a phytophagous insect, *Saccharimonadales* may possibly contribute to the breakdown of plant-derived carbohydrates, thus likely supporting the insect's ability to process difficult-to-digest compounds present in its plant-based diet (Sun *et al.*, 2022; Yang *et al.*, 2021). *TM7a* is a genus closer to Saccharimonadales (same family) that could be an obligate symbiont or endophyte (He *et al.*, 2015; Zhou *et al.*, 2024), which could share the same niche and possibly play a role in carbohydrates breakdown, but his function has not been well characterized. We note that *A. obliqua* occasionally utilizes guava as a host (Birke and Aluja, 2011), a fruit with high levels of polyphenols, which are complex aromatic compounds that can be toxic to many organisms such as the phylogenetically related *A. ludens* (Ochoa-Sánchez *et al.*, 2022). *Sphingobacterium* and *Sphingobium* play crucial roles in degrading these complex lipids and aromatic compounds, possibly enabling *A. obliqua* to feed on guava (Asaf *et al.*, 2020; Verma *et al.*, 2014; Zhang *et al.*, 2024).

In conclusion, our comparative study contrasting the gut microbiota of newly emerged adults of five economically important *Anastrepha* species, yielded exciting results. Particularly, our finding that females and males exhibited different microbiotas is noteworthy as it likely points out to a differential, sex-related screening process during metamorphosis. We acknowledge that the possible microbiota functions that we discuss were inferred from the literature, and thus further in-depth investigations applying metagenomic, metatranscriptomic and experimental validation approaches are still needed to better understand the mechanisms at play. Further research is also needed to compare the gut microbiota of newly emerged adults with those that had a chance to forage for food, oviposition sites and mates, to determine if the microbiota at 'birth' prevails or if it is largely substituted by ambient bacteria ingested after adult emergence. It remains unclear whether the microbiota present at emergence persists or is replaced by bacteria acquired during the adult stage. Understanding this transition could reveal critical roles for teneral adult microbiota in mature adult development and behavior (Hammer and Moran, 2019). We need to also dwell in much more depth into the functional roles bacteria play in the guts of flies within *Anastrepha* (e.g., Ochoa-Sánchez *et al.*, 2022). In this sense the inspiring work of the early pioneers cited in the introduction, added to the elegant work by Boaz Yuval and his collaborators in Israel (e.g., Behar *et al.*, 2008, 2005; Ben-Yosef *et al.*, 2010, 2015; Yuval, 2017) and studies like the one by Ceja-Navarro *et al.* (2015), should lead us in the right direction.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0007485325100527.

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