Archaea, the unseen kingdom in the gut microbiome of *Anastrepha obliqua*

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Research Article

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Abstract

The fruit fly *Anastrepha obliqua* is an economically important pest. The sterile insect technique is used to control this pest; it involves mass production and release of sterile flies to reduce the reproduction of the wild population. As noted in different tephritidae, the performance of sterile males may be affected by the assimilation of nutrients under mass-rearing conditions. In the wild, the fly's life cycle suggests the acquisition of different organisms that could modulate its fitness and physiology. For *A. obliqua*, there is no information regarding microorganisms other than bacteria. This study analyzed bacteria, fungi, and archaea communities in the *A. obliqua* gut through denaturing gradient gel electrophoresis (DGGE) profiles of 16S (using different set of primers for bacteria and archaea) and 18S ribosomal DNA markers. We found that wild flies presented higher microbial diversity than laboratory samples related to fructose assimilation. In contrast, laboratory microbial species suggest that microorganisms have led to a specialized metabolism to process nutrients associated to an artificial diet. We showed that by employing different primer sets aimed at the same molecular marker but targeting diverse hypervariable regions of bacteria and archaea 16S rDNA, it was possible to identify species that have not been previously described in this fruit fly. Here, the archaea kingdom is suggested as an important player in fly metabolism. This report of the intestinal microbial (bacteria, archaea, and fungi) composition of *A. obliqua*, contributes to our understanding of the role of microorganisms in the development and physiology of the flies.

Introduction

Fruit flies (Diptera: Tephritidae) encompass ~ 70 species, which infect more than 30 fruit species, leading to worldwide economic impacts (Qin et al. 2015). In Mexico, despite being cosmopolitan, the Mediterranean fruit fly *Ceratitis capitata* has been controlled by the sterile insect technique (SIT). However, the fruit fly *Anastrepha obliqua* (Macquart) is a pest that causes losses of fruit crops; from January to July 2018 there were losses of 22 million tons of mango (*Mangifera indica*) and Spondias (*Spondias purpurea* and *S. mombin*) fruits (SENASICA 2018). SIT is a biological technique without adverse impact on biodiversity and the environment, that involves the systematic mass release of irradiated sterile adult competitive and flying males, which induce sterility in the wild population, by preventing offspring (KNIPLING 1959; Montoya and Toledo 2010).

Different efforts had been made to optimize the efficiency of SIT by enhancing the quality of sterile insects (Ami et al. 2010). In this sense, nutrient assimilation is a critical factor for the quality of the released sterile insects. Composition of the artificial diet, enzymatic metabolic machinery, and microorganisms harbored in the fly's gut modulate assimilation, contributing to fly fitness (Rivera-Ciprian et al. 2017; Rempoulakis et al. 2018). Additionally, the wildlife cycle of the fruit fly suggests the acquisition of different microorganisms, which exploit nutrients from the natural diet, modulating its biology (Ben-Yosef et al. 2014). Therefore, the fly's gut ecology must be determinative in the modulation of fly fitness.

The microorganisms present within a host, known as microbiota, are generally well known for modulating host health and fitness (Thaiss et al. 2016). In fruit flies, most of the microbial diversity studies have focused on elucidating the role of bacteria housed in the gut of different fly genera, such as *Ceratitis*, *Drosophila*, *Bactrocera*, *Helaeomyia*, and *Anastrepha* (Kuzina et al. 2001; Juneja and Lazzaro 2009; Ben-Yosef et al. 2014). Regarding these, bacterial microbiota such as *Klebsiella oxytoca*, *K. pneumoniae*, *Citrobacter freundii*, *Enterobacter* sp., and *Providencia rettgeri* have been evaluated as probiotics to enhance fly fitness (Ami et al. 2010; Hamden et al. 2013; Augustinos et al. 2015; Roque-Romero et al. 2020). Therefore, manipulation of resident bacterial populations could influence host fitness, possibly by generating stronger sterile males, which will compete better against wild flies (Ami et al. 2010; Kyritsis et al. 2017). In addition, fungi and actinomycetes studies have focused on evaluating their role as insecticides, leading to the proposed use of different strains, such as *Beauveria bassiana*, *Metarhizium anisopliae*, and *Bacillus cereus* to improve biocontrol of *C. capitata* (Imoulan and Elmezziane 2014; Navarro-Llopis et al. 2015; Ruiiu et al. 2015; Samri et al. 2017). Interestingly, for the same fly species, different microorganisms have been proposed to present a functional role, indicating the importance of elucidating the gut ecology of the fly.

For the related fly, *Anastrepha ludens*, bacteria such as *Citrobacter*, *Enterobacter*, *Klebsiella*, *Providencia*, and *Pseudomonas* have been found in its intestinal tract (Kuzina et al. 2001). To date, studies on *Anastrepha* spp. have mainly focused on bacteria, and have not investigated other associated microorganisms, which could also modulate physiology and fitness. Thus, knowing the ecology of the fly intestine would provide a better understanding of microbiota–insect interactions, multi-species community structure, and its effect on the host. To determine the microbiota diversity associated with the gut of *A. obliqua*, we employed 16S and 18S PCR-denaturing gradient gel electrophoresis (DGGE) to describe the diversity of fungi, bacteria, and archaea present in the intestinal tract of wild and laboratory flies. Moreover, we used cloning techniques of 16S-ribosomal DNA (rDNA) coupled with phylogenetic analyses to gain a better insight into the species harbored in the fly intestine. This knowledge will provide the basis for further studies of the microbiota in the digestive tract of *A. obliqua*, which could improve the success of sterile insect release.

Materials And Methods

Biological material

The larvae and adult *A. obliqua* flies were obtained from a mass-rearing colony at Moscafrut Facility in Metapa de Domínguez, Chiapas, Mexico. Third instar larvae were harvested and fed a diet containing 19% corn cob powder (Maformu, Cd. Guzmán, Jalisco, Mexico), 5.3% corn flour (Maíz Industrializado del Sureste, Arriaga, Chiapas, Mexico), 7% torula yeast (Lake States, Div. Rhineland Paper, Rhineland, WI, USA), 9.2% sugar (Ingenio Huixtla, Chiapas, Mexico), 0.4% sodium benzoate (Cia. Universal de Industrias, S.A. de C.V., Mexico), 0.2% nipagin (Mallinckrodt Specialty, Chemicals Co., St. Louis, MO, USA), and 0.44% citric acid (Anhidro Acidulantes FNEUM, Mexana S. A. de C.V., Morelos, Mexico) (Orozco-Dávila et al. 2017). Lab

adult specimens were collected after emerging from the pupae stage on vermiculite incubated at approximately 14 days and fed with hydrolyzed protein:sugar (in a 1:3 ratio), and water until collection at 12 days of age. Wild larvae were obtained from infested mango fruits provided by the Junta Local de Sanidad Vegetal del Soconusco, Chiapas. Third instars were obtained from infested mango fruits collected in the surroundings of the town of Tuxtla Chico, Chiapas, Mexico. Wild adult flies were caught during the harvest in mango orchards in the same town using modified MacPhail traps baited with hydrolyzed protein as an attractant to catch live adults (Enkerlin 2018). Flies were taxonomically identified using the keys previously described (FAO/OIEA 2018). All samples were washed twice with 70% ethanol. Dissections were performed to extract the digestive tract under sterile conditions in a laminar flow hood and stored in 70% ethanol at -20°C until processing.

DNA extraction

Before extraction, samples (0.3 g) of the midgut were washed twice with 700 µl of 20 mM EDTA. DNA was extracted following the phenol-chloroform protocol (Sambrook 2001) with some modifications. Briefly, 500 µl of 2 mM EDTA pH 8, 500 µl phenol pH 8.0, 0.25 g of glass beads, and 60 µl 20% SDS were added to macerated midguts and homogenized for 4 min. DNA purification was performed with chloroform and centrifuged at 13,000 x *g*. Then, 2.7 M sodium acetate and two equal volumes of absolute cold ethanol were added to the aqueous phase, which was incubated for 40 min at -70°C to precipitate DNA, centrifuged 35 min at 16,000 x *g*, washed with 70% ethanol, decanted, and allowed to dry at 37°C. The DNA pellet was resuspended in 50 µl water with 1.5 µg/µl RNase and incubated for 1 h at 37°C. The obtained genomic DNA was purified using the Soil Microbe DNA kit MiniPrep ZR™ (Zymo Research, USA) according to the manufacturer’s instructions. Each sample was analyzed by 0.8% agarose gel electrophoresis to verify quality and purity.

DNA extraction from legs was performed using a single laboratory adult specimen that was frozen at -20°C. The legs were dissected, placed in a 1.5 ml tube, and washed with 70% ethanol five times. DNA extraction was performed as described above.

PCR-DGGE analysis

Amplification of molecular markers was performed using the primers listed in Table 1, as follows. Bacterial 16S rDNA fragments were performed with primers F984GC and R1378 (Nübel et al. 1996). Amplification of Gram-positive bacteria (Actinomycetes) 16S rDNA was performed with a nested PCR employing the primer F243 and R1378 for the first reaction to Gram positive population enrichment, and F984GC and R1378 in the second (Heuer et al. 1997). Archaea identification was performed using a nested amplification using primers 21F and 958R (DeLong 1992) in the first PCR amplification; Parch519F and Arch915R (Coolen et al. 2004). To verify the presence of fungi, amplicons were obtained with primers NS1 (White et al. 1990) and GCfung (May et al. 2001). The primers alignment and PCR conditions are found at Online Resource 1.

Table 1
Primers were used to amplify the molecular markers employed in this study

Label	Organism / marker	Sequence	Reference
F984GC	Bacteria 16S*	CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGCGCAACGCGAAGAACCTTAC	Nübel et al. 1996
R1378	Bacteria 16S	CGGTGTGTACAAGGCCCGGGAACG	Nübel et al. 1996
F243	Gram positive bacteria/Actinomycetes 16S	GGATGAGCCCGCGGCCTA	Heuer et al. 1997
21F	Archaea 16S	TTCCGGTTGATCCYGCCGGA	DeLong 1992
958R	Archaea 16S	CCGGCGTTGAMTCCAATT	DeLong 1992
Parch519F	Archaea* 16S	CAGCCGCCGCGGTAA	Coolen et al. 2004
Arch915R	Archaea* 16S	CGCCCGCCGCGCCCGCGCCCGGCCCGCCCGCCCGTCTCCCCGCCAATTCTT	Coolen et al. 2004
NS1	Fungi 18S	GTAGTCATATGCTTGTCTC	White et al. 1990
GCfung	Fungi 18S	GCCCGCCGCGCCCCGCGCCCGGCCCGCCCGCCCGCCCGATTCCTCGTTACCG	May et al. 2001
* used for nested PCR as indicated at main text and Online Resource 1.			

For DGGE, PCR products were quantified using Molecular Kodak Imaging software, and loaded in equal concentrations directly on a 0.8% polyacrylamide gel with a 20–50% denaturing gradient of urea and formamide, and electrophoresed in 1% TAE buffer (40 mM TAE, 2 mM Tris-acetate, and 1 mM Na₂EDTA, pH 8.5). Electrophoresis was performed in the D-Code™ Universal Mutation Detection System (BioRad, Hercules, CA, USA) at 90 V for 8.5 h, with a constant temperature of 60°C. Subsequently, the gels were stained with SYBR Gold. Each DGGE was repeated three times in each experiment.

The DGGE band profiles indicate the different microbial communities from the fly midgut (Online Resource 2). The profiles were compared using the Jaccard index (J), which allows analysis of the biodiversity found in a sample where the maximum value indicates a greater diversity. This was calculated according to the following formula: $J = \frac{nAB}{(nA + nB - nAB)} - 1$, where nAB is the number of bands in common between lanes A and B, nA is the total number of bands in lane A, and nB is the total number of bands in lane B.

The Shannon index (H') allows comparisons of community similarity between two samples, where a value of 1.0 (or 100%) corresponds to communities that share an identical pattern, and a value of 0 indicates that no difference exists. This index was calculated using the following equation: $H' = - \sum (ni/N) (\log ni/N)$, where H' is the diversity, and ni/N is the number of individuals of the species given by band intensity (ni) to the total subjects (N, the total intensity of all bands of the same sample); *i.e.*, the relative abundance of species. Band intensity reflects the abundance of the same (Eichner et al. 1999) and theoretically represents a genus, in this case, analyzed by Molecular Kodak Imaging software.

PCR-DGGE analysis

The 16S rDNA amplicons for bacteria, and archaea were obtained as described above. The purified PCR products were cloned into a p-JET vector with CloneJet PCR Cloning Kit (Thermo Scientific, Waltham, MA, USA) and transformed into *E. coli* DH5-α cells (Sambrook 2001). Clones were sequenced using pJET primers by capillary sequencing at Macrogen Inc. (Korea). The sequence chromatograms were visually inspected and manually trimmed using AliView software (Larsson 2014). To identify closely related 16S rRNA genes, the remaining sequences were analyzed with the BlastN tool at 99% identity against the 16S archaea and bacteria database at NCBI. Those sequences were submitted to NCBI under the accession numbers shown at Table 2. Multiple sequence alignment was performed using ClustalW (Thompson et al. 1994). And the best suitable model was employed to construct the phylogenetic tree using Molecular Evolutionary Genetics Analysis (MEGA6) (Kimura 1980; Tamura et al. 2013). The accuracy of the tree topology was performed by 1000 bootstrap replicates (Felsenstein 1985).

Table 2
NCBI accession number of the sequences identified in this study.

Sample code	NCBI Number	Sample code	NCBI Number	Sample code	NCBI Number
LA-B6	MK729487	WL-B4	MK729503	LL_Ar3	MK729519
LA-B2	MK729488	WL-B6	MK729504	LL_Ar4	MK729520
LA-B6_2	MK729489	WA-Ar2	MK729505	LL_Ar5	MK729521
LA-B8	MK729490	WA-Ar8	MK729506	LL_Ar6	MK729522
LL-A15	MK729491	WA-Ar9	MK729507	LL_Ar7	MK729523
LL-B1	MK729492	WA-Ar13	MK729508	LL_Ar9	MK729524
LL-B2	MK729493	WA-Ar14	MK729509	LL_Ar10	MK729525
LL-B3	MK729494	WA_q27	MK729510	LA_Ar1	MK729526
LL-B4	MK729495	WL_Ar4	MK729511	LA_Ar2	MK729527
LL-B6	MK729496	WL_Ar5	MK729512	LA_Ar3	MK729528
WA-B3	MK729497	WL_Ar6	MK729513	LA_Ar4	MK729529
WA-A2	MK729498	WL_Ar7	MK729514	LA_Ar5	MK729530
WL-Ac2	MK729499	WL_Ar8	MK729515	LA_Ar8	MK729531
LA-B3	MK729500	WL_Ar9	MK729516	LA_Ar9	MK729532
WL-B1	MK729501	LL_Ar1	MK729517	LA_Ar10	MK729533
WL-B2	MK729502	LL_Ar2	MK729518		
Sample codes correspond at the label assigned to our samples; NCBI number is the assigned number after submission. LL-Laboratory larvae (mass-reared), LA-Laboratory Adult (mass-reared); WL-Wild larvae, WA-Wild adult.					

Geneious diversity analysis.

The 16S rDNA clones of bacteria and archaea communities obtained from wild and laboratory adult and larvae flies were loaded into Geneious software version 11.1 (Kearse et al. 2012). Subsequently, all submitted NCBI sequences as well as those which display robust electropherogram (Online Resource 3) were manually curated and merged to gain a general view of the global biological diversity harbored at *A. obliqua*. Clean sequences were then analyzed using the 16S diversity tool in Geneious software.

Results And Discussion

Bacteria communities

In related fruit-fly pests such as *Anastrepha obliqua*, *A. ludens*, and *Ceratitis capitata* both, their gut enzymatic activity, and the presence of gram-positive and gram-negative bacteria modulate the food bio-assimilation capability leading effects on host fitness (Rivera-Ciprian et al. 2017; Kuzina et al. 2001; Ami et al. 2010; Gallo-Franco and Toro-Perea 2020). Because of this, we got interested in disclosing *A. obliqua*'s bacterial microbiota diversity. For this, DNA was extracted from the digestive tract of wild adult and larvae (WA and WL, respectively) and laboratory (LA and LL, respectively) flies, and samples were subjected to Gram-Positive enrichment and general 16S rDNA bacterial PCR amplification followed by DGGE. We observe different patterns of migration between developmental stages and groups suggesting different bacterial communities. Besides, our DGGE Gram-positive enriched experiments over all samples recovered just three communities in the midgut of wild larvae fly samples. To confirm this, we tested a minimum amount of DNA for the absence of actinomycetes in other stages of the fly. We could only detect actinomycetes up to a minimum concentration of 0.14 ng of DNA, suggesting that if there are Gram-positive, they are found in undetectable amounts in the samples analyzed by DGGE (with the primers used) as has been reported for taxa of low abundance (González et al. 2011). It has been demonstrated that the detection limit of PCR-DGGE is 104–108 CFU/ml (Ercolini 2004), suggesting that the communities in our samples are below these limits or the primers were inefficient for actinomycetes in this fly. Indeed, amplification of 16S rDNA for Gram-positive showed smear bands (data not shown) in all fly stages, which were subsequently used for cloning.

In general, population index analyses show that adult flies harbor higher diversity than larvae. Specifically, Shannon index (H') analyses (Table 3) showed that adult wild flies presented the higher bacterial diversity ($H'=1.22$), as could be expected in a native environment varied diet setting a more diverse group of microorganisms. The wild larvae showed lower diversity (1.07 H') than the adult, as its development is limited within the host fruit. Furthermore, Jaccard index (J) analysis (Table 3) showed that LW and AW share 53% of their bacterial communities, suggesting a bacterial core-conserved community or vertical transmission of bacterial species as observed in *C. capitata* (Behar et al. 2008).

Table 3
Diversity (H') and Similarity (J) indexes of the microbial communities found in the midgut of *A. obliqua*.

Sample	Bacteria			Archaea			Fungi	
	H'	J %	J %*	H	J %	J %*	H	J %
WA	1.22	53	36	1.0	77	78	0.8	62
WL	1.07		52	0.77		85	0.67	
LA	0.95	63	36	0.8	83	78	0	0
LL	0.94		52	0.77		85	0	
Shannon (H') and Jaccard (J) indexes were determined from DGGE as described in the methodology. Diversity values are shown at H' column for wild Adult (WA), wild Larvae (WL), laboratory Adult (LA), and laboratory Larvae (LL). Similarity between WA and WL, and between LA and LL populations are shown (J % column). Similarity between adults and between larvae samples independently of their environmental origin are indicated at J %* columns.								

Regarding mass-rearing flies, larvae and adult samples showed low diversity ($H' = 0.94$ and 0.95 , respectively) than wild flies and shared 63% of their communities. It is worth mentioning that the adult wild flies were collected as adults from mango orchards and not from fruits infested with larvae letting remain in lab conditions until emergence, so the wild gut microbiome was better represented. Comparison of mass-rearing and wild flies showed that larvae samples share 52%, while adult flies share only 36% of their microorganism communities. Since the mass-rearing *A. obliqua* colony has been maintained for more than 150 generations, this allows us to suggest that it must contain bacterial core microbiota from both natural and mass-rearing environments, which could contribute to the metabolic function of the flies.

Upon bacteria diversity evaluation, we were interested in identifying the species that could be harbored in the fly midgut. With this in mind, we cloned, sequenced, and analyzed ribosomal genetic markers from our samples. We obtained 30 bacteria (using general primers) and 23 Gram-positive enriched population sequenced clones; after analysis, we kept 15 bacteria and 3 actinomycetes unique sequences. Thus, the taxonomic classification of these sequences was restricted because of the low throughput cloning and sequencing methods.

The phylogenetic analysis of bacteria cloned sequences showed two main groups (Fig. 1) composed of Gram-positive and negative bacteria. In general, *Enterobacteriaceae* is the most common group found in the intestinal tract of fruit flies (Kuzina et al. 2001; Behar et al. 2005, 2008) as shown here for all samples. Our results showed that the majority of the mass-rearing larvae sequences were clustered within an *Enterobacteriaceae* and *Pseudomonadaceae*, that group is composed of *Pseudomonas*, *Providencia*, *Klebsiella*, *Serratia*, and *Morganella*, but were undetected in mass-reared and irradiated adults (Fig. 1 compare light-dark blue circles). Indeed, probiotic administration of the missed strains (i.e., *Klebsiella*, *Providencia*, and *Enterobacter*) due to the irradiation process improves mating capabilities and fly development on related species (Ami et al. 2010; Augustinos et al. 2015; Liu et al. 2016; Roque-Romero et al. 2020), supporting the importance of those strains in the metabolism of the fly. Outstandingly, *Pseudomonas spp.* is present in artificial diets and induce beneficial effects during larval development and in adult flies (Augustinos et al. 2015; Rempoulakis et al. 2018). Besides, it has been identified in adult wild *C. capitata* and adult laboratory *A. ludens* (Kuzina et al. 2001; Behar et al. 2008) with impactions in modulate the endemic *Enterobacteriaceae* population in the midgut (Behar et al. 2008). However, here, *Pseudomonas spp.* was identified at larvae mass-reared sequences but lost at adult samples, suggesting a role in the fly. In addition, four mass-reared larvae sequences cluster into the *Morganella morganii* sequence. This strain severely affects the egg and larvae development of *Anastrepha ludens*, a related species (Salas et al. 2017). Thus, this bacteria species could produce a profound impact on the mass-reared samples with respect to the wild in regards to its

fitness. Additional to Gram negative bacteria, we identify one Larvae sequence related to the Gram positive *Aerococcus viridians* and *Enterococcus faecalis*. All those bacteria species have been differentially described in wild adult's caught, as well as larvae and adult mass-reared flies of *Drosophila melanogaster*, *C. capitata*, *Bactrocera* spp, and *A. ludens*, respectively (Juneja and Lazzaro 2009; Ami et al. 2010; Wang et al. 2014; Raza et al. 2020). Currently, it is documented that bacterial species are related to the diet nutrient metabolisms in flies since different compounds such as sugar, fruits, or olive are used in the diet, suggesting that those bacterial strains contribute to nutrient assimilation (Ben-Yosef et al. 2014; Raza et al. 2020).

The **mass-rearing adult** sequences clustered within metabolically specialized species of bacteria, such as members of the *Desulfovibrionaceae* family and *Lentisphaerae* phylum (i.e., *Victivallis* and *Oligosphaera* spp). Sulfate-reducing bacteria (*Desulfovibrionaceae* family) are found in humans (Loubinoux et al. 2002), where they modulate the metabolism of primary fermenter bacteria in the intestine to regulate energy supply. In insects, *Desulfovibrionaceae* family bacteria are naturally found in the beetle *Amblyopoda palpalis* (Koneru et al. 2016). *Lentisphaerae*-isolated bacteria are related to sugar fermentation (Qiu et al. 2013). The presence of most of the adult sequences in this bacteria group suggests that adult laboratory flies develop a specialized microbiota related to the mass-rearing process. In the Gram-positive bacterial group, one mass-rearing adult sequence was related to the *Rarobacter* species which use torula yeast as a complement to its carbohydrate's metabolism (Shimoi and Tadenuma 1991). Thus, it might play an important metabolic role within the fly, since artificial diets contain different compounds, such as torula yeast (Montoya and Toledo 2010). Altogether, in addition to the irradiation process, the mass-rearing conditions and biological processes within the fly could also be responsible for controlling the bacteria load. These results suggest the importance of the microbial species described herein modulating the metabolism of mass-rearing *A. obliqua* fruit flies, although their functions during the nutrient assimilation process have yet to be determined. Also, suggest that microbiota and host interactions mediate the proper metabolic conditions to modulate fly fitness.

Regarding wild samples (Fig. 1, triangles), larvae sequences were related to the *Enterobacteriaceae* (*Escherichia* and *Kosakonia*) family. *Kosakonia sacchari* is a nitrogen-fixing bacterium reported in *Saccharum officinarum* L, and uses sugars such as fructose and glucose (Chen et al. 2014). The wild adult sequences were related to *Tatumella* and *Pluralibacter*, this last and *Enterobacter cloacae* were detected in *A. ludens* (Kuzina et al. 2001). Presence or addition as probiotics of those strains induces an enhanced fitness in mass-rearing sterile flies (Ami et al. 2010), supporting its role in wild fitness success. Altogether, we present evidence of the Gram-positive and negative bacteria diversity harbored in the midgut of wild and mass-rearing *A. obliqua* flies. These results are consistent with the microbial communities reported in *Anastrepha* related flies (Ventura et al. 2018; Gallo-Franco and Toro-Perea 2020; Roque-Romero et al. 2020). The identification of different species suggests molecular crosstalk between these microorganisms and the host. It remains to decipher their kind of interaction. Additionally, results reflect the advantage of using different primers set to study bacterial diversity

Archaea communities

Archaea are distributed in extreme and moderate habitats in which they play a significant role because of their metabolic differences compared to bacteria, and eukaryotic organisms (Bräsen et al. 2014; Könneke et al. 2014). For insects such as cockroaches, termites, and beetles, which can survive on a low-nutrient diet, archaea of the class *Halobacteria* and *Methanomicrobia* provide the proper conditions in the intestine (e.g., CH₄) for the development of other bacteria that modulate host metabolism (Donovan et al. 2004; Ceja-Navarro et al. 2014). Due to this importance, we were interested in analyzing the diversity of archaea communities in the gut of *A. obliqua*. The adult flies (wild, H' = 1 and mass-rearing, H' = 0.8) have high diversity compared to larval stages (wild and mass-rearing, H' = 0.77) (Table 3). The trend, as in bacterial communities, is that the wild adult exhibits greater diversity than wild larvae and mass-rearing samples, possibly due to a greater variety in their diet. The Jaccard index showed that wild specimens have similar archaea communities (77%), whereas the adult and larvae mass-rearing flies share 83% of their communities. Besides, a comparison between adult and larvae stages also showed that they share more than 70% of their communities. Specifically, the WA and LA samples share 78%, while WL and LL share 85% of their archaea communities. Archaea are endowed with particular paths to modify metabolic pathways which are related to sugar fermentation (Bräsen et al. 2014) suggesting a role in the host metabolism processing. Overall, we detect the presence of archaea species for the first time in this *Anastrepha* species. Our data suggest that archaea may be essential throughout the cycle life of the fly and likely could be determined by the mother and diet, as in bacteria.

To gain a better perspective of the archaea species present in the midgut of *A. obliqua*, we got 29 clean sequences from archaea PCR amplicons. Then, identity was assessed at both archaea and bacteria NCBI databases since primers are ambiguous to bacteria (Coolen et al. 2004). With the gathered information, we construct a phylogenetic tree that discerns archaea and bacteria clades (Fig. 2). Outstandingly, bacterial species detected with archaea-directed primers were different from the species identified with bacteria primers (compare Fig. 1 with bacteria clade in 2).

Mass-rearing samples clustered at the archaea clade and one LA sequence clustered at the bacteria clade within the *Enterococcus* branch. Interestingly, nine samples from mass-rearing larvae were related to *Halalkalicoccus* species (*Halobacteriaceae* family), which can grow on fructose and mannose in the laboratory (Xue et al. 2005; Poehlein et al. 2016). Laboratory adult samples were related to strains that use inorganic compounds as an energy source but also organic compounds, such as yeast (i.e., *Nitrososphaera* spp, and *Thermofilum* spp) (Tournai et al. 2011; Toshchakov et al. 2015). With basis of the nutritional content of the artificial diet (including torula yeast) employed for mass-reared flies and the microbial species found, our results suggest that those strains could be gained via the diet, modulating the bio-assimilation process.

In the wild samples, larvae sequences clustered within the *Haloterrigena*, *Natronorubrum*, *Natrinema*, and *Haloferax* branch (*Halobacteriaceae* family). These species are related to phosphorus solubilization and can grow in the presence of fructose (Yadav et al. 2015). Interestingly, wild larvae were collected from mangoes, and it is possible that these species could be enriched, as the mango has a high content of fructose and phosphorus.

Therefore, these strains could be ingested with the natural diet, or acquired through vertical transmission, contributing to the host's sugar assimilation. Finally, wild adult sequences clustered within the bacteria clade (i.e. *Ralstonia*, *Herbaspirillum*, and *Ochrobactrum*), and no archaea were detected. Isolated *Ralstonia* and *Herbaspirillum* strains are associated with copper biosequestration and degradation of chlorophenol, respectively (Im et al. 2004; Yang et al. 2010). In mango crops, copper and chlorophenol are used in distinct formulations to avoiding different diseases caused by fungus and bacteria (Czaplicka 2004; Sarwar 2015). In flies, depending upon the dose, copper compound leads to positive and negative effects. Positively, copper works by activating superoxide dismutase to regulate oxidative stress, modulating the genotoxic effects of reactive oxygen species and therefore, fly survival increases; while copper intoxication leads to the opposite outcome (Matsuo et al. 1997; Arcaya et al. 2013; Southon et al. 2013). Meanwhile, chlorophenol has detrimental outcomes in different organisms (Czaplicka 2004). This suggests that the species harbored in the *A. obliqua* intestine could modulate fly fitness by regulating copper and oxidative metabolism.

To the best of our knowledge, this is the first record of archaea in the digestive tract of *A. obliqua*. Archaea are important organisms for metabolism in the intestines of insects (Ceja-Navarro et al. 2014). It has also been described that archaea produce metabolites that are used by insects to locate fruits (Piñero et al. 2015). Thus, archaea and the rest of microorganisms could promote food pre-digestion, change the nutritional content, or improve digestion in the fly gut, but also suppress the toxic effects of chemical compounds used in crops. Altogether, the results presented here suggest that archaea and bacteria strains, which could be ingested during natural or artificial feeding, could modulate metabolic pathways in tephritidae through the digestion and assimilation of nutrients. Therefore, the differences between archaea and bacteria specimens detected among our samples can highlight their importance in the midgut.

Fungi diversity in wild and laboratory flies

Fungi provide sustenance and dwellings for insects, while insects offer material for fungal decomposition, protect growing spaces, and allow for transportation to new locations. In the intestine of *D. melanogaster*, 45 different species of fungi have been identified, and this diversity is related to the feeding environment (Stefanini 2018). For the fruit fly *C. capitata*, isolated species of fungi, as well as their metabolites, have been suggested to act as biocontrol agents (Ortiz-Urquiza et al. 2009; Imoulán and Elmezziane 2014; Navarro-Llopis et al. 2015; Ruiu et al. 2015). For *Bactrocera* spp, the yeast of the genera *Hanseniaspora* and *Pichia* are fundamental in larval survival (Piper et al. 2017). Therefore, fungi must also play an essential role in the intestine of *A. obliqua*. Consequently, we evaluated the presence of fungi in the midguts of all samples.

Fungal communities observed in the intestine of wild adults had greater diversity than wild larvae samples. The wild adult fly exhibited greater diversity ($H' = 0.8$) than wild larvae ($H' = 0.67$), sharing 62% of their communities. In the case of the laboratory samples, we observe similar DGGE pattern as the used control (Online Resource 2), restricting the H' and J analyses (Table 1). Despite the efforts we did not obtain clones possible due to the lower concentration of fungi DNA related to it host DNA. Nevertheless, our DGGE results suggest the presence of fungi at *A. obliqua* at midgut, mainly at wild samples, and that adults have more diversity than larvae.

It is known that the fungal community can play essential roles in insect guts and has been shown to produce toxins that are pathogenic for insects (Kostovcik et al. 2015). As beneficial guests, fungal communities could act as suppliers of organic ingredients, essential vitamins, and enzymes that promote digestion (Vega and Dowd 2005). Fungi could also improve metabolism via sugar fermentation and nitrogen-fixing, as well as participate in pheromone production by synthesizing steroids, as has been shown in bark beetles (Klepzig et al. 2009). Some of these functions may occur in the digestive tract of wild larvae and adult flies, which could explain the differences found between wild and laboratory samples.

16S rDNA hypervariable region diversity

Our study aimed to increase our knowledge regarding *A. obliqua* microbiota. Through this process, we found that some biases must be overcome to determine microbial diversity with the best possible accuracy. These biases start with the selection of DNA extraction methods, primer design, and PCR yield, being determinative to identify the microbiome composition in a sample (Guillén-Navarro et al. 2015; Abellan-Schneyder et al. 2021). We showed that with the use of different primer sets aimed at the same target (diverse hypervariable regions of bacteria and archaea 16S rDNA), it was possible to identify species that have not been previously described in this fruit fly. Next-generation sequencing methods using 16S rDNA would allow us to increase the number of species identified, but could introduce biases due to different factors, one of which is the use of primers targeted to only one or two of nine 16S rDNA hypervariable regions, which limits species identification in complex bacterial populations (Laursen et al. 2017; Bukin et al. 2019). Thus, the phyla *Proteobacteria*, *Actinobacteria*, and *Deinococcus* have been favored as the most abundant in *Anastrepha ludens*, *A. obliqua*, *A. serpentina*, and *A. striata* through 16S pyrosequencing, with primers targeted to the V3 hypervariable region (Ventura et al. 2018). In this work, we systematically amplify 16S rDNA hyper variable regions of V6-V8 to Gram-negative, V1-V8 > V6-V8 (nested) to gram positive (nested), and V1-V6 > V4-V5 (nested) to archaea (Online Resource 1). With this, we observe that V6-V8 hyper variable region captured with primers to gram-positive bacteria, can also identify gram-negative bacteria. Outstandingly, the V4-V5 archaea regions also retrieve blast to specialized bacteria. Despite, other hyper variable regions are used to evaluate microbial diversity (V2-V3), our experimental approach provide important understanding of the microbial composition in this fly (Bukin et al. 2019). Our results also broaden our knowledge of the microbiota of *A. obliqua* with a 28% global abundance of the Archaea kingdom (Fig. 3 and Online Resource 4, 5).

Conclusion

In this work, we present the inter-kingdom diversity harbored in the midgut of *A. obliqua*, expanding the intricate microbial crosstalk, which could be related to different biological outcomes (e.g., bio-assimilation) in larvae and adult wild or laboratory flies. It is important to note that the techniques based on the analysis of rRNA genes amplified by PCR may not represent a complete and accurate picture of the microbial community. This study is only the beginning of our understanding of the intestinal ecosystem of *Anastrepha*. We have determined some predominant populations, which could significantly modulate the fitness and physiology in mass-rearing and wild *A. obliqua* flies through an inter-kingdom communication of such communities. Archaea and metabolic specialized bacteria were identified here for the first time, suggesting an important role to fly fitness. Further studies are required to understand how these microorganisms affect metabolic processes, sexual behavior, and other aspects of the fly's physiology. This knowledge will eventually improve the mass rearing technology of *A. obliqua*, and focus on the discovery of microorganisms and enzymes of nutritional interest for fruit flies.

Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

KGN and HE idealized and designed the research. LVGF and GZR perform the experiments. KGN, GRA and LVGF analyze the data. KGN and GRA wrote and edit the manuscript.

Data Availability

The identified sequences were submitted at the NCBI repository with the identifiers from MK729487 to MK729533, see Table 2.

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Figures

Figure 1

Phylogenetic tree of bacteria species identified in the intestine of *Anastrepha obliqua*. Gram-negative and positive bacterial communities are delimited in blue and red, respectively. The 16S ribosomal DNA (rDNA) bacterial clones obtained from wild adult and larvae flies are shown as WL_ (pink triangles) and WA_ (red triangles); the laboratory samples as LL_ (blue light circles) and LA_ (blue circles). The tree (439 positions) was constructed using maximum composite likelihood (MCL) with a Kimura 2-parameter model. The percentage higher than 70% of 1000 bootstrap resampling is shown next to the branches. Evolutionary analyses were conducted using MEGA6

Figure 2

Phylogenetic tree of archaea communities identified in the intestine of *A. obliqua*. Two main clades can be seen, archaea (green) and bacterial (blue) communities. The 16S rDNA bacterial clones obtained from wild adult and larvae flies are shown as WL_ (pink triangles) and WA_ (red triangles); the laboratory samples as LL_ (blue light circles) and LA_ (blue circles). DAMBE software was employed to merge equal sequence clones. The neighbor-joining method to a matrix of pairwise distances estimated using the MCL approach with a Kimura 2-parameter model was used to construct the tree (406 positions). The percentage higher than 70% of 1000 bootstrap resampling of trees in which the associated taxa clustered together is shown next to the branches. Evolutionary analyses were conducted using MEGA6.

Figure 3

Microbial community profile identified in the intestine of *A. obliqua*. The 16S rDNA bacterial, actinomycete, and archaea clones obtained from wild and laboratory adult and larvae flies were curated, merged, and analyzed in Geneious software to determine 16S biodiversity. Most of the recovered sequences (~85%) were relative to bacteria showing three main clades (proteobacteria, firmicutes, and actinomycetes) ~25% were relative to Archaea

Supplementary Files

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