CHAPTER 4.4.

INSECT SYMBIOSIS IN SUPPORT OF THE STERILE INSECT TECHNIQUE

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SUMMARY

Recent estimations from various studies suggest that the total number of insect species ranges between $2.8\text{--}30 \times 10^6$; an estimate of 5.5×10^6 is probably closer to reality (Stork et al. 2015; Stork 2018). Insect species have established sophisticated symbiotic associations with a plethora of diverse microorganisms including bacterial species. These symbiotic associations can drastically influence the biology, ecology, and evolution of insect hosts, including insect species of agricultural, veterinary, and human-health importance (Margulis and Fester 1991; Zilber-Rosenberg and Rosenberg 2008). Despite the widespread presence of symbiosis, only in the last decades have technological advances enabled us to take a first glance at the complexity of such interactions, at least at the genomic level. Although studies increasingly describe and assess different possible effects of insects' symbionts on their hosts, including in mosquitoes, tsetse flies, and other pest and disease-vector species, this chapter summarizes the knowledge, gained mainly in the last two decades, on the structure and importance of gut symbiotic communities of tephritid fruit flies of economic importance, focusing mainly on their beneficial impact and prospects for improvement in the sterile insect technique (SIT).

1. INTRODUCTION

Defining symbiosis and its types has been quite challenging. It was first defined as “the living together of unlike organisms” (De Bary 1879). However, the complexity underlying this term is extensive, and additional terms have been proposed to further elaborate this widely diverse phenomenon. For practical purposes, it is important to follow generally accepted criteria to define symbiotic relationships. Although not fully determined and quite dynamic, such criteria are necessary to categorize and study the different aspects of symbiosis.

A symbiotic relationship (and more specifically, a symbiont) may fall into more than one category, and categorization depends also on the perspective or the aim of the researcher studying the symbiont or the symbiotic relationship. Some of the prevailing terms and classifications of symbiosis are summarized in Table 1. Although the clear majority of insect symbiotic relationships can still be considered as unknown, there are some well-studied examples among different insects of economic, veterinary or human health importance. Their elucidation has been achieved due to numerous studies which have revealed the existence of specific bacteria that clearly are important in certain insect species; some of them are summarized below.

- *Buchnera aphidicola* in aphids: This gammaproteobacterium is a primary symbiont of aphids that provides a clear nutritional benefit to its host. It is an Enterobacteriaceae-like bacterium, but a long-standing symbiotic relationship, estimated to have started $160\text{--}260 \times 10^6$ years ago, has led to the gradual loss of important genes from the *B. aphidicola* genome and the development of an obligatory symbiotic relationship with aphids (van Ham et al. 2003; Andersson 2006; Pérez-Brocal et al. 2006; Douglas 2009, 2013; Akman Gündüz and Douglas 2012).
- *Wigglesworthia glossinidia*: Like *B. aphidicola*, this bacterium is a primary obligatory symbiont of tsetse flies, offering primarily a nutritional benefit to its host. It has also lost many genes, resulting in a very small genome and dependency on the tsetse host (Aksoy 1995; Chen et al. 1999; Akman et al. 2002; Pais et al. 2008; Weiss and Aksoy 2011; Bing et al. 2017).

Table 1. Different types and classifications of symbiosis

Term	Definition/examples
Mutualism vs commensalism vs parasitism	Mutualism is when both partners benefit from the relationship, commensalism when one benefits while the other partner is not affected, and parasitism is the relationship where one partner benefits at the expense of the other.
Obligatory vs facultative	In an obligatory symbiosis, partners cannot survive without each other. In a facultative symbiosis, the partners benefit from living with each other, but they can still live without each other. Alternatively, symbionts can be defined as primary (obligatory) or secondary (facultative). As an example, <i>Wigglesworthia glossinidia</i> is considered as a primary endosymbiont of tsetse flies.
Ectosymbiosis vs endosymbiosis	Depending on whether the symbiont lives on the outer surface or within the body of the host, symbiosis is defined as ecto- and endosymbiosis, respectively. In the case of endosymbiosis, if the symbiont lives either within the host's cells or outside them, it can be considered as intra- or extracellular symbiont, respectively. As examples, <i>Wolbachia</i> is considered as intracellular, while the different Enterobacteriaceae localized in the gut are primarily extracellular.
Tissue specificity	A symbiont may have a "universal" presence in the host or may be restricted to specific tissues. This may also indicate the specific role of the symbiont. In this respect, Enterobacteriaceae are considered mainly as "gut symbionts", while the alphaproteobacterium <i>Wolbachia</i> is considered as a "reproductive symbiont", although additional functions have been reported for it.
Type of benefit gained	Symbionts can be beneficial for their hosts in different ways. Among them, those delivering nutritional, reproductive, and immunity/defense advantages, are receiving much attention.
Autochthonous (or resident) vs allochthonous (or transient) symbiosis	One of the most difficult categories to define is whether we are dealing with "actual" symbiosis, which implies the establishment of long-standing symbiotic relationships, or the observed symbiotic communities are, at least partially, defined by chance (meaning by the random acquisition of symbionts from the environment that are not able to sustain themselves in the host without their continuous supply from the environment). Such relationships, although they may be beneficial for the host, will be eliminated after transfer to a different "ecological niche" (a laboratory can be considered as a different niche as well). In such observed changes, the question regarding symbionts that are no longer present is: are these microorganisms actual symbionts that were no longer beneficial in the new environment, or do they represent transient symbionts that disappeared since there was no longer an external continuous supply from the environment?
Type of partner	The usual assumption is that it is bacteria that have established relationships with their eukaryotic host. Although probably bacteria cover most of such symbiotic relationships, there are also symbiotic relationships involving archaea, viruses, and eukaryotic microorganisms (such as fungi and yeast).

- *Candidatus* Erwinia dacicola: This bacterium is considered as a primary obligatory symbiont for the olive fruit fly *Bactrocera oleae* (Rossi). It is important for helping *B. oleae* larvae feed on a "challenging" environment, such as unripe olives. It is also a characteristic example of the confusing limits of obligatory and secondary symbionts, since it is no longer necessary for domesticated olive flies not reared on olives. Therefore, studying only laboratory populations would lead to the conclusion that this bacterium is not a symbiont of *B. oleae* (Capuzzo 2005; Estes et al. 2009, 2012; Kounatidis et al. 2009; Ben-Yosef et al. 2014).

- *Wolbachia pipientis*: This group of Alphaproteobacteria is widespread in insects. It is found mainly in reproductive tissues, and is usually referred to as a “reproductive parasite” due to its ability to manipulate reproductive functions, leading to diverse phenotypes including parthenogenesis, feminization, male-killing, and cytoplasmic incompatibility (CI). It is a facultative intracellular symbiont that has developed preferential relationships with many insect species. Although referred to mainly as a reproductive parasite, there are studies indicating that *Wolbachia* also has a role in nutrition, metabolism, and immunity (Werren et al. 2008; Saridaki and Bourtzis 2010; LePage and Bordenstein 2013). Current knowledge on the status of *Wolbachia* in tephritids, regarding both presence and possible effects, has been summarized recently by Mateos and her colleagues (Mateos et al. 2020). In addition, it has been suggested that the SIT and *Wolbachia* symbiosis can be combined (SIT/IIT, Incompatible Insect Technique) into a safe and biosecure approach for the population suppression of *Aedes* mosquito species transmitting major human pathogenic diseases such as dengue, chikungunya, Zika, and yellow fever (Zheng et al. 2019; Lees et al., this volume).

2. MAPPING SYMBIOTIC DIVERSITY

An important step towards utilizing insect symbionts is mapping their diversity. This rather descriptive step is crucial, since we need to know the identities of the symbionts so that later on we can decide what roles symbionts have and which ones are important. Characterizing symbionts and symbiotic communities has been laborious, but recently technological advances in sequencing technology have enabled their quick identification, at least at the DNA level. However, the complete characterization of a symbiont requires a combination of approaches, and is still a labour-consuming task (Table 2).

Prior to DNA-based characterization, the classification of bacteria was based only on phenotypic characteristics. These include morphological, biochemical, and cultivation characteristics, including the ability to grow in selective media, production of characteristic substances that can be identified easily, and resistance/sensitivity to a variety of antimicrobials. As a prerequisite for classification, the bacteria had to be cultivable.

Systematic genotyping started with molecular methods that also required a cultivation-dependent step, especially in the time before the introduction of the Polymerase Chain Reaction (PCR). Sequencing of the 16S *rRNA* gene (or others), and related methods (such as the Denaturing Gradient Gel Electrophoresis (DGGE), Restriction Fragment Length Polymorphism (RFLP), and PCR-RFLP) based methodologies (Krafsur and Ouma, this volume), were utilized and are still being used. Reduction in the cost of Sanger sequencing, and advances in bioinformatic analysis during previous decades, enabled the development of Multi Locus Sequence Typing (MLST) and Multi Locus Sequence Analysis (MLSA) approaches, based on a combination of selected genes, universal for a species or closely related species. However, these methods are still time- and labour-consuming, as well as costly, permitting only a limited number of samples to be analysed and a rather restricted look into the genetic diversity of bacteria. A brief history on the transition from the

era of sole microscopy to the NGS-supported (Next Generation Sequencing) DNA genotyping is provided by Escobar-Zepeda et al. (2015).

Table 2. Symbiont classification approaches

Criteria		Approach	Outcome
Phenotypic	Morphological	Microscopic	Characterize the morphology of a selected bacterium
		Macroscopic	Characterize the morphology of a selected bacterial colony
	Biochemical	Growth	Characterize the growth profile of a selected bacterium under different conditions (aerobic vs anaerobic vs microaerophilic, temperature, pH, selective growth media)
		Antibiotic resistance	Characterize the resistance of a selected bacterium to different antimicrobials
		Metabolic pathways	Characterize the presence/absence of specific enzymes through their effect on metabolism
Genotypic	Single gene (16S <i>rRNA</i> gene)	PCR-RFLP	Characterize genetic diversity based on differences in sequences that are recognized by restriction enzymes; mainly used for 16S <i>rRNA</i> gene
		DGGE	Characterize genetic diversity based on differences in the DNA sequence of single genes that alter melting point and electrophoretic mobility
		Sanger sequencing	Characterize genetic diversity of single gene; it can be effective without cultivation in the presence of species-specific primers and absence of multiple genetically close strains
		NGS sequencing	Characterize the genetic diversity of mixed samples, based on a single gene; massive parallel sequencing that generates thousands or millions of short reads (amplicon sequencing)
	MLST	Sanger sequencing	Characterize genetic diversity of the DNA sequence of different genes; not suitable for mixed bacterial samples; it can be effective without cultivation in the presence of species-specific primers and absence of multiple genetically close strains
	Metagenomics	Genome sequencing	Characterize the genetic diversity based on the whole genome (complete or draft) in complex samples; more effective with cultivated material
	Single cell genomics	Genome sequencing	Characterize the genetic diversity based on the whole genome (complete or draft), derived from a single cell

The recent advances in sequencing technologies, such as High Throughput Sequencing, have enabled quick and massive parallel sequencing at a reduced cost, thus allowing a better look into bacterial communities and not just isolated bacteria. These technologies have been widely used in the last decade for the simultaneous analysis of the 16S *rRNA* gene profile of environmental samples, producing thousands

to millions of reads per sample, based on the set-up and the needs of the researcher. Still, the major limitation of this approach was the reading length, which is restricted to a few hundred base pairs, thus compromising the robustness of 16S *rRNA* gene-based taxonomy.

These sequencing advances, along with advances in molecular biology and bioinformatic analysis, now enable a faster analysis of bacteria or bacterial communities of interest at a reduced cost. This is extremely important because single gene or MLST approaches cannot fully resolve the genetic diversity among bacteria of interest, especially considering that even strains of the same species can have quite diverse properties. Based on these advances, metagenomic approaches that can analyse almost the complete genomic content of communities of organisms, and single cell genomics (that is the ability to have the complete genome of a single cell), are offering opportunities for the analysis of symbiotic communities that did not seem realistic one or two decades ago.

3. EXPLOITING INSECT GUT SYMBIONTS TO ENHANCE THE SIT

There are several studies describing many different possible effects of insects' symbionts on their hosts, as well as their potential role to enhance SIT application. There are studies in mosquito species (for recent reviews see Guégan et al. (2018), Strand (2018), and Scolari et al. (2019) and references therein) and tsetse species (less) (for reviews see Snyder and Rio (2013) and Wang et al. (2013) and references therein), but most of the data derive from experiments on fruit flies in the family Tephritidae (Niyazi et al. 2004; Behar et al. 2008; Ben Ami et al. 2010; Gavriel et al. 2011; Hamden et al. 2013; Sacchetti et al. 2014; Augustinos et al. 2015; Kyritsis et al. 2017; Cai et al. 2018; Khaeso et al. 2018; Ventura et al. 2018; Akami et al. 2019; Augustinos et al. 2019).

3.1. Mapping Symbiotic Diversity in Fruit Flies of Economic Importance

Numerous studies have been performed on different tephritids. Among them, most studies are on the Mediterranean fruit fly *Ceratitis capitata* (Wiedemann), which is a model for SIT application, and *B. oleae*. Recently, other species, mainly of the *Anastrepha*, *Bactrocera*, and *Zeugodacus* genera, have also been targeted. Studies that address the structure of symbiotic communities in tephritids are summarized in Table 3. As already stated, the first steps are descriptive; many studies deal with identifying which bacterial species are found in which hosts. The next step is to describe the relative abundances, and how bacterial symbiotic communities change under different conditions. Subsequently comes the question of functional roles; the mere analysis of presence and/or abundance of certain symbionts cannot answer such questions.

3.1.1. Gut Symbiotic Communities in the Mediterranean Fruit Fly

Starting with the Mediterranean fruit fly, many studies have been performed on the characterization of its gut symbiotic communities (Table 3). In more recent years,

beginning in 2005, gut symbiotic communities were described for this species under different conditions. *Klebsiella oxytoca*, an Enterobacteriaceae diazotroph common on plants and able to fix atmospheric nitrogen, was identified as a major symbiont of natural populations (Behar et al. 2005). Minor but persistent *Pseudomonas* communities were also identified using PCR-based assays (Behar et al. 2008).

The effect of irradiation on the structure of gut symbiotic communities was also documented (Ben Ami et al. 2010), and communities of reduced variability were identified in long-established laboratory populations and strains of SIT importance (Ben Ami et al. 2010; Augustinos et al. 2015). Although not strictly referring to gut communities, the reduced diversity of symbionts in a line of the Mediterranean fruit fly VIENNA 7 genetic sexing strain (GSS), reared in Australia (~99% *Enterobacter* sp.) (Morrow et al. 2015), is in accordance with these two studies. Utilization of NGS approaches enabled a more detailed look at the symbiotic communities of both wild and laboratory populations and at different developmental stages (Aharon et al. 2013; Malacrinò et al. 2018).

3.1.2. Gut Symbiotic Communities in the Olive Fruit Fly

Among tephritids, the olive fruit fly is probably the species that most clearly demonstrates how advances in sequencing and bioinformatics have enlarged our ability to study and understand insect symbiotic communities (Table 3). More than a century ago, Petri described cultivable endosymbionts of *B. oleae* (Petri 1909). In 2005, *Candidatus* *Erwinia dacicola* was identified as the main symbiont of olive fruit fly's natural populations, while later other potential symbionts were identified in natural and laboratory populations of the species (Capuzzo 2005; Estes et al. 2009, 2012; Kounatidis et al. 2009; Ben-Yosef et al. 2014; Augustinos et al. 2019; Koskinioti et al. 2019).

Interestingly, it was also recognized that the primary symbiont of natural populations, *Candidatus* *Erwinia dacicola*, is almost absent from artificially reared populations (Estes et al. 2009, 2012; Kounatidis et al. 2009). The presence of abundant and minor components of the communities could be better described, at least to the resolution of genus level (Ben-Yosef et al. 2014, 2015). Using single cell genomics and NGS sequencing, the draft genome of *Candidatus* *Erwinia dacicola* was obtained, starting from DNA of a single cell, which is important for bacterial species that cannot be cultivated (Blow et al. 2016).

3.1.3. Gut Symbiotic Communities in Some Anastrepha, Bactrocera, and Zeugodacus Species

Except for the Mediterranean fruit fly and the olive fruit fly discussed above, there are only a few studies in other tephritids (Table 3). Using classical microbiological approaches, a small number of bacterial taxa has been characterized in *Anastrepha ludens* (Loew) (Kuzina et al. 2001), *Bactrocera dorsalis* (Hendel) (Pramanik et al. 2014; Gujjar et al. 2017; Khaeso et al. 2018; Khan et al. 2019), *Bactrocera zonata* (Saunders) (Naaz et al. 2016), *Zeugodacus cucurbitae* (Coquillett) (Hadapad et al. 2016; Gujjar et al. 2017), and *Zeugodacus tau* (Walker) (Prabhakar et al. 2013; Khan et al. 2014).

Table 3. Efforts for mapping gut associated bacteria species in tephritids of economic importance

Species	Approach	Material used	Main outcome	Reference
<i>Bactrocera oleae</i>	Direct PCR and sequencing of the 16S <i>rRNA</i> gene of extracts	Wild material collected as pupae	<i>Candidatus</i> Erwinia dacicola is the main symbiont of wild populations	Capuzzo 2005
	Microscopy, culture dependent, and culture independent approaches	Wild material kept on olives in the lab	<i>Candidatus</i> Erwinia dacicola is the main symbiont <i>Enterobacter</i> sp., <i>Citrobacter</i> sp., and <i>Raoultella</i> sp. also present	Estes et al. 2009
	16S <i>rRNA</i> gene libraries and sequencing	Wild material collected as pupae and laboratory colonies	<i>Acetobacter tropicalis</i> is present in laboratory colonies and wild sample; <i>Enterococcus</i> sp. and <i>Paenibacillus</i> sp. also detected; <i>Candidatus</i> Erwinia dacicola detected only in natural populations	Kounatidis et al. 2009
	16S <i>rRNA</i> gene PCR and <i>Candidatus</i> Erwinia dacicola specific assay	Wild material collected as pupae and laboratory colonies	<i>Candidatus</i> Erwinia dacicola spread in all wild populations <i>Candidatus</i> Erwinia dacicola present in laboratory colonies reared on olives; <i>Candidatus</i> Erwinia dacicola is rare in laboratory colonies reared on artificial diet; Presence of different Enterobacteriaceae in laboratory populations	Estes et al. 2012
	16S <i>rRNA</i> gene libraries and sequencing	Wild material collected as pupae (only oesophageal bulbs)	Two haplotypes of <i>Candidatus</i> Erwinia dacicola	Savio et al. 2012
	High throughput sequencing -- 16S <i>rRNA</i> gene	Wild material of different developmental stages	High prevalence of <i>Candidatus</i> Erwinia dacicola; Low presence of <i>Pectobacterium</i> , <i>Pseudomonas</i> , and <i>Kluyvera</i>	Ben-Yosef et al. 2014
	High throughput sequencing – 16S <i>rRNA</i> gene	Wild material kept on olives in the laboratory	High prevalence of <i>Candidatus</i> Erwinia dacicola in wild flies but not in mass-reared flies; Low presence of <i>Pantoea</i> and <i>Burkholderia</i> both in wild flies and mass-reared flies; High prevalence of <i>Providencia</i> in mass-reared flies but not in wild flies	Ben-Yosef et al. 2015

Table 3. Continued

Species	Approach	Material used	Main outcome	Reference
<i>Bactrocera oleae</i>	Single cell genomics and metagenomics	Wild material	Draft genome of <i>Candidatus</i> <i>Erwinia dacicola</i>	Blow et al. 2016
	High throughput sequencing – 16S <i>rRNA</i> gene	Wild material plus 1 st generation in the laboratory	Detection of different OTUs including <i>Candidatus</i> <i>Erwinia dacicola</i> , <i>Providencia</i> , <i>Enterobacter</i> , and <i>Klebsiella</i> , Prevalence of <i>Candidatus</i> <i>Erwinia dacicola</i>	Koskinioti et al. 2019
	454 pyrosequencing	Laboratory population, different developmental stages, age, and sex	Mainly Enterobacteriaceae, <i>Morganella</i> prevailing, developmental stage and age influence structure of gut microbiota community	Augustinos et al. 2019
<i>Bactrocera cacuminata</i>	16S <i>rRNA</i> gene libraries and sequencing	Wild material	Dominant Gammaproteobacteria Mainly Enterobacteriaceae (<i>Enterobacter</i> , <i>Serratia</i> , <i>Klebsiella</i> , <i>Escherichia</i>)	Thaochan et al. 2015
<i>Bactrocera carambolae</i>	Illumina sequencing	Wild material	Gammaproteobacteria dominant	Yong et al. 2017
<i>Bactrocera dorsalis</i>	PCR of the 16S <i>rRNA</i> gene, DGGE, sequencing	Laboratory colonies and wild material	Dominant Gammaproteobacteria Diverse symbiotic community	Wang et al. 2011
	Culture dependent, morphological and biochemical characterization	Laboratory colonies	11 genera identified Dominant Enterobacteriaceae community (<i>Citrobacter</i> , <i>Enterobacter</i> , <i>Serratia</i> , <i>Klebsiella</i> , <i>Morganella</i>)	Pramanik et al. 2014
	Pyrosequencing	Wild material	172 OTUs identified Effect of developmental stage on symbiotic communities Firmicutes and Proteobacteria dominant	Andongma et al. 2015
	Illumina sequencing	Wild material	Gammaproteobacteria dominant	Yong et al. 2017
	Culture dependent, morphological, biochemical, 16S <i>rRNA</i> gene sequencing	Laboratory colonies	Mainly different Enterobacteriaceae genera (<i>Providencia</i> , <i>Klebsiella</i> , <i>Enterobacter</i> , <i>Citrobacter</i>)	Gujjar et al. 2017
	Culture dependent, 16S <i>rRNA</i> gene sequencing	Laboratory colonies	13 bacterial species Firmicutes and Proteobacteria more abundant	Khaeso et al. 2018

Table 3. Continued

Species	Approach	Material used	Main outcome	Reference
<i>Bactrocera dorsalis</i>	Illumina sequencing plus culture dependent 16S <i>rRNA</i> gene sequencing	Laboratory colonies	Dominance of Enterobacteriaceae, presence of other minor communities that increase their relative abundance after irradiation	Cai et al. 2018
	Culture dependent 16S <i>rRNA</i> gene sequencing, morphological characterization	Laboratory colonies	Dominance of Enterobacteriaceae, reduced diversity	Khan et al. 2019
	Illumina sequencing	Laboratory colonies and wild material	Differences due to developmental stage, age, and origin (wild versus laboratory)	Hadapad et al. 2019
<i>Bactrocera minax</i>	454 pyrosequencing	Wild material	Proteobacteria dominant Genera of Enterobacteriaceae the most represented (<i>Enterobacter</i> , <i>Klebsiella</i> , <i>Serratia</i> , <i>Citrobacter</i>)	Wang A. et al. 2014
	454 pyrosequencing	Wild material	Proteobacteria dominant Genera of Enterobacteriaceae the most abundant	Andongma et al. 2019
<i>Bactrocera tryoni</i>	16S <i>rRNA</i> gene libraries and sequencing	Wild material	Proteobacteria dominant, mainly Gammaproteobacteria	Thaochan et al. 2015
	16s <i>rRNA</i> gene NGS amplicon sequencing	Laboratory colonies and wild material	Presence of Acetobacteraceae, Enterobacteriaceae, and Leuconostocaceae in wild samples Differences between laboratory and wild populations Presence of <i>Asaia</i> sp. in samples deriving from wild	Deutscher et al. 2018
<i>Bactrocera zonata</i>	Culture dependent, morphological, biochemical, 16S <i>rRNA</i> gene sequencing	Wild material	Three dominant bacterial genera (<i>Klebsiella</i> , <i>Microbacterium</i> , <i>Rhodococcus</i>)	Naaz et al. 2016
<i>Ceratitis capitata</i>	PCR-DGGE analysis and sequencing	Wild material	Identification of different Enterobacteriaceae (<i>Enterobacter</i> , <i>Citrobacter</i> , <i>Klebsiella</i> , <i>Pectobacterium</i>) <i>Klebsiella</i> is the most dominant Variability among developmental stages	Behar et al. 2005

Table 3. Continued

Species	Approach	Material used	Main outcome	Reference
<i>Ceratitis capitata</i>	Specific assays for targeting minor symbiotic communities (16S <i>rRNA</i> gene)	Wild material	Presence of a minor, previously undetected, <i>Pseudomonas</i> community	Behar et al. 2008
	Culture dependent approach, 16S <i>rRNA</i> gene, DGGE, sequencing	Mass-reared Vienna 8 GSS	Effect of irradiation on gut symbiotic communities Differences between laboratory VIENNA GSS strain and the wild population Different Enterobacteriaceae present (<i>Salmonella</i> , <i>Citrobacter</i> , <i>Providencia</i> , <i>Morganella</i> , <i>Enterobacter</i> , <i>Pectobacterium</i> , <i>Klebsiella</i>) Presence of a stable <i>Pseudomonas</i> community	Ben Ami et al. 2010
	In situ, metabolic profiles, culture, pyrosequencing	Wild material	Change in the diversity, size and distribution of symbionts in different developmental stages Communities of different Enterobacteriaceae	Aharon et al. 2013
	Culture dependent approach, 16S <i>rRNA</i> gene sequencing	Mass-reared Vienna 8 GSS, different developmental stages and ages	Three genera identified (<i>Enterobacter</i> , <i>Providencia</i> , <i>Acinetobacter</i>) Changes in the composition of the symbiotic communities with development	Augustinos et al. 2015
	Illumina MiSeq sequencing, 16S <i>rRNA</i> gene	Wild material from different hosts	More than 3000 OTUs present, mainly Firmicutes and Proteobacteria Significant differences among different developmental stages Effect of the host fruit on the profile of the communities	Malacrinò et al. 2018
<i>Anastrepha fraterculus</i>	454 pyrosequencing	Laboratory populations of two morphospecies, different developmental stage, age, and sex	Mainly Enterobacteriaceae, <i>Morganella</i> prevailing, developmental stage and age influence structure of gut microbiota community	Augustinos et al. 2019
<i>Anastrepha grandis</i>	454 pyrosequencing	Laboratory population, different develop. stage, age, and sex	Mainly Enterobacteriaceae, <i>Morganella</i> prevailing, developmental stage and age influence structure of gut microbiota community	Augustinos et al. 2019

Table 3. Continued

Species	Approach	Material used	Main outcome	Reference
<i>Anastrepha ludens</i>	Culture dependent, metabolic profile	Laboratory colonies	18 species in 13 genera Enterobacteriaceae prevailing community (<i>Enterobacter</i> , <i>Providencia</i> , <i>Serratia</i>)	Kuzina et al. 2001
	454 pyrosequencing	Wild material	Mainly Enterobacteriaceae	Ventura et al. 2018
	454 pyrosequencing	Laboratory population, different developmental stages, age, and sex	Mainly Enterobacteriaceae, <i>Morganella</i> prevailing, developmental stage and age influence structure of gut microbiota community	Augustinos et al. 2019
<i>Anastrepha obliqua</i>	454 pyrosequencing	Wild material	Mainly Enterobacteriaceae	Ventura et al. 2018
<i>Anastrepha serpentina</i>	454 pyrosequencing	Wild material	Mainly Enterobacteriaceae	Ventura et al. 2018
<i>Anastrepha striata</i>	454 pyrosequencing	Wild material	Mainly Enterobacteriaceae	Ventura et al. 2018
<i>Zeugodacus cucurbitae</i>	Culture dependent, morphological, biochemical, 16S <i>rRNA</i> gene sequencing	Wild material	Nine genera identified, mainly Enterobacteriaceae (<i>Enterobacter</i> , <i>Klebsiella</i> , <i>Citrobacter</i> , <i>Providencia</i>)	Hadapad et al. 2016
	Culture dependent, 16S <i>rRNA</i> gene sequencing	Laboratory population	<i>Bacillus</i> and <i>Morganella</i>	Gujjar et al. 2017
	Illumina sequencing	Laboratory colonies and wild material	Differences due to developmental stage, age, and origin (wild versus laboratory)	Hadapad et al. 2019
<i>Zeugodacus tau</i>	Culture dependent, morphological, biochemical, 16S <i>rRNA</i> gene sequencing	Laboratory populations	Three species (<i>Klebsiella oxytoca</i> , <i>Pantoea agglomelans</i> , <i>Staphylococcus</i> sp.)	Prabhakar et al. 2013
	Culture dependent, morphological, biochemical, 16S <i>rRNA</i> gene sequencing	Laboratory population	Mainly Enterobacteriaceae identified (<i>Proteus</i> , <i>Klebsiella</i> , <i>Erwinia</i>) but also others	Khan et al. 2014

Characterization based on construction of 16S rRNA gene libraries and/or Sanger sequencing has been performed in *Anastrepha fraterculus* (Wiedemann) (Juárez et al. 2019), *Bactrocera cacuminata* (Hering) (Thaochan et al. 2015), *B. dorsalis* (Wang et al. 2011; Khaeso et al. 2018), *Bactrocera minax* (Enderlein), (Andongma et al. 2019), and *Bactrocera tryoni* (Froggatt) (Thaochan et al. 2015).

Finally, using NGS approaches, the structure of gut symbiotic communities was resolved in higher resolution for some species, including both laboratory and natural populations. Among them are natural populations of *A. ludens* (Loew), *A. obliqua* (Macquart), *A. serpentina* (Wiedemann), *A. striata* Schiner (Ventura et al. 2018), *Bactrocera carambolae* Drew and Hancock (Yong et al. 2017), *B. dorsalis* (Andongma et al. 2015; Yong et al. 2017), *Bactrocera minax* (Enderlein) (Wang A. et al. 2014), *Z. cucurbitae* (Hadapad et al. 2019), as well as laboratory populations of two morphotypes of *A. fraterculus*, *A. grandis* (Macquart), and *A. ludens* (Augustinos et al. 2019), *B. dorsalis* (Hadapad et al. 2019), and *Z. cucurbitae* (Hadapad et al. 2019).

3.2. Gut Symbiotic Communities – Limitations

Despite the advances in sequencing approaches that result in enormous data sets, we must keep in mind that such sequencing-based studies are descriptive in principle since a) the resolution provided does not easily go beyond genus level and, b) the “actual” bacteria are not isolated and cultivated, resulting in reduced usefulness regarding possible applications. Moreover, the differences in the methodological set-up (samples collection, DNA extraction, sequencing platform, number of sequences analysed, and algorithms applied for analysis of sequences) make comparisons among studies difficult.

Although the focus until now has been on bacteria, they are not the only symbiotic players in the insect microbiota. A few recent studies are investigating other symbionts that could be important. For example, there is a fungal survey of olive fruit fly wild populations, based on a metabarcoding analysis of ITS2 (Malacrinò et al. 2017), and the cultivation-based identification (coupled with sequencing of selected genes) of yeast in natural populations of *B. tryoni* (Deutscher et al. 2017).

4. GUT SYMBIONTS IN THE INSECT MICROBIOTA – ROLE ASSESMENT

It is rather difficult to document the role and importance of symbionts for their host, mainly because:

- a. Symbionts may play a role in specific environments but may be redundant, unnecessary or outcompeted in others. *Candidatus* *Erwinia dacicola* plays a critical role in adult olive fruit flies by providing amino acids or converting inaccessible nitrogen. This symbiont is also important when olive fruit fly larvae feed on unripe olives. In such cases, poor sampling, or transfer of insects to a new environment for experiments, can compromise the results and interpretation.
- b. Symbiotic communities probably function in a “redundant” way, meaning that some of the symbiotic bacteria may be important, but their absence can be compensated by others, based on availability and chance. Provided that we are

dealing with “actual” and not transient symbionts, this may explain why different bacterial species can be found in the same host under different circumstances.

- c. There may be changes in preferential relationships during ontogeny. This is also difficult to prove and monitor, but may explain differences observed among developmental stages. During development, communities comprising different bacterial operational taxonomic units (OTUs) during development, or presenting quantitative differences, are commonly found. This means that the same OTUs are present but with significantly different relative abundances. This is what makes interpretation of data from natural populations difficult, where only a limited view of the life cycle is available.
- d. Bacteria with the highest representation are not necessarily the most important symbiont. Sometimes, underrepresented members of the community may provide something crucial. Based on the methodological approaches used, such symbionts may remain cryptic and their role underestimated.
- e. It is difficult to measure directly the effect of a symbiont. This is because it has to be isolated to test possible properties and functions or it must be studied as a part of the whole symbiotic association (but then it is not clear if a certain effect can be attributed to this specific symbiont).

To provide answers that are as robust as possible, different approaches are followed, and even better, a combination of them. However, all these approaches make certain compromises which must be considered both prior to planning and during the interpretation of results. Some of the main approaches used to estimate the importance of gut-related bacteria to their host are summarized below:

- *Characterization of the symbiotic communities of a species across its range:* As in all experiments, proper sampling is crucial. In this case, proper sampling means collecting material that represents different geographic origins, different seasons, and different hosts. In addition, different developmental stages, sex, and age are parameters that may shape the profile of symbiotic communities. Approaches that take into consideration the above could determine the “core microbiome” of an insect host. Obviously, ideal sampling targeting the symbiotic profile of a species can be a very laborious task.
- *Providing an excess amount of a cultivated symbiont under artificial-diet rearing:* Providing a symbiont through feeding can demonstrate its beneficial effects. However, this does not necessarily mean that these effects are present under standard conditions. Moreover, the positive effect may not have a linear correlation with the quantity; in such cases the effect will not be demonstrated by artificial accumulation of the symbiont.
- *Creation and test of aposymbiotic (symbiont-free) organisms:* This approach is useful because it provides evidence based on the negative (or positive) effects identified after the removal of symbionts. It can be done through antibiotic feeding, which results in the suppression or elimination of the symbiotic community. However, there are certain limitations. It is difficult to attribute an observed effect to a specific symbiont, since the whole community is influenced. Also, antibiotic treatment can influence non-symbiont mediated metabolic pathways (such as those of mitochondria), which makes the interpretation of results questionable. In such experiments, giving time for some generations to

develop without the effect of the antibiotic would reduce the possibility that the observed results are associated with anything but the removal of the symbionts.

- *Creation of aposymbiotic organisms and reintroduction of selected bacteria:* Although useful, this approach is partially compromised by the fact that an important effect may be identified, but this is in an artificial system and not in the framework of studying the host organism together with its symbionts as a single entity. That means that this effect could be substantially different in the presence of the other autochthonous symbionts.
- *Cultivation of symbionts and assessment of possible effects in vitro:* Cultivation in artificial growth media is necessary to find the metabolic pathway through which a symbiont affects its host. *In vitro* cultivation provides evidence about the potentially useful metabolites produced. However, it is not possible to assess *in vitro* whether the symbiont will produce the same effect under the complex *in vivo* interactions of its host and all associated symbionts.

5. SYMBIONTS AND THEIR POTENTIAL APPLICATION TO ENHANCE THE SIT

Symbionts can be useful in different ways to boost SIT application (Deutscher et al. 2019). The first application is what we refer to as “probiotic diets”. A probiotic diet can be beneficial in different ways. Based on the experimental set-up and the aim of the researcher, a valid research goal is to determine how diets involving symbionts are beneficial. Symbionts can be provided as live material; they can be used as a source of nutrients or to establish their beneficial properties. Live or dead symbionts can also act as food sources for resident symbionts, thus offering an indirect positive effect. Another possible application is to explore the attractancy potential of symbionts, e.g. to make sterile males more attractive for mating with wild females or to include them as a lure in a “trap package” (Pereira et al., this volume). The focus of this chapter is mainly on probiotic applications of gut-associated bacteria.

5.1. Positive Effects of Symbiont-Enriched Diets

Most of the studies have been performed on the Mediterranean fruit fly because it has been a model for the development of the SIT in tephritids (Table 4). This also means that there are universal protocols for rearing and handling, and laboratory rearing is quite easy and optimized. More importantly, the application of the SIT, involving the mass-production of the target species, makes these studies worthwhile. The potential positive effect of symbiont-enriched diets can be adopted easily in fly-rearing facilities, and may have a significantly favourable financial impact. However, the logistics aspect related to the large-scale production of probiotics still needs to be addressed.

Table 4. Summary of probiotic studies in Tephritidae and the main findings

Species	Bacteria	Provision	Effect	Reference
<i>Ceratitis capitata</i>	Enterobacteriaceae mix (<i>Enterobacter agglomerans</i> , <i>Klebsiella pneumoniae</i>)	Adult diet	Positive on male mating competitiveness of irradiated males (diet-dependent) Positive on male longevity of irradiated males (diet and age-dependent) Positive on sexual calling of irradiated males (diet-dependent)	Niyazi et al. 2004
	Enterobacteriaceae mix isolated from Mediterranean fruit fly (<i>Enterobacter</i> spp., <i>Klebsiella oxytoca</i> , <i>Citrobacter freundii</i> , <i>Pectobacterium cypripedi</i> , <i>Pantoea</i> spp.)	Adult diet	Positive on adult longevity (nutritionally restricted flies)	Behar et al. 2008
	<i>Klebsiella oxytoca</i> (Mediterranean fruit fly gut isolated)	Adult diet	Positive on mating latency time (irradiated, nutritionally restricted males)	Ben Ami et al. 2010
	<i>Klebsiella oxytoca</i> (Mediterranean fruit fly gut isolated)	Adult diet	Positive on mating competitiveness of irradiated males Positive on mating receptivity of nutritionally restricted females Positive on longevity in starvation of nutritionally restricted females	Gavriel et al. 2011
	Enterobacteriaceae mix of unknown isolation source: <i>Enterobacter</i> spp., <i>Klebsiella pneumoniae</i> , <i>Citrobacter freundii</i>	Larval diet	Positive on pupal weight Positive on flight ability (irradiated males) Positive on adult size Positive on mating competitiveness of irradiated males Positive on sperm transfer	Hamden et al. 2013
	<i>Enterobacter</i> sp. AA26	Larval diet	Replacement or partial replacement of yeast with dead <i>Enterobacter</i> can: positively affect pupal and adult recovery positively affect immature development duration (decrease) positively affect female pupal weight positively affect longevity under stress	Kyritsis et al. 2019

Table 4. Continued

Species	Bacteria	Provision	Effect	Reference
<i>Ceratitis capitata</i>	<i>Enterobacter</i> sp. AA26 (Mediterranean fruit fly gut isolated)	Larval diet	Positive on immature developmental duration (decrease) Positive on immature stages survival	Augustinos et al. 2015
	<i>Enterobacter</i> sp. AA26, <i>Klebsiella oxytoca</i>	Larval and adult diet	<i>Klebsiella oxytoca</i> has a positive on immature developmental duration (decrease) as a larval diet probiotic <i>Klebsiella oxytoca</i> has a positive on flight ability as a larval diet probiotic	Kyritsis et al. 2017
<i>Bactrocera oleae</i>	<i>Pseudomonas putida</i> (olive fly gut isolated)	Adult diet	Positive on the fecundity of nutritionally restricted females	Sacchetti et al. 2014
<i>Bactrocera dorsalis</i>	Various preparations of the identified bacteria	Larval diet	Effect on immature developmental duration (reduction or increase) Positive effect on pupal weight	Khaeso et al. 2018
	<i>Klebsiella oxytoca</i> and <i>Citrobacter coseri</i> separate live feeding	Larval diet	Feeding with live bacteria can restore ecological fitness of males that are sterilized through irradiation	Cai et al. 2018
	<i>Proteus</i> sp.	Larval diet	Improved adult emergence, male ratio, and survival under stress	Khan et al. 2019
<i>Bactrocera tryoni</i>	<i>Klebsiella</i>	Adult diet	No effect on fecundity	Meats et al. 2009
	<i>Asaia</i> sp., <i>Enterobacter</i> sp., <i>Lactobacillus</i> sp., <i>Leuconostoc</i> sp.	Larval diet	<i>Enterobacter</i> sp. and <i>Asaia</i> sp. shortened immature development duration	Shuttleworth et al. 2019
<i>Zeugodacus tau</i>	<i>Klebsiella</i> and <i>Proteus</i>	Adult diet	No effect on ovariole number or fecundity	Khan et al. 2014
<i>Anastrepha obliqua</i>	Mix of endogenous gut symbionts	Adult diet	No effect on male longevity	Rull et al. 2015

5.1.1. Probiotic Supplements for the Adult Diet of the Mediterranean Fruit Fly

Probiotics can be added to the larval or adult diets (or both). A positive effect of adult diets (enriched with an Enterobacteriaceae mix) on the mating competitiveness, longevity, and sexual calling of irradiated males was described by Niyazi et al. (2004). A different Enterobacteriaceae mix had positive effects on adult longevity

(Behar et al. 2008). Later, a positive impact (of the addition of *K. oxytoca* to the adult diet of the VIENNA 8 GSS) on the mating latency time of irradiated males was found by Ben Ami et al. (2010). By enriching the adult diet with the same isolate, and for the same Mediterranean fruit fly strain, positive effects on the mating competitiveness of irradiated males, and on the mating receptivity and longevity of females under starvation, were described (Gavriel et al. 2011) (see also Bakri et al., this volume; Barclay, this volume; Hendrichs and Robinson, this volume; Itô et al., this volume; Lance and McInnis, this volume; Marec et al., this volume; Parker, Mamai et al., this volume; Robinson, this volume; Vreysen, this volume; Whitten and Mahon, this volume). However, in an independent study a few years later, using the same bacterial isolate and the VIENNA 8^{D53+} strain, a positive effect on the mating competitiveness of irradiated males was not detected (Kyritsis et al. 2017).

5.1.2. Probiotic Supplements for the Larval Diets of the Mediterranean Fruit Fly

Regarding probiotic enrichment of larval diets, the addition of three Enterobacteriaceae strains, isolated from sources other than the Mediterranean fruit fly, showed positive effects on pupal weight, adult size, flight ability, and the mating competitiveness and sperm transfer of irradiated males (Hamden et al. 2013). The addition of an indigenous bacterial symbiont *Enterobacter* sp. AA26 decreased the duration of immature development and increased the survival of immature stages, leading to increased productivity in the fly strain. However, no positive effects on the mating competitiveness of irradiated males were observed (Augustinos et al. 2015).

The use of *K. oxytoca* as an additive to the larval diet led to a decrease in the duration of immature development, and also had positive effects on flight ability (Kyritsis et al. 2017). Finally, although not strictly probiotic, the partial or full replacement of yeast (the costliest diet ingredient) with dead *Enterobacter* sp. AA26, in the form of dry biomass, gave promising results regarding the reduction or elimination of yeast from the larval diet, which could lead to a decreased cost. More specifically, replacement had a positive effect on the survival of immature stages, on pupal weight, and on adult survival under starvation. At the same time, no effect was observed regarding the sex ratio, fecundity, flight ability, and male mating competitiveness, showing that it is an approach worthy of further investigation (Kyritsis et al. 2019; Stathopoulou et al. 2021).

5.1.3. Probiotic Supplements for the Larval and Adult Diets of Tephritids Other than the Mediterranean Fruit Fly

Several recent studies have described the effects of adding a bacterial inoculant to the larval diet of a fruit fly other than the Mediterranean fruit fly. In *B. dorsalis* and *B. tryoni*, adding various bacterial cultures either reduced or increased the immature developmental duration, and had a positive or no effect on pupal weight, emergence, sex ratio, flight ability or survival under stress (Khaeso et al. 2018; Khan et al. 2019; Shuttleworth et al. 2019). Also, studies were done on using probiotic-enriched adult diets. Adding *Pseudomonas putida* to an olive fruit fly adult diet had positive effects on female fecundity (Sacchetti et al. 2014), but adding *Klebsiella* sp. in a *B. tryoni* adult diet, and *Klebsiella* sp. combined with *Proteus* sp. in a *Z. tau* diet, had no effect

on fecundity (Meats et al. 2009; Khan et al. 2014). In a comprehensive analysis of virgin olive fruit fly females naturally infected with their symbionts, treated with antibiotics or treated with antibiotics and then provided with probiotic supplements of symbionts isolated from the olive fruit fly or Mediterranean fruit fly, it was shown that the first group with the natural microbiome (primarily consisting of *Candidatus* *Erwinia dacicola*) attempted oviposition significantly more times than the treated flies (Jose et al. 2019). Finally, adding a mix of endogenous symbionts in an *A. obliqua* diet had no effect on male longevity (Rull et al. 2015). In a recent report on *B. dorsalis* it was shown that a probiotic supplement of the gut-isolated symbiont *Klebsiella oxytoca* (BD177) can restore the biological quality of insects irradiated at the pupal stage (Cai et al. 2018).

5.2. Non-Probiotic Applications of Symbionts, and Alternative Documented Positive Effects

Besides probiotic diets, there are other ways to exploit putative positive effects of symbionts (Table 5). Moreover, feeding in different stages is not the only starting point to assess a potentially beneficial effect. Comparisons between symbiotic and aposymbiotic olive fruit flies have shown that aposymbiotic flies have decreased fecundity, and their larvae cannot develop on unripe olives (Ben-Yosef et al. 2014, 2015). NGS characterization of symbiotic communities, along with the metabolic profile of *Citrobacter* sp., and accompanying insecticide resistance assays in *B. dorsalis*, demonstrated that this bacterium enhances its host insecticide resistance (Cheng et al. 2017). The use of aposymbiotic and reinfected *B. dorsalis* females revealed that symbionts influence mate selection (Damodaram et al. 2016), while aposymbiotic *B. dorsalis* flies exhibit a different foraging behaviour than symbiotic ones (Akami et al. 2019). Also, it is worth noting that commensal microbiota were shown recently to modulate larval foraging behaviour, development rate, and pupal production in *B. tryoni* (Morimoto et al. 2019). Finally, using bacterial cultures of different species in attractancy assays showed that *A. ludens*, *B. dorsalis*, *B. zonata*, *Rhagoletis mendax* Curran, and *Z. cucurbitae* adults can be attracted by various bacterial cultures (Maccollom et al. 1992; Reddy et al. 2014; Wang H. et al. 2014; Hadapad et al. 2016).

6. SYMBIONTS AS UNIVERSAL PROBIOTICS: REALITY OR ILLUSION?

Until now, results are encouraging but sometimes contradicted by other studies. Variations and inconsistencies among studies show that “universal probiotics” for the SIT-important species are feasible, at least for Tephritidae, but not yet applicable. The main origins of controversy are described below:

- *Obvious ones*: For several reasons it is not easy to compare directly the findings of different studies: the experiments are conducted with different insect species or strains, and use different bacterial species/isolates and feeding strategies (larval stages versus adults). Moreover, not all studies answer the same questions, e.g. they shed light on different parameters that are considered important in SIT application (such as rearing productivity or biological quality).

Table 5. Summary of non-probiotic effects of symbiotic communities on Tephritidae

Species	Material	Methodology	Main effect	Reference
<i>Ceratitis capitata</i>	Laboratory colony	Symbiotic vs aposymbiotic flies	Aposymbiotic females showed an accelerated oviposition rate under restricted diets Symbionts affect mating latency time	Ben-Yosef et al. 2008b
	Laboratory colony	Symbiotic vs aposymbiotic flies	Aposymbiotic adults have decreased longevity under restricted diets	Ben-Yosef et al. 2008a
<i>Bactrocera oleae</i>	Wild material	Symbiotic vs aposymbiotic flies	Aposymbiotic females have decreased fecundity under various restricted diets	Ben-Yosef et al. 2010
	Wild material	Symbiotic vs aposymbiotic flies	Aposymbiotic females have decreased fecundity under various restricted diets	Ben-Yosef et al. 2014
	Wild material	Symbiotic vs aposymbiotic flies	Gut microbiota are important for larvae when developing in unripe olives, but not in ripe olives	Ben-Yosef et al. 2015
<i>Bactrocera dorsalis</i>	Wild material	Bacterial cultures as baits	Adults are attracted to bacterial isolates (autoclaved)	Wang H. et al. 2014
	Laboratory colony	Aposymbiotic and reinfected females	Symbionts influence mate selection	Damodaram et al. 2016
	Laboratory colony	NGS characterization of symbiotic communities, culture and metabolic profile of <i>Citrobacter</i>	<i>Citrobacter</i> sp. enhances insecticide resistance	Cheng et al. 2017
	Laboratory colony	Symbiotic vs aposymbiotic flies	Suppression of the microbiome results in changes of foraging behaviour of males and females	Akami et al. 2019
<i>Bactrocera tryoni</i>	Laboratory colony	Assessment of the effect of vertically and horizontally acquired bacteria to development	The presence of both vertically and horizontally acquired bacteria improves larval foraging behaviour, development rate, and pupal production	Morimoto et al. 2019
<i>Bactrocera zonata</i>	Laboratory colony	Attractancy assays for bacterial cultures	<i>Enterobacter</i> sp. and <i>Klebsiella</i> sp. are highly attractive for adults	Reddy et al. 2014
<i>Zeugodacus cucurbitae</i>	Laboratory colony	Cultivation of bacteria and attractancy assays	Adults are attracted to bacterial isolates	Hadapad et al. 2016
<i>Rhagoletis mendax</i>	Wild material	Traps with <i>P. agglomerans</i>	Adults are more attracted to traps baited with <i>P. agglomerans</i>	Maccollom et al. 1992

Even in cases where the same questions are asked, the methodological set-up may be quite different, e.g. differences in rearing practices, and how male mating competitiveness is evaluated in laboratory, semi-field or field conditions. For all these reasons, results may vary or in some cases even be contradictory.

- *Differences in rearing practices:* Even in cases where the same insect strains (or strains sharing the same origin) and similar bacterial isolates have been used, results derived from different laboratories could be different. An important parameter influencing data interpretation is how each laboratory defines “standard (or control) rearing conditions”. In the larval diet, different substrates that can influence the nutritional value of the control diet are being used. Diets such as wheat-bran, carrot-based or liquid diets are employed in various laboratories, and the percentage of yeast and other ingredients may vary as well. Diet has a strong effect on shaping the associated microbiota, as was shown recently in *B. tryoni*, *C. capitata*, and *Z. cucurbitae* (Asimakis et al. 2019; Augustinos et al. 2019; Woruba et al. 2019). These aspects could strongly affect the results, and consequently the probiotic role assigned to the bacteria; some probiotic supplements could be beneficial only under specific conditions, e.g. in nutritionally poor diets.
- *Developmental stage specificity and dynamics:* Comparing the findings of related studies shows that the same (or similar) bacterial taxa do not consistently have the same effects. Bacterial taxa show great variations in the various developmental stages (Ben Ami et al. 2010; Aharon et al. 2013; Andongma et al. 2015; Augustinos et al. 2015). Over the last decade, many studies have tried to shed light on the characterization of gut symbiotic communities of tephritids, either conducted with long-time laboratory-adapted fly populations or with ones recently introduced into the laboratory. These studies suggest that gut symbiotic communities, although relatively simple in their structure, consisting of few bacterial taxa, show great variations in the different developmental stages at lower taxonomic levels (Ben Ami et al. 2010; Aharon et al. 2013; Andongma et al. 2015; Augustinos et al. 2015). These differences are usually evident at the taxonomic level of bacterial species or genus, may reflect the development of preferential relationships, and may explain why specific bacteria do not “retain” their beneficial effects when provided in different feeding stages (such as larval stages versus adults).

From a different point of view, it is possible that different isolates belonging to the same bacterial species may not have the same effect. This can be related to either “real” differential properties of these strains, due to restricted but crucial genetic variability, or their “false” identification as being genetically close, due to the genotyping methodology used. Advances, that allow the complete metagenomic analysis at a relatively low cost, are expected to solve such potential misidentification issues.

- *Methodological and experimental set-up:* A major issue when trying to compare results regarding the effect of adult probiotic feeding on SIT-important parameters, such as male mating competitiveness and longevity under stress conditions, comes from experimental assumptions and limitations. The use of live instead of dead bacteria, the nutritional profile of the control diet, and the wild

populations used for mating competitiveness studies are important parameters that may affect both the results and their interpretation.

7. CONCLUSIONS

Insects have established coevolved complex and sophisticated symbiotic associations with diverse microorganisms, including bacterial species. Recent advances in molecular biology and microbiology, sequencing methods, and bioinformatics have enabled the characterization of these symbiotic interactions, indicating the importance of the symbionts in many aspects of insect host biology, ecology, and evolution (including nutrition, mating behaviour, reproduction, and immunity). These advances in insect symbiosis enable us to exploit these symbiotic associations to support improving SIT applications against insect pests of agricultural, veterinary, and human health importance. In particular, there appears to be considerable potential in harnessing insect gut-associated microbiota to improve mass-rearing cost-effectiveness, as well as the biological quality (including male mating competitiveness) of the resulting sterile insects for SIT operational programmes.

8. REFERENCES

(Note: References dated 2018 and earlier may be hyperlinked)

- Aharon, Y., Z. Pasternak, M. Ben Yosef, A. Behar, C. Lauzon, B. Yuval, and E. Jurkevitch. 2013. Phylogenetic, metabolic, and taxonomic diversities shape Mediterranean fruit fly microbiotas during ontogeny. *Appl. Envir. Microbiology* 79: 303–313. <https://aem.asm.org/content/aem/79/1/303.full.pdf>
- Akmi, M., A. A. Andongma, C. Zhengzhong, J. Nan, K. Khaeso, E. Jurkevitch, C.-Y. Niu, and B. Yuval. 2019. Intestinal bacteria modulate the foraging behavior of the oriental fruit fly *Bactrocera dorsalis* (Dip.: Teph.). *PLOS ONE* 14(1): e0210109. <https://doi.org/10.1371/journal.pone.0210109>
- Akman, L., A. Yamashita, H. Watanabe, K. Oshima, T. Shiba, M. Hattori, and S. Aksoy. 2002. Genome sequence of the endocellular obligate symbiont of tsetse flies, *Wigglesworthia glossinidia*. *Nature Genetics* 32: 402–407. <https://www.nature.com/articles/ng986>
- Akman Gündüz, E., and A. E. Douglas. 2012. Symbiotic bacteria enable insect to use a nutritionally inadequate diet. *Proceedings of the Royal Society B. Biological Sciences* 276: 987–991.
- Aksoy, S. 1995. *Wigglesworthia* gen. nov. and *Wigglesworthia glossinidia* sp. nov., taxa consisting of the mycetocyte-associated, primary endosymbionts of tsetse flies. *Int. J. Syst. Evol. Micro.* 45: 848–851.
- Andersson, S. G. E. 2006. The bacterial world gets smaller. *Science* 314(5797): 259–260.
- Andongma, A. A., L. Wan, Y. C. Dong, P. Li, N. Desneux, J. A. White, and C. Y. Niu. 2015. Pyrosequencing reveals a shift in symbiotic bacteria populations across life stages of *Bactrocera dorsalis*. *Scientific Reports* 5: 9470. Pages 1–6. <http://dx.doi.org/10.1038/srep09470>
- Andongma, A. A., L. Wan, Y. C. Dong, Y. L. Wang, J. He, and C. Y. Niu. 2019. Assessment of the bacteria community structure across life stages of the Chinese citrus fly, *Bactrocera minax* (Diptera: Tephritidae). *BMC Microbiology* 19(Suppl. 1): 285. <https://doi.org/10.1186/s12866-019-1646-9>
- Asimakis, E. D., M. Khan, P. Stathopoulou, C. Cáceres, K. Bourtzis, and G. Tsiamis. 2019. The effect of diet and radiation on the bacterial symbiome of the melon fly, *Zeugodacus cucurbitae* (Coquillett). *BMC Biotechnology* 19(Suppl. 2): 88. <https://doi.org/10.1186/s12896-019-0578-7>
- Augustinos, A. A., G. A. Kyritsis, N. T. Papadopoulos, A. M. M. Abd-Alla, C. Cáceres, and K. Bourtzis. 2015. Exploitation of the medfly gut microbiota for the enhancement of sterile insect technique: use of *Enterobacter* sp. in larval diet-based probiotic applications. *PLOS ONE* 10(9): e0136459. <https://doi.org/10.1371/journal.pone.0136459>
- Augustinos, A. A., G. Tsiamis, C. Cáceres, A. M. M. Abd-Alla, and K. Bourtzis. 2019. Taxonomy, diet, and developmental stage contribute to the structuring of gut-associated bacterial communities in tephritid pest species. *Frontiers in Microbiology* 10: 2004. <https://doi.org/10.3389/fmicb.2019.02004>

- Behar, A., B. Yuval, and E. Jurkevitch. 2005. Enterobacteria-mediated nitrogen fixation in natural populations of the fruit fly *Ceratitis capitata*. *Molecular Ecology* 14: 2637–2643.
- Behar, A., B. Yuval, and E. Jurkevitch. 2008. Gut bacterial communities in the Mediterranean fruit fly (*Ceratitis capitata*) and their impact on host longevity. *Journal of Insect Physiology* 54: 1377–1383.
- Ben Ami, E., B. Yuval, and E. Jurkevitch. 2010. Manipulation of the microbiota of mass-reared Mediterranean fruit flies *Ceratitis capitata* (Diptera: Tephritidae) improves sterile male sexual performance. *ISME Journal* 4: 28–37. DOI: 10.1038/ismej.2009.82
- Ben-Yosef, M., A. Behar, E. Jurkevitch, and B. Yuval. 2008a. Bacteria-diet interactions affect longevity in the medfly - *Ceratitis capitata*. *Journal of Applied Entomology* 132: 690–694.
- Ben-Yosef, M., E. Jurkevitch, and B. Yuval. 2008b. Effect of bacteria on nutritional status and reproductive success of the Mediterranean fruit fly *Ceratitis capitata*. *Physio. Entomol.* 33: 145–154.
- Ben-Yosef, M., Y. Aharon, E. Jurkevitch, and B. Yuval. 2010. Give us the tools and we will do the job: symbiotic bacteria affect olive fly fitness in a diet-dependent fashion. *Proceedings of the Royal Society B. Biological Sciences* 277: 1545–1552. <http://rspb.royalsocietypublishing.org/content/277/1687/1545>
- Ben-Yosef, M., Z. Pasternak, E. Jurkevitch, and B. Yuval. 2014. Symbiotic bacteria enable olive flies (*Bactrocera oleae*) to exploit intractable sources of nitrogen. *J. Evolutionary Biology* 27: 2695–2705.
- Ben-Yosef, M., Z. Pasternak, E. Jurkevitch, and B. Yuval. 2015. Symbiotic bacteria enable olive fly larvae to overcome host defenses. *Royal Society Open Science* 2: 150170.
- Bing, X., G. M. Attardo, A. Vigneron, E. Aksoy, F. Scolari, A. Malacrida, B. L. Weiss, and S. Aksoy. 2017. Unravelling the relationship between the tsetse fly and its obligate symbiont *Wigglesworthia*: transcriptomic and metabolomic landscapes reveal highly integrated physiological networks. *Proc. Royal Soc. B. Biol. Sci.* 284: 1857. <http://rspb.royalsocietypublishing.org/content/284/1857/20170360>
- Blow, F., A. Gioti, D. Starns, M. Ben-Yosef, Z. Pasternak, E. Jurkevitch, J. Vontas, and A. C. Darby. 2016. Draft genome sequence of the *Bactrocera oleae* symbiont “*Candidatus* Erwinia dacicola”. *Genome Announcements* 4(5):e00896-16. <https://mra.asm.org/content/4/5/e00896-16>
- Bourtzis, K., R. S. Lees, J. Hendrichs, and M. J. B. Vreysen. 2016. More than one rabbit out of the hat: radiation, transgenic and symbiont-based approaches for sustainable management of mosquito and tsetse fly populations. *Acta Tropica* 157: 115–130. <https://doi.org/10.1016/j.actatropica.2016.01.009>
- Cai, Z., Z. Yao, Y. Li, Z. Xi, K. Bourtzis, Z. Zhao, S. Bai, and H. Zhang. 2018. Intestinal probiotics restore the ecological fitness decline of *Bactrocera dorsalis* by irradiation. *Evol. Appl.* 11: 1946–1963.
- Capuzzo, C., G. Firrao, L. Mazzon, A. Squartini, and V. Girolami. 2005. ‘*Candidatus* Erwinia dacicola’, a coevolved symbiotic bacterium of the olive fly *Bactrocera oleae* (Gmelin). *International Journal of Systematic and Evolutionary Microbiology* 55: 1641–1647.
- Chen, X., S. Li, and S. Aksoy. 1999. Concordant evolution of a symbiont with its host insect species: molecular phylogeny of genus *Glossina* and its bacteriome-associated endosymbiont, *Wigglesworthia glossinidia*. *J. Molecular Evolution* 48: 49–58. <https://link.springer.com/article/10.1007/PL00006444>
- Cheng, D., Z. Guo, M. Riegler, Z. Xi, G. Liang, and Y. Xu. 2017. Gut symbiont enhances insecticide resistance in a significant pest, the oriental fruit fly *Bactrocera dorsalis* (Hendel). *Microbiome* 5: 13.
- Damodaram, K. J. P., A. Ayyasamy, and V. Kempraj. 2016. Commensal bacteria aid mate-selection in the fruit fly, *Bactrocera dorsalis*. *Microbial Ecology* 72: 725–729.
- De Bary, A. 1879. De la symbiose. *Revue Internationale des Sciences* 3: 301–309.
- Deutscher, A. T., O. L. Reynolds, and T. A. Chapman. 2017. Yeast: an overlooked component of *Bactrocera tryoni* (Diptera: Tephritidae) larval gut microbiota. *J. Econ. Entomology* 110: 298–300.
- Deutscher, A. T., C. M. Burke, A. E. Darling, M. Riegler, O. L. Reynolds, and T. A. Chapman. 2018. Near full-length 16S rRNA gene next-generation sequencing revealed *Asaia* as a common midgut bacterium of wild and domesticated Queensland fruit fly larvae. *Microbiome* 6: 85.
- Deutscher, A. T., T. A. Chapman, L. A. Shuttlesworth, M. Riegler, and O. L. Reynolds. 2019. Tephritid-microbial interactions to enhance fruit fly performance in sterile insect technique programs. *BMC Microbiology* 19(Suppl. 1): 287. <https://doi.org/10.1186/s12866-019-1650-0>
- Douglas, A. E. 2009. The microbial dimension in insect nutritional ecology. *Functional Ecol.* 23: 38–47.
- Douglas, A. E. 2013. Microbial brokers of insect-plant interactions revisited. *J. Chem. Ecol.* 39: 952–961.
- Escobar-Zepeda, A., A. Vera-Ponce de León, and A. Sanchez-Flores. 2015. The road to metagenomics: from microbiology to DNA sequencing technologies and bioinformatics. *Frontiers in Genetics* 6: 348.
- Estes, A. M., D. J. Hearn, J. L. Bronstein, and E. A. Pierson. 2009. The olive fly endosymbiont, “*Candidatus* Erwinia dacicola,” switches from an intracellular existence to an extracellular existence during host insect development. *Applied and Environmental Microbiology* 75: 7097–7106.

- Estes, A. M., D. J. Hearn, H. J. Burrack, P. Rempoulakis, and E. A. Pierson. 2012. Prevalence of *Candidatus* Erwinia dacicola in wild and laboratory olive fruit fly populations and across developmental stages. *Environmental Entomology* 41: 265–274. <https://doi.org/10.1603/EN11245>
- Gavriel, S., E. Jurkevitch, Y. Gazit, and B. Yuval. 2011. Bacterially enriched diet improves sexual performance of sterile male Mediterranean fruit flies. *Journal of Applied Entomology* 135: 564–573.
- Guégan, M., K. Zouache, C. Démichel, G. Minard, V. Tran Van, P. Potier, P. Mavingui, and C. Valiente Moro. 2018. The mosquito holobiont: fresh insight into mosquito-microbiota interactions. *Microbiome* 6: 49. <https://doi.org/10.1186/s40168-018-0435-2>
- Gujjar, N. R., S. Govindan, A. Verghese, S. Subramaniam, and R. More. 2017. Diversity of the cultivable gut bacterial communities associated with the fruit flies *Bactrocera dorsalis* and *Bactrocera cucurbitae* (Diptera: Tephritidae). *Phytoparasitica* 45: 453–460.
- Hadapad, A. B., C. S. Prabhakar, S. C. Chandekar, J. Tripathi, and R. S. Hire. 2016. Diversity of bacterial communities in the midgut of *Bactrocera cucurbitae* (Diptera: Tephritidae) populations and their potential use as attractants. *Pest Manag. Science* 72: 1222–1230. <https://doi.org/10.1002/ps.4102>
- Hadapad, A. B., S. K. G. Shettigar, and R. S. Hire. 2019. Bacterial communities in the gut of wild and mass-reared *Zeugodacus cucurbitae* and *Bactrocera dorsalis* revealed by metagenomic sequencing. *BMC Microbiology* 19(Suppl. 1): 282. <https://doi.org/10.1186/s12866-019-1647-8>
- Hamden, H., M. M. Guerfali, S. Fadhl, M. Saidi, and C. Chevrier. 2013. Fitness improvement of mass-reared sterile males of *Ceratitis capitata* (Vienna 8 strain) (Diptera: Tephritidae) after gut enrichment with probiotics. *J. Economic Entomology* 106: 641–647. <https://doi.org/10.1603/EC12362>
- Jose, P. A., M. Ben-Yosef, E. Jurkevitch, and B. Yuval. 2019. Symbiotic bacteria affect oviposition behavior in the olive fruit fly *Bactrocera oleae*. *Journal of Insect Physiology* 117: 103917. <https://doi.org/10.1016/j.jinsphys.2019.103917>
- Juárez, M. L., L. E. Pimper, G. E. Bachmann, C. A. Conte, M. J. Ruiz, L. Goane, P. M. Pereyra, F. Castro, J. Salgueiro, J. L. Cladera, P. E. Fernández, K. Bourtzis, S. B. Lanzavecchia, M. T. Vera, and D. F. Segura. 2019. Gut bacterial diversity and physiological traits of *Anastrepha fraterculus* Brazilian-1 morphotype males are affected by antibiotic treatment. *BMC Microbiology* 19(Suppl. 1): 283. <https://doi.org/10.1186/s12866-019-1645-x>
- Khaeso, K., A. A. Andongma, M. Akami, B. Souliyanonh, J. Zhu, P. Krutmuang, and C. Y. Niu. 2018. Assessing the effects of gut bacteria manipulation on the development of the oriental fruit fly, *Bactrocera dorsalis* (Diptera: Tephritidae). *Symbiosis* 74: 97–105.
- Khan, M., A. A. Mahin, M. K. Pramanik, and H. Akter. 2014. Identification of gut bacterial community and their effect on the fecundity of pumpkin fly, *Bactrocera tau* (Walker). *Journal of Entomology* 11: 68–77.
- Khan, M., K. Seheli, M. A. Bari, N. Sultana, S. A. Khan, K. F. Sultana, and M. A. Hossain. 2019. Potential of a fly gut microbiota incorporated gel-based larval diet for rearing *Bactrocera dorsalis* (Hendel). *BMC Biotechnology* 19(Suppl. 2): 94. <https://doi.org/10.1186/s12896-019-0580-0>
- Koskinioti, P., E. Ras, A. A. Augustinos, G. Tsiamis, L. W. Beukeboom, C. Cáceres, and K. Bourtzis. 2019. The effects of geographic origin and antibiotic treatment on the gut symbiotic communities of *Bactrocera oleae* populations. *Ent. Exper. Appl.* 167(is. 3): 197–208. <https://doi.org/10.1111/eea.12764>
- Kounatidis, I., E. Crotti, P. Sapountzis, L. Sacchi, A. Rizzi, B. Chouaia, C. Bandi, A. Alma, D. Daffonchio, P. Mavragani-Tsipidou, and K. Bourtzis. 2009. *Acetobacter tropicalis* is a major symbiont of the olive fruit fly (*Bactrocera oleae*). *Appl. Environmental Microbiology* 75: 3281–3288.
- Kuzina, L. V., J. J. Peloquin, D. C. Vacek, and T. A. Miller. 2001. Isolation and identification of bacteria associated with adult laboratory Mexican fruit flies, *Anastrepha ludens* (Diptera: Tephritidae). *Current Microbiology* 42: 290–294. <https://link.springer.com/article/10.1007/s002840110219>
- Kyritsis, G. A., A. A. Augustinos, C. Cáceres, and K. Bourtzis. 2017. Medfly gut microbiota and enhancement of the sterile insect technique: similarities and differences of *Klebsiella oxytoca* and *Enterobacter* sp. AA26 probiotics during the larval and adult stages of the VIENNA 8^{DS3+} genetic sexing strain. *Frontiers in Microbiology* 8: 2064. <https://doi.org/10.3389/fmicb.2017.02064>
- Kyritsis, G. A., A. A. Augustinos, S. Ntougias, N. T. Papadopoulos, K. Bourtzis, and C. Cáceres. 2019. *Enterobacter* sp. AA26 gut symbiont as protein source for Mediterranean fruit fly mass-rearing and sterile insect technique applications. *BMC Microbiology* 19(Suppl. 1): 288. <https://doi.org/10.1186/s12866-019-1651-z>
- LePage, D., and S. R. Bordenstein. 2013. *Wolbachia*: can we save lives with a great pandemic? *Trends in Parasitology* 29: 385–393. <https://doi.org/10.1016/j.pt.2013.06.003>
- Maccollom, G. B., C. R. Lauzon, R. W. Weires, Jr., and A. A. Rutkowski. 1992. Attraction of adult apple maggot (Diptera: Tephritidae) to microbial isolates. *J. of Economic Entomology* 85: 83–87.

- Malacrinò, A., L. Schena, O. Campolo, F. Laudani, S. Mosca, G. Giunti, C. P. Strano, and V. Palmeri. 2017. A metabarcoding survey on the fungal microbiota associated to the olive fruit fly. *Microbial Ecology* 73: 677–684. <https://doi.org/10.1007/s00248-016-0864-z>
- Malacrinò, A., O. Campolo, R. F. Medina, and V. Palmeri. 2018. Instar- and host-associated differentiation of bacterial communities in the Mediterranean fruit fly *Ceratitidis capitata*. *PLOS ONE* 13(3): e0194131. <https://doi.org/10.1371/journal.pone.0194131>
- Margulis, L., and R. Fester. 1991. Symbiosis as a source of evolutionary innovation: speciation and morphogenesis. MIT Press. <https://mitpress.mit.edu/books/symbiosis-source-evolutionary-innovation>
- Mateos, M., H. Martinez Montoya, S. B. Lanzavecchia, C. Conte, K. Guillén, B. M. Morán-Aceves, J. Toledo, P. Liedo, E. D. Asimakis, V. Doudoumis, G. A. Kyritsis, N. T. Papadopoulos, A. A. Augustinos, D. F. Segura, and G. Tsiamis. 2020. *Wolbachia pipientis* associated with tephritid fruit fly pests: from basic research to applications. *Frontiers in Microbiology* 11: 1080. <https://doi.org/10.3389/fmicb.2020.01080>
- Meats, A., K. Streamer, and A. S. Gilchrist. 2009. Bacteria as food had no effect on fecundity during domestication of the fruit fly, *Bactrocera tryoni*. *Journal of Applied Entomology* 133: 633–639.
- Morimoto, J., B. Nguyen, S. T. Tabrizi, I. Lundbäck, P. W. Taylor, F. Ponton, and T. A. Chapman. 2019. Commensal microbiota modulates larval foraging behaviour, development rate and pupal production in *Bactrocera tryoni*. *BMC Micro.* 19, S 1: 286. <https://doi.org/10.1186/s12866-019-1648-7>
- Morrow, J. L., M. Frommer, D. C. A. Shearman, and M. Riegler. 2015. The microbiome of field-caught and laboratory-adapted Australian tephritid fruit fly species with different host plant use and specialisation. *Microbial Ecology* 70: 498–508. <https://doi.org/10.1007/s00248-015-0571-1>
- Naaz, N., J. S. Choudhary, C. S. Prabhakar, M. Sudarshan Maurya. 2016. Identification and evaluation of cultivable gut bacteria associated with peach fruit fly, *Bactrocera zonata* (Diptera: Tephritidae). *Phytoparasitica* 44: 165–176. <https://doi.org/10.1007/s12600-016-0518-1>
- Niyazi, N., C. R. Lauzon, and T. E. Shelly. 2004. Effect of probiotic adult diets on fitness components of sterile male Mediterranean fruit flies (Diptera: Tephritidae) under laboratory and field cage conditions. *Journal of Economic Entomology* 97: 1570–1580. <https://doi.org/10.1603/0022-0493-97.5.1570>
- Pais, R., C. Lohs, Y. Wu, J. Wang, and S. Aksoy. 2008. The obligate mutualist *Wigglesworthia glossinidia* influences reproduction, digestion, and immunity processes of its host, the tsetse fly. *Applied and Environmental Microbiology* 74: 5965–5974. <https://aem.asm.org/content/74/19/5965>
- Pérez-Brocal, V., R. Gil, S. Ramos, A. Lamelas, M. Postigo, J. M. Michelena, F. J. Silva, A. Moya, and A. Latorre. 2006. A small microbial genome: the end of a long symbiotic relationship? *Science* 314(5797): 312–313. <http://science.sciencemag.org/content/314/5797/312>
- Petri, L. 1909. Ricerche sopra i batteri intestinali della mosca olearia. *Memorie della Regia Stazione di Patologia Vegetale di Roma*. Rome, Italy.
- Prabhakar, C. S., P. Sood, S. S. Kanwar, P. N. Sharma, A. Kumar, and P. K. Mehta. 2013. Isolation and characterization of gut bacteria of fruit fly, *Bactrocera tau* (Walker). *Phytoparasitica* 41: 193–201.
- Pramanik, M. K., A. A. Mahin, M. Khan, and A. B. Miah. 2014. Isolation and identification of mid-gut bacterial community of *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae). *Research Journal of Microbiology* 9: 278–286. <https://scialert.net/abstract/?doi=jm.2014.278.286>
- Reddy, K., K. Sharma, and S. Singh. 2014. Attractancy potential of culturable bacteria from the gut of peach fruit fly, *Bactrocera zonata* (Saunders). *Phytoparasitica* 42: 691–698.
- Rull, J., R. Lasa, C. Rodriguez, R. Ortega, O. E. Velazquez, and M. Aluja. 2015. Artificial selection, pre-release diet, and gut symbiont inoculation effects on sterile male longevity for area-wide fruit-fly management. *Ent. Experimentalis et Applicata* 157: 325–333. <https://doi.org/10.1111/eea.12371>
- Sacchetti, P., B. Ghiardi, A. Granchietti, F. M. M. Stefanini, and A. Belcari. 2014. Development of probiotic diets for the olive fly: evaluation of their effects on fly longevity and fecundity. *Annals of Applied Biology* 164: 138–150. <https://doi.org/10.1111/aab.12088>
- Saridaki, A., and K. Bourtzis. 2010. *Wolbachia*: more than just a bug in insects genitals. *Current Opinion in Microbiology* 13: 67–72. <https://doi.org/10.1016/j.mib.2009.11.005>
- Savio, C., L. Mazzon, I. Martinez-Sañudo, M. Simonato, A. Squartini, and V. Girolami. 2012. Evidence of two lineages of the symbiont ‘*Candidatus* Erwinia dacicola’ in Italian populations of *Bactrocera oleae* (Rossi) based on 16S rRNA gene sequences. *International Journal of Systematic and Evolutionary Microbiology* 62(Issue 1): 179–187. <https://doi.org/10.1099/ijs.0.030668-0>
- Scolari, F., M. Casiraghi, and M. Bonizzoni. 2019. *Aedes* spp. and their microbiota: a review. *Frontiers in Microbiology* 10: 2036. <https://doi.org/10.3389/fmicb.2019.02036>

- Shuttleworth, L. A., M. A. M. Khan, T. Osborne, D. Collins, M. Srivastava, and O. L. Reynolds. 2019. A walk on the wild side: gut bacteria fed to mass-reared larvae of Queensland fruit fly [*Bactrocera tryoni* (Froggatt)] influence development. BMC Biotechnology 19(Suppl. 2): 95. <https://doi.org/10.1186/s12896-019-0579-6>
- Snyder, A. K., and R. V. M. Rio. 2013. Interwoven biology of the tsetse holobiont. Journal of Bacteriology 195: 4322–4330. DOI: 10.1128/JB.00487-13 <https://jb.asm.org/content/195/19/4322>
- Stathopoulou, P. M., E. Asimakis, and G. Tsiamis. 2021. *Enterobacter*: one bacterium multiple functions, pp. 917–945. In J. Hendrichs, R. Pereira and M. J. B. Vreysen (eds.), Area-wide integrated pest management. Development and field application. CRC Press, Boca Raton, FL, USA.
- Stork, N. E. 2018. How many species of insects and other terrestrial arthropods are there on earth? Annual Review of Entomology 63: 31–45. <https://doi.org/10.1146/annurev-ento-020117-043348>
- Stork, N. E., J. McBroom, C. Gely, and A. J. Hamilton. 2015. New approaches narrow global species estimates for beetles, insects, and terrestrial arthropods. Proc. Nat. Acad. Sci. 112(24): 7519–7523.
- Strand, M. R. 2018. Composition and functional roles of the gut microbiota in mosquitoes. Current Opinion in Insect Science 28: 59–65. <https://doi.org/10.1016/j.cois.2018.05.008>
- Thaochan, N., R. A. I. Drew, A. Chinajariyawong, A. Sunpapao, and C. Pornsuriya. 2015. Gut bacterial community structure of two Australian tropical fruit fly species (Diptera: Tephritidae). Songklanakarin Journal of Science and Technology 37: 617–624.
- van Ham, R. C. H. J., J. Kamerbeek, C. Palacios, C. Rausell, F. Abascal, U. Bastolla, J. M. Fernández, L. Jiménez, M. Postigo, F. J. Silva, J. Tamames, E. Viguera, A. Latorre, A. Valencia, F. Morán, and A. Moya. 2003. Reductive genome evolution in *Buchnera aphidicola*. Proc. National Academy of Sciences of the USA 100(2): 581–586. <https://doi.org/10.1073/pnas.0235981100>
- Ventura, C., C. I. Briones-Roblero, E. Hernández, F. N. Rivera-Orduña, and G. Zúñiga. 2018. Comparative analysis of the gut bacterial community of four *Anastrepha* fruit flies (Dip.: Tephritidae) based on pyrosequencing. Current Microbiol. 75: 966–976. <https://doi.org/10.1007/s00284-018-1473-5>
- Wang, A., Z. Yao, W. Zheng, and H. Zhang. 2014. Bacterial communities in the gut and reproductive organs of *Bactrocera minax* (Diptera: Tephritidae) based on 454 pyrosequencing. PLOS ONE 9(9): e106988. <https://doi.org/10.1371/journal.pone.0106988>
- Wang, H., L. Jin, and H. Zhang. 2011. Comparison of the diversity of the bacterial communities in the intestinal tract of adult *Bactrocera dorsalis* from three different populations. Journal of Applied Microbiology 110: 1390–1401. <https://doi.org/10.1111/j.1365-2672.2011.05001.x>
- Wang, H., L. Jin, T. Peng, H. Zhang, Q. Chen, and Y. Hua. 2014. Identification of cultivable bacteria in the intestinal tract of *Bactrocera dorsalis* from three different populations and determination of their attractive potential. Pest Management Science 70: 80–87. <https://doi.org/10.1002/ps.3528>
- Wang, J., B. L. Weiss, and S. Aksoy. 2013. Tsetse fly microbiota: form and function. Frontiers in Cellular and Infection Microbiology 3: 69. <https://doi.org/10.3389/fcimb.2013.00069>
- Weiss, B., and S. Aksoy. 2011. Microbiome influences on insect host vector competence. Trends in Parasitology 27: 514–522. <https://doi.org/10.1016/j.pt.2011.05.001>
- Werren, J. H., L. Baldo, and M. E. Clark. 2008. *Wolbachia*: master manipulators of invertebrate biology. Nat. Rev. Micro. 6: 741–751. <http://babec.org/files/resources/wolbachia/nrmicro1969.pdf>
- Woruba, D. N., J. L. Morrow, O. L. Reynolds, T. A. Chapman, D. P. Collins, and M. Riegler. 2019. Diet and irradiation effects on the bacterial community composition and structure in the gut of domesticated teneral and mature Queensland fruit fly, *Bactrocera tryoni* (Diptera: Tephritidae). BMC Microbiology 19(Suppl. 1): 281. <https://doi.org/10.1186/s12866-019-1649-6>
- Yong, H. S., S. L. Song, K. O. Chua, and P. E. Lim. 2017. Microbiota associated with *Bactrocera carambolae* and *B. dorsalis* (Insecta: Tephritidae) revealed by next-generation sequencing of 16S rRNA gene. Meta Gene 11: 189–196. <https://doi.org/10.1016/j.mgene.2016.10.009>
- Zheng, X., D. Zhang, Y. Li, C. Yang, Y. Wu, X. Liang, Y. Liang, X. Pan, L. Hu, Q. Sun, X. Wang, Y. Wei, J. Zhu, W. Qian, Z. Yan, A. G. Parker, J. R. L. Gilles, K. Bourtzis, J. Bouyer, M. Tang, B. Zheng, J. Yu, J. Liu, J. Zhuang, Z. Hu, M. Zhang, J. T. Gong, X. Y. Hong, Z. Zhang, L. Lin, Q. Liu, Z. Hu, Z. Wu, L. A. Baton, A. A. Hoffmann, and Z. Xi. 2019. Incompatible and sterile insect techniques combined eliminate mosquitoes. Nature 572: 56–61. <https://doi.org/10.1038/s41586-019-1407-9>
- Zilber-Rosenberg, I., and E. Rosenberg. 2008. Role of microorganisms in the evolution of animals and plants: the hologenome theory of evolution. FEMS Microbiology Reviews 32(5): 723–735. <https://doi.org/10.1111/j.1574-6976.2008.00123.x>