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Review of the role of gut microbiota in mass rearing of the olive fruit fly, Bactrocera oleae, and its parasitoids

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Key words: sterile insect technique, SIT, area-wide integrated pest management, IPM, Tephritidae, Diptera, Ceratitis capitata

Abstract

The olive fruit fly, Bactrocera oleae (Rossi) (Diptera: Tephritidae), is the major insect pest in commercial olive (Olea europaea L., Oleaceae) production worldwide. Its population management is largely based on the use of insecticides. However, concerns about the impact of insecticides on the environment and human health along with increasing resistance development calls for novel and environment-friendly approaches for population management. Integrated pest management programmes with a sterile insect technique (SIT) component and parasitoids are currently considered for the control of B. oleae. A major challenge for the development of such tools is mass rearing of both host and parasitoids. In this review, we consider the role of endogenous microbiota and its potential exploitation for improving the efficacy, quality, and cost effectiveness of mass rearing B. oleae as well as their parasitoids.

Introduction

Tephritid fruit flies (Diptera), particularly species belonging to the genera Anastrepha, Bactrocera, Ceratitis, Dacus, Rhagoletis, and Zeugodacus, are among the most important pests for the horticultural industry in tropical, subtropical, and temperate regions (Hendrichs et al., 2015). The olive fruit fly, Bactrocera oleae (Rossi) (Diptera: Tephritidae), is the single major insect pest in commercial olive production (Olea europaea L., Oleaceae) (Haniotakis, 2005). Female oviposition creates damage to table olives, but most damage to the fruit is caused by B. oleae larvae, which are specialist feeders on olives. The losses caused by B. oleae are substantial and frequently exceed 30% of total olive production (Weems & Nation, 1999; Bueno & Jones, 2002). A recent detailed account of the impact of the damage caused by B. oleae infestation has been presented by Malheiro et al. (2015).

Symbiotic bacteria play a major role in several aspects of insect biology, ecology, and evolution, affecting among others nutrition, immunity, reproduction, behaviour, and pest status (Engel & Moran, 2013; Wingfield et al., 2016; Hosokawa et al., 2017). Here, we review the current knowledge in respect to the population control of the olive fruit fly with emphasis on the importance of insect-associated microbiota for the mass production of high-quality insects required for large-scale area-wide integrated pest management (AW-IPM) projects. These include the application of the sterile insect technique (SIT) and augmentative release of parasitoids. After providing the most relevant current knowledge regarding SIT and available parasitoids for B. oleae, the mass-rearing challenges are described in detail for both pest control methods. The final part of the review explores the potential for practical application of gut microbes to improve rearing of B. oleae and its parasitoids. Throughout the review, B. oleae is compared to its relatives, in particular to the successfully mass-reared Mediterranean fruit fly (medfly), Ceratitis capitata (Wiedemann). As knowledge on bacteria, fungi, and other microbes in parasitoids of B. oleae is scarce, available knowledge from other host–parasitoid systems is used as a source of suggestions for potential research foci.
Olive fruit fly pest control: SIT and parasitoid release

Current control methods against *B. oleae* are largely based on the use of insecticides, bait sprays (GF-120), and mass trapping (Haniotakis, 2005). Intensive use of insecticides and baits sprays may have undesirable effects such as insecticide resistance in *B. oleae* or killing of non-target insects, whereas mass trapping has a lower efficiency (Vassiliou et al., 1985; Haniotakis, 2005; Stewart & Johnson, 2008; Daane & Johnson, 2010; Kakani et al., 2010). Generally, pesticide use can be reduced and the effectiveness of IPM can be increased by the integration of various pest control techniques and their application in an area-wide manner (Carlson & Wetstein, 1993; Klassen, 2005; Hendrichs et al., 2007). An example of a successful and cost-beneficial AW-IPM programme is that against the medfly; this programme has eliminated the pest from the USA and Mexico, thus protecting a multi-billion USD horticultural industry (Salcedo-Baca et al., 2009; Enkerlin et al., 2015). This programme has been based on an integrated approach combining surveillance with ground or aerial bait sprays, fruit stripping, mass trapping, SIT, and GIS-aided predictive models (Klassen, 2005; Enkerlin et al., 2015).

SIT and the application of parasitoids can be complementary approaches, as natural enemies (parasitoids) generally work better when the host density is high, whereas SIT is generally more efficient at low densities according to theoretical models and field studies (Barclay, 1987; Bloem et al., 1998). The integration of SIT and parasitoids as components of AW-IPM programmes for the population suppression of *B. oleae* has been suggested (Nestel et al., 2016).

SIT depends on the mass rearing of a target species, the sterilization of these mass-reared insects (ideally males) using ionizing irradiation, and the handling, transport, and release of large numbers of sterilized males over the release site. These sterile males compete with wild males for mating with the wild females and these matings produce no offspring, resulting in a reduction in pest population growth. Continuous releases of sterile males at high ratios to wild males can effectively suppress the target population (Klassen, 2005; Klassen & Curtis, 2005). Previous work has shown that SIT can also be a promising tool for the population control of *B. oleae* (Economopoulos et al., 1977; Estes et al., 2011). However, as reviewed previously, the main problem for the deployment of large-scale SIT applications against *B. oleae* has been the lack of a standardized procedure for the mass rearing of high-quality sterile males (Economopoulos & Zervas, 1982; Estes et al., 2011).

Parasitoids are important biological control agents in AW-IPM programmes against major agricultural pests including several fruit fly species (Montoya et al., 2007). Compared to other natural enemies of *B. oleae* (such as ants and generalist ectoparasitoids), parasitoid wasps are more specialized, which is beneficial in biological control (Daane et al., 2015). Parasitoids have the potential to significantly contribute to the population control of *B. oleae*. For instance, in South Africa, the damage of *B. oleae* is minimal due to the resident natural enemy fauna (Hancock, 1989), of which parasitic wasps are the main component (Walton et al., 2005). Several species of parasitoids targeting olive flies are available (Hoelmer et al., 2011; Wharton & Yoder, 2016) and some species have been released and tested (Table 1). However, their potential as a component of AW-IPM has not yet been realised for several reasons, among which inefficient mass rearing.

Olive fruit fly mass rearing

Although *B. oleae* can be reared on artificial diet under laboratory conditions, its large-scale mass rearing, needed for SIT, has proven challenging (Manoukas, 1975; Estes et al., 2011). When small-scale rearing is upgraded to mass rearing, the quality, fecundity, production stability, and costs are important factors in determining the insect quality and thus the project’s success (Calkins & Parker, 2005; Parker, 2005). The same is also true for parasitoids whose large-scale mass rearing depends on the efficient, high-quality, and cost-effective mass production of their hosts.

In respect to the mass rearing of *B. oleae*, the bottleneck lies in the larval stage. For *B. oleae* culturing, it is not practical, and certainly not cost effective, to provide fresh olives year-round, so the larvae need to be produced on an artificial diet. Artificial diets are commonly used in fruit fly rearing facilities, but are usually the most expensive component of the rearing procedure (Parker, 2005). They are also difficult to design as there are many factors that can influence insect quality, such as pH and preservatives, nutritional elements, moisture, texture, and microbes (Cohen, 2003; Lance & McInnis, 2005). The fact that *B. oleae* larvae are specialists on olives makes it difficult to grow this life stage efficiently as it is generally more difficult to find a suitable diet for specialist feeders (Parker, 2005). It has indeed proven particularly difficult to develop an efficient artificial diet for *B. oleae* larvae that produces enough pupae because they are very sensitive to diet changes (e.g., different batches of the same dried yeast) compared to other fruit flies (Tzanakakis, 1989).

The diet used nowadays for *B. oleae* is still very similar to the ones developed in the 1960s and 1970s...
<table>
<thead>
<tr>
<th>Family</th>
<th>Species</th>
<th>Host stage attacked</th>
<th>Positive</th>
<th>Negative</th>
<th>Type of study</th>
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<tr>
<td>Braconidae</td>
<td><em>Fopius arisanus</em> (Sonan)</td>
<td>Egg and first instar</td>
<td>Just oviposition can also kill, did better than <em>D. kraussii</em> and <em>P. concolor</em>, will not attract Tephritidae that feed in inflorescences or galls</td>
<td>Prefers medfly, may attack other fruit-infesting tephritids, high rates of mortality with release, so better for augmentation, seems difficult to rear on <em>B. oleae</em>, strongly outcompetes other parasitoids on medfly</td>
<td>Tested in laboratory (Calvitti et al., 2002; Sime et al., 2008), and on laboratory medfly (Wang &amp; Messing, 2003)</td>
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<td></td>
<td><em>Bracon celer</em> (Szépligeti)</td>
<td>Third instar</td>
<td>Mainly targeted on <em>B. oleae</em> and medfly, most abundant with high parasitism rates in commercial olives, longer ovipositor than <em>Psyttalia</em> spec., has been reared from medfly</td>
<td>Did reproduce in non-target species, idiobiont ectoparasitoids tend to have a broader host range, difficult to rear and import</td>
<td>Laboratory tests in California, USA (Sime et al., 2006b; Nadel et al., 2009)</td>
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<td></td>
<td><em>Diachasmimorpha kraussi</em> (Fullaway)</td>
<td>Second and third instar</td>
<td>Long ovipositor, easy to rear, much experience from other hosts, found in <em>B. oleae</em> after release against medfly in Israel</td>
<td>Broad host range, <em>B. oleae</em> seems not preferred as host, did not establish in field trial, sensitive to higher temperatures</td>
<td>Laboratory study (Sime et al., 2006a), field study Israel (Argov et al., 2009)</td>
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<td></td>
<td><em>Diachasmimorpha longicaudata</em> (Ashmead)</td>
<td>Second and third instar</td>
<td>Long ovipositor, easy to rear, much experience from other hosts</td>
<td>Broad host range, <em>B. oleae</em> seems not preferred as host, sensitive to higher temperatures, and also more to lower temperatures</td>
<td>Laboratory study (Sime et al., 2006a)</td>
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<td></td>
<td><em>Psyttalia (Opius) concolor</em> (Szépligeti)</td>
<td>Second and third instar</td>
<td>Established in Europe, easy to collect and rear on medfly, much knowledge</td>
<td>Does not easily establish, needs inundation, short ovipositor thus not good in large olives, high temperature is negative for longevity but strain variation is present</td>
<td>Life-history tests in laboratory (Canale &amp; Loni, 2006 (medfly); Sime et al., 2006c; Yokoyama et al., 2008; Wang et al., 2009b; Canale &amp; Benelli, 2012), introduced in field in Europe (Delrio et al., 2005; Miranda et al., 2008), Turkey (Hepdurgun et al., 2009), and in California, USA (Yokoyama et al., 2006, 2008; Daane et al., 2015)</td>
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<td>Family</td>
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<td>Host stage attacked</td>
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<td><em>Psyttalia</em></td>
<td><em>Psyttalia (Opius) lounsburyi</em></td>
<td>Second and third instar</td>
<td>Specialist on <em>B. oleae</em> tested in quarantine studies</td>
<td>Short ovipositor and therefore less effective than <em>P. humilis</em>, not efficient at higher temperatures, established in cooler coastal parts in California, USA</td>
<td>Field cage tests and release in California, USA (Daane et al., 2008, 2011; Wang et al., 2009a, 2011, 2012, 2013; Daane &amp; Johnson, 2010)</td>
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<td></td>
<td><em>(Silvestri)</em></td>
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<td>Field cage tests and releases in California, USA (Yokoyama et al., 2010, 2011, 2012; Daane et al., 2011, 2015; Wang et al., 2011, 2013)</td>
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<td></td>
<td><em>Psyttalia humilis</em></td>
<td>Second and third instar</td>
<td>Can reproduce on <em>B. oleae</em> in the field, but limited</td>
<td>Short ovipositor</td>
<td>Tested in quarantine in California, USA (Sime et al., 2007)</td>
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<td></td>
<td><em>(Silvestri)</em></td>
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<td>Field cage tests in California, USA (Kapaun et al., 2010)</td>
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<td><em>(formerly cf. concolor)</em></td>
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<td></td>
<td><em>Psyttalia ponerophaga</em></td>
<td>Second and third instar</td>
<td>Only found from <em>B. oleae</em>, broader temperature range, good to survive in hotter regions of California, USA, could survive relatively well on local <em>B. oleae</em></td>
<td>Short ovipositor, cannot be reared efficiently yet, performs similar to <em>Diachasmimorpha</em> species although it is a specialist</td>
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<td><em>(Silvestri)</em></td>
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<tr>
<td>Pteromalidae</td>
<td><em>Pteromalus nr. myopitae</em></td>
<td>Third instar</td>
<td>Found in California, USA</td>
<td>Difficult to rear, cage tests not successful</td>
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<td><em>(Graham)</em></td>
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1 A complete list of parasitoids found in *B. oleae* is given by Hoelmer et al. (2011), complemented with the parrot database (Wharton & Yoder, 2016).
(Economopoulos & Tzanakakis, 1967; Estes et al., 2011). Although the larvae are able to survive on this artificial diet, their survival rate from larval to pupal stage is variable, especially in mass rearing (Ahmad et al., 2016). In addition, the yield is not high enough with ca. 2,000 pupae kg⁻¹ diet (Estes et al., 2011). In comparison, in the largest SIT production facility of the medfly (the Moscamed project) 10,000 pupae kg⁻¹ are produced (Cáceres, 2002; Cáceres et al., 2002). This medfly mass rearing facility shows the enormous scale at which these insects can be reared, with a capacity to produce more than 2 million sterile males per week (Enkerlin et al., 2015). Besides variable and relatively low yields, the costs for the *B. oleae* larval diet are relatively high (compared to medfly diet) as expensive hydrolysed proteins and anti-pathogen ingredients are required in the current formulation (Economopoulos & Tzanakakis, 1967; Yokoyama, 2015; C. Cáceres-Barrios, pers. obs.). Therefore, it is important to find other ingredients that can provide a balance between cost and quality (Ahmad et al., 2014).

### Parasitoid mass rearing

Mass rearing of parasitoids is a major component of the biological control industry (van Lenteren, 2000). There are several critical factors in the mass rearing of parasitoids that may be affected upon laboratory domestication, such as environmental conditions for adults and for infested host larvae and pupae (temperature, humidity, photoperiod, microbial pathogens – particularly fungi), host suitability (species, developmental stage, age, quality, diet, symbionts), parasitoid density (food, competition), superparasitism, diapause, genetic diversity, and microbial flora. It has been shown, for example, that density as well as diet during rearing can affect fitness parameters in parasitoids (Harvey et al., 1995; Loni, 2003; Zboralski et al., 2016). The problems of superparasitism and competition between wasp larvae in the host can be remedied by limiting the exposure density and ovipositing time of the wasps to achieve an optimal number of parasitoid eggs per host (Loni, 2003). In egg parasitoids, special methods are needed for egg exposure (Bautista et al., 1999). Other potential problems, which have been observed in many parasitoids, are skewed sex ratios and lack of mating (Waage et al., 1985; Bautista et al., 1999; Montoya et al., 2011).

The quality of parasitoid wasps is important in terms of size, longevity, fecundity, progeny sex ratio, and parasitism rate (Messing et al., 1993; Eben et al., 2000; Yokoyama et al., 2012). Besides quality, field efficacy is also essential, for which flight (Messing et al., 1997) and host localization (Eben et al., 2000) are important parameters. As for most biological control agents, a strain of a beneficial parasitoid would ideally be entirely consisting of long-lived females parasitizing many hosts (Hoffmann et al., 2001). An additional challenge for the mass rearing of parasitoids is that the host species is also mass reared efficiently to produce the substrate for the parasitoids to develop in. For AW-IPM projects with a SIT component, the ideal practical method for parasitoid rearing is to use the host that one targets for sterile male releases. There are several indications that using the target pest species as a host may be critical for the production of high-quality parasitoids (Hoffmann et al., 2001; Daane et al., 2015).

Rearing parasitoids on *B. oleae* is currently challenging and expensive due to the lack of a robust and cost-efficient rearing system of the host. However, a closely related species can be used as an alternative host, e.g., the parasitoids *Psyttalia concolor* (Szépligeti), *Psyttalia humilis* (Silvestri), and *Diaclassmonopora longicaudata* (Ashmead) can be successfully reared on medfly (Yokoyama et al., 2006; Ovruski et al., 2011; Daane et al., 2015). This is an advantage when insufficient *B. oleae* larvae are available, but may lead to wasps that are less efficient on *B. oleae*. This is illustrated by the search behaviour of *P. concolor* that has proven more efficient after previous exposure to *B. oleae* larvae (Canale & Benelli, 2012). It remains unclear whether this host habituation effect is due to the host larva itself or the used larval medium (olive pulp).

It is a general practice that host medfly larvae are irradiated before exposing them to parasitoids (Cancino et al., 2012; Yokoyama et al., 2012). This practice causes the medflies to die before emergence, thus eliminating the risk of releasing fertile hosts in the target area, as well as eliminating the need to sort the parasitoid-infested from the non-infested hosts before the parasitoids are released into the field. Both gamma and x-ray irradiation do not seem to have major negative impacts on the quality of medfly (Cancino et al., 2012) and *Anastrepha fraterculus* (Wiedemann) (Bachmann et al., 2015) for use in rearing of *D. longicaudata*. Although a decrease in the number of mature eggs in adult females was observed, this did not affect the number of progeny in *P. humilis* (Yokoyama et al., 2010, 2012). Alternative methods of removing the residing non-parasitized hosts, when irradiation treatment is not available, can be (1) selection on pupal size with a pupal sizer or pneumatic air separator (Bautista et al., 1999), but this is time consuming and can result in too many parasitized pupae being discarded; (2) separation based on size difference between adult emerged parasitoid and host with a mesh (Bautista et al., 1998), which usually works well but is less convenient on a large scale, and (3) use of developmental time differences, such as...
unparasitized pupae typically emerging earlier (Bautista et al., 1999).

Other main problems in B. oleae parasitoid rearing are (1) insufficient production of host insect numbers (Yokoyama et al., 2010; Daane et al., 2015), (2) low parasitoid quality as a result of rearing procedures or laboratory domestication (Delrio et al., 2005), (3) low emergence rates under laboratory rearing conditions (Loni, 2003), and (4) low parasitoid infestation in the field after release (Yokoyama et al., 2010). These studies reveal that rearing procedures for B. oleae parasitoids are not yet optimized and that much can be improved for increasing their quantity, quality, field survival, and host targeting.

**Beneficial symbionts in olive fruit fly and related species**

Like all animal species, insects are masters in establishing sophisticated symbiotic associations with a variety of bacteria and fungi affecting all aspects of their biology, including nutrition, immunity, reproduction, ecology, and evolution (Bourtzis & Miller, 2003, 2006, 2008; Vega & Blackwell, 2005; Zchori-Fein & Bourtzis, 2011; Engel & Moran, 2013). The insect gut contains a variety of symbiotic microorganisms, which provide various benefits that enhance the fitness of their hosts. In principle, these symbiotic microorganisms may be exploited to enhance mass rearing by helping insects digest their diet or by providing them with crucial nutritional elements. Nutrient provisioning (especially nitrogen) is an important function of symbionts because they are able to digest food or waste components by hydrolysis, making them available to their insect host (Engel & Moran, 2013). Sometimes this benefit to the host is accompanied by detoxification of insecticides and plant defence chemicals, enabling their host to live in unique habitats (Kikuchi et al., 2012; Engel & Moran, 2013; Hammer & Bowers, 2015).

Besides digestive functions, insect gut symbionts can have an array of other functions (see Engel & Moran, 2013, for an overview). They can provide protection against parasites via competition or immune priming, called colonization resistance (Vollaard & Clasener, 1994). Microorganisms can also be involved in the production of certain signalling compounds such as cuticular hydrocarbons that act as pheromones; in Drosophila melanogaster Meigen the gut microbiota affect mate choice and flies are attracted to individuals with a similar microbial ecology (Sharon et al., 2010). In the same species, bacteria are responsible for cell renewal and growth promotion (Storelli et al., 2011). These non-digestive bacteria are also interesting for biological control because they potentially make flies more attractive and thus better candidates for SIT. They also provide opportunities for attractants in bait sprays or traps, as proven in B. oleae (Scarpati et al., 1996) and related fruit flies (Gow, 1954; Drew, 1987; Robacker et al., 2009).

Tephritid species have established symbiotic associations with a variety of bacterial and fungal species (Petri, 1909; Mazzon et al., 2008; Andongma et al., 2015; Angelinos et al., 2015; Ben-Yosef et al., 2015; Morrow et al., 2015; Hadapad et al., 2016). Although less is known about the function of fungi compared to bacteria, inactivated yeasts are applied successfully to the artificial diet of tephritids (Cohen, 2003) and are common attractors in tephritid baits (Bortoli et al., 2016). This indicates that yeast is important for nutrition in wild tephritids as well, just as yeast and yeast-like fungi are in Drosophilidae (Vega & Blackwell, 2005; Hamby & Becher, 2016). Associated cultivable yeasts have been identified in Bactrocera tryoni (Froggatt) (Deutscher et al., 2017) and the total gut fungal microbiome has been investigated in wild B. oleae (Malacrinò et al., 2015).

Medfly is the model species in the Tephritidae family. Bacterial communities vary between medfly strains and populations, and can vary among life stages (Aharon et al., 2013), particularly when exposed to different environments. These shifts in community composition may allow medfly to feed on various host plants (Aharon et al., 2013). Medfly symbionts are typically taken up from the environment, but can also be passed on via vertical transmission (Behar et al., 2008a; Ben Ami et al., 2010). Besides passing on the bacteria, the female medfly also provides eggs with an antibiotic substance during oviposition (Marchini et al., 1991), which is probably meant to ward off pathogenic bacteria and select for the beneficial symbionts. The medfly is known to be associated with bacterial species predominantly from the Enterobacteriaceae family (Enterobacter, Klebsiella, and Pectobacterium species). The core bacteria of medfly are diazotrophic (atmospheric nitrogen fixators) and pectinolytic (hydrolyser of pectin substances in plants) and seem to help by accelerating fruit decay and providing nitrogen for the larva as soon as they are inoculated by the adult by oviposition (Behar et al., 2005, 2008a). Bacteria also affect survival depending on the nutrients the fly receives (Behar et al., 2008b). The benefit of medfly symbionts can be diet dependent: when enough food is present some are beneficial by accumulating fats and improve mating success, but when food is scarce those bacteria may have a negative effect (Behar et al., 2008b). It has also been demonstrated that mass rearing and irradiation may adversely affect bacterial communities in medfly, by increasing the density of potentially pathogenic Pseudomonas species (Ben Ami et al., 2010).
Similar to related tephritid species, *B. oleae* possesses several symbiotic-supporting devices (Petri, 1909; Girolami, 1973, cited in Sacchetti et al., 2014), with ceca connected to the larval midgut which can grow and store bacteria, an oesophageal bulb with the same capabilities in adults, and additionally an ovipositor diverticulum in adult females. These specialized organs suggest that the fly and the symbiont(s) have a tight evolutionary bond and are in close symbiosis, in which the bacteria provide benefits for the survival of the fly and vice versa. Even though the exact transmission mechanism has not yet been elucidated, the presence of the ovipositor diverticulum and the bacteria covering the egg suggest vertical transmission (Stammer, 1929; Sacchetti et al., 2008; Estes et al., 2009).

Various bacterial species have been reported in association with *B. oleae* over the years (Table 2). Recent studies have clearly demonstrated that the major symbiont of *B. oleae* is *Candidatus Erwinia dacicola*, a non-cultivable γ-proteo-bacterium, which is present both intra- and extracellularly (Capuzzo et al., 2005; Estes et al., 2009, 2012; Kounatidis et al., 2009; Savio et al., 2012; Ben-Yosef et al., 2014, 2015). In addition to *Ca. E. dacicola*, several bacterial species have been detected in laboratory and natural populations of olive fruit fly. These are summarized in the review by Estes et al. (2011), but additional studies were done since by Savio et al. (2012), Estes et al. (2012), and Ben-Yosef et al. (2014, 2015). In wild flies, these symbionts are mostly found in low densities (Ben-Yosef et al., 2015), and are suggested to be transient (Estes et al., 2011).

The most dominant species are members of Enterobacteriaceae, for instance, *Enterobacter* (Stamopoulos & Tzanetakis, 1988; Estes et al., 2009), *Klebsiella* (Tsiroupolos, 1983; Konstantopoulou et al., 2005), *Pantoea* (Ben-Yosef et al., 2015), and *Serratia* (Tsiroupolos, 1983; Konstantopoulou et al., 2005), which are commonly associated with fruit digestion in other fruit fly species (Drew & Lloyd, 1991; Behar et al., 2008a; Ben-Yosef et al., 2010, 2015).

It has been reported that *B. oleae* (as well as its close tephritid relatives) cannot survive under sterile laboratory conditions unless its artificial larval diet contains hydrolysed proteins (Hagen et al., 1963). This clearly suggests that microorganisms are somehow essential and play a role in digestion in natural populations. The main function of the *B. oleae* symbionts seems to be to provide the fly with the ability to digest unripe olives. This is evident when the bacteria are removed with antibiotics, which causes inability to digest unripe olives and non-hydrolysed proteins (Hagen, 1966; Ben-Yosef et al., 2015). The bacteria seem to produce essential amino acids by converting proteins and non-essential amino acids (Ben-Yosef et al., 2010) and additionally help to overcome the olive’s protective compound oleuropein in unripe olives (Ben-Yosef et al., 2015), as supported by a recent transcriptomic study (Pavlidi et al., 2017). In addition, *B. oleae* without symbionts becomes more prone to infections by pathogenic microbes (Cavalloro & Girolami, 1968, cited in Estes et al., 2011), suggesting a protective function of the symbionts.

**Effects of rearing environment on olive fruit fly symbionts**

Laboratory-reared flies maintained on artificial diets tend to have a smaller oesophageal bulb (Cavalloro & Girolami, 1968, cited in Estes et al., 2011), and have a lower diversity in their associated bacterial community. In particular, they carry fewer members of Enterobacteriaceae and appear to lose their *Ca. E. dacicola* (Tsiroupolos, 1983; Belcari et al., 2003; Konstantopoulou et al., 2005; Estes, 2009; Estes et al., 2009, 2012; Kounatidis et al., 2009; Ben-Yosef et al., 2015). In contrast, the genera *Acetobacter*, *Morganella*, and *Paenibacillus* are only found in laboratory flies with the most abundant species belonging to *Providencia* and *Acinetobacter* (Kounatidis et al., 2009; Ben-Yosef et al., 2015). These differences in the gut-associated microbial communities are likely to be caused by the different environment in the laboratory which may (1) lack certain important natural substances required for the maintenance of the key bacterial symbiotic species, and (2) include antibiotics and preservatives that may cause the elimination of the beneficial species. Genetic factors and bottlenecks are also likely to play an important role during the domestication process and the adaptation of wild *B. oleae* into a non-natural environment. In most cases, the laboratory populations originate from a small number of individuals resulting in a laboratory strain that may be genetically different from wild *B. oleae* populations (Zygouridis et al., 2014). A small founder *B. oleae* population may also mean a smaller founder population of symbionts.

In current *B. oleae* laboratory rearing, antimicrobial agents are indispensable to prevent the growth of pathogenic fungi or bacteria. This potentially influences the gut-associated bacteria community. For example, eggs are often washed with 2% Clorox (0.11% sodium hypochlorite) solution (Tsitsipis, 1975; Estes et al., 2011) but this may also remove the bacterial layer on the eggs deposited, preventing the vertical transmission of the naturally associated symbionts. In addition, the larval medium contains the antimicrobial elements nipagin and potassium sorbate. Nipagin or Methyl 4-hydroxybenzoate [\(\text{CH}_3(\text{C}_6\text{H}_4(\text{OH})\text{COO})\)] (NCBI PubChem, 2016) is a methylparaben used as an antimicrobial agent in foods (preservative against yeasts and moulds) and cosmetics (topical antibiotics).
<table>
<thead>
<tr>
<th>Year</th>
<th>SIT and rearing milestones</th>
<th>Bacteria ID studies</th>
<th>Microbiology milestones</th>
</tr>
</thead>
<tbody>
<tr>
<td>1909</td>
<td>First bacteria identified$^1$</td>
<td>Petri (1909)</td>
<td>Intensive study of symbiotic device, description of vertical transmission</td>
</tr>
<tr>
<td>1956</td>
<td>Wild adult flies$^1$</td>
<td>Hellmuth (1956)</td>
<td></td>
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<tr>
<td>1963</td>
<td>Elaborate study on <em>B. oleae</em> rearing under laboratory conditions for SIT</td>
<td>Hagen et al. (1963)</td>
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<tr>
<td>1966</td>
<td>Successful sterilisation and first field trial with sterilized flies</td>
<td>Tzanakakis et al. (1966); Orphanidis et al. (1966)</td>
<td>Symbiont needed for protein and unripe olive digestion; <em>Acidophilus</em> spec. cannot replace all functions of the original symbiont but can provide two essential amino acids</td>
</tr>
<tr>
<td>1973</td>
<td>Antibiotics inhibit larval development</td>
<td>Tzanakakis &amp; Stavrinides (1973)</td>
<td></td>
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<tr>
<td>1975</td>
<td>Current larval diet defined</td>
<td>Tsisipis (1975)</td>
<td></td>
</tr>
<tr>
<td>1977</td>
<td>Laboratory adults$^1$</td>
<td>Haniotakis &amp; Avtiz (1977)</td>
<td></td>
</tr>
<tr>
<td>1982</td>
<td>Summary of research on SIT, mentioning its problems</td>
<td>Economopoulos &amp; Zervas (1982)</td>
<td></td>
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<tr>
<td>1988</td>
<td>Wild and laboratory adults$^1$</td>
<td>Stamopoulos &amp; Tzanetakis (1988)</td>
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<tr>
<td>1991</td>
<td>Wild adults</td>
<td></td>
<td></td>
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<tr>
<td>1999</td>
<td>Antibiotics influence allele frequencies in laboratory flies</td>
<td>Konstantopoulou et al. (1999)</td>
<td>Host–bacterial interactions affect development and detection of <em>Adh</em> alleles</td>
</tr>
<tr>
<td>2003</td>
<td>Wild and laboratory adults and phylloplane</td>
<td>Belcari et al. (2003)</td>
<td></td>
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<tr>
<td>2005</td>
<td>Wild adults$^{2,3}$</td>
<td>Capuzzo et al. (2005)</td>
<td>Uncultivable <em>Ca. Erwinia dacicola</em> found as the main symbiont</td>
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</table>

$^1$ Refers to specific studies and milestones related to the bacteria and their identification and impact on the research milestones.
<table>
<thead>
<tr>
<th>Year</th>
<th>SIT and rearing milestones</th>
<th>Bacteria ID studies</th>
<th>Microbiology milestones</th>
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<tbody>
<tr>
<td>2007</td>
<td>Laboratory and wild pupae</td>
<td>Konstantopoulou et al. (2005)</td>
<td>Host–bacterial interactions affect development and detection of Adh alleles</td>
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<td></td>
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<td>Fly attraction to microbes</td>
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<td></td>
<td>Laboratory adults and</td>
<td>Chrysargyris (2008)</td>
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<td>2008</td>
<td>wild pupae</td>
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<td></td>
<td>Laboratory pupae and</td>
<td>Estes et al. (2009)</td>
<td>Ca. E. dacicola goes intracellular during larval metamorphosis, and is dominant in all life stages in wild and olive-reared flies</td>
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<tr>
<td></td>
<td>adults, wild larvae,</td>
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<td></td>
<td>pupae, and adults</td>
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<td>Fewer, other, or no symbionts found in laboratory flies compared to wild flies</td>
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<td>2009</td>
<td>Laboratory adults and</td>
<td>Estes (2009)</td>
<td>Acetobacter tropicalis is present in Greek natural and laboratory populations</td>
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<td>wild larvae</td>
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<td>2011</td>
<td>Reviewing previous</td>
<td>Estes et al. (2011)</td>
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<td>knowledge with intent to</td>
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<td></td>
<td>try SIT again</td>
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<tr>
<td>2012</td>
<td>Laboratory pupae and</td>
<td>Estes et al. (2012)</td>
<td>Probiotic diet has positive effect on adult females</td>
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<td>adults olive and</td>
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<td></td>
<td>non-olive reared, wild</td>
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<td></td>
<td>larvae, pupae, and adults</td>
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<td></td>
<td>Wild adults</td>
<td>Savio et al. (2012)</td>
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<td>2014</td>
<td>Adult diet without</td>
<td>Rempoulakis et al. (2014)</td>
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<td>antibiotics is not</td>
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<td>problematic in non-</td>
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<td>mass-rearing situation</td>
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<tr>
<td></td>
<td>Wild larvae, pupae, and</td>
<td>Ben-Yosef et al. (2014)</td>
<td>Symbionts can also create essential amino acids from urea</td>
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<tr>
<td></td>
<td>adults</td>
<td></td>
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<tr>
<td>Year</td>
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<sup>1</sup> Isolation of bacteria through culturing plus morphological and/or biochemical identification of cultivable bacteria.

<sup>2</sup> Isolation of bacteria through culturing plus 16S rRNA gene-based identification, including Sanger sequencing and/or PCR-RFLP and/or DGGE of cultivable bacteria.

<sup>3</sup> Culture-independent approach, using a 16S rRNA gene-based characterization: it includes total DNA extraction, PCR, cloning, and screening of libraries, through Sanger sequencing and/or PCR-RFLP and/or DGGE.

<sup>4</sup> Culture-independent approach, using a 16S rRNA gene-based characterization: it includes total DNA extraction, preparation of 16S rRNA libraries (not cloning), and Next Generation Sequencing.

<sup>5</sup> Culture-independent approach, based on whole-genome characterization: it uses single-cell genomics technology to assemble whole bacteria genomes.

RFLP, Restriction Fragment Length Polymorphism (detects differences in the gene by the presence of fragments of different lengths after digestion of the sequence with one or more restriction endonucleases); DGGE, Denaturing Gradient Gel Electrophoresis (detects differences in DNA fragments induced by denaturing conditions within a sequence according to their mobility on an agarose gel).
Potassium sorbate \( [\text{C}_6\text{H}_7\text{K}_2\text{O}_2] \) is a potassium salt of sorbic acid used as a food preservative to inhibit, retard, or arrest the process of fermentation, acidification, or other deterioration of foods (NCBI PubChem, 2016). Both these substances may have an important impact on the \( B. \text{oleae} \)-associated microbiota. Nipagin has been shown to cause changes in the cultivable microbiota community in \( B. \text{oleae} \), where its presence on culture plates caused most cultivable wild-associated bacteria to be removed and laboratory-associated bacteria to be inhibited (Konstantopoulou et al., 1999). Streptomycin is also added to the adult diet (Hagen et al., 1963). It is a broad-spectrum bactericidal antibiotic that inhibits the synthesis of proteins by interacting with the bacterial 16S rRNA gene (NCBI PubChem, 2016). It is likely that those antimicrobials are the cause of the difference in the cultivable microbiome between laboratory-reared and wild \( B. \text{oleae} \) (Konstantopoulou et al., 1999, 2005). It is, however, not known yet at what stage of the domestication process the symbionts are affected.

**Potential of gut symbionts for olive fruit fly rearing**

There are several possible options to bring back the beneficial symbionts into the mass rearing of \( B. \text{oleae} \). One option would be to remove antibiotics from the adult and larval diets, especially during domestication, as demonstrated by two recent studies. Removal of streptomycin from the adult diet did not cause extra diet spoilage and had no negative effect on \( B. \text{oleae} \) production, at least up to the eighth generation (Dimou et al., 2010; Rempoulakis et al., 2014). However, the long-time laboratory colony that was reared on antibiotics still performed better than the F8 generation wild-derived flies on diet without antibiotics, probably due to a longer laboratory adaptation (Dimou et al., 2010). Moreover, these experiments were performed on a relatively small scale (few thousands eggs per cage per day), whereas most problems appear when \( B. \text{oleae} \) strains are put under mass-rearing conditions (hundreds of thousands of eggs per cage per day) (Manoukas, 1975; Estes et al., 2011; Ahmad et al., 2016). It would be interesting to repeat this antibiotic removal on a larger scale, including quality tests involving male mating competitiveness which is important for SIT applications. At the same time, the microbiome composition should be monitored to see whether particular symbionts, such as \( Ca. \) E. dacicola, may survive the antibiotic treatment.

Besides removing the antibiotics, another option to promote symbiont survival in the host would be to change the diet because the larval diet is likely to influence the gut conditions. The best solution would be to find a food source that is selective for beneficial bacteria and against pathogens in the larvae (Cohen, 2003). The olive is such a selective medium because wild and laboratory flies reared on olives keep their \( Ca. \) E. dacicola (Estes et al., 2012). There are quite some differences in nutritional values and chemical composition when comparing the current artificial larval diet to olives in terms of lipids, amino acid ratio, and kNA ratio (Manoukas, 1984). For example, oleuropein, which is naturally present in olives and makes the olive difficult to digest without bacteria (Ben-Yosef et al., 2015), has interesting antimicrobial properties (Bisignano et al., 1999). Through these antimicrobial properties, oleuropein might create a selective environment in the larval gut for the well-adapted symbiotic bacteria like \( Ca. \) E. dacicola. Oleuropein-rich olive waste, olive leaf extracts, stored waste products of olive oil production, or chemical substances related to oleuropein have also been shown to exhibit antimicrobial effects (Medina et al., 2011). Besides many phenolic compounds, the main natural polyphenolic compound in olive mill waste water is hydroxytyrosol, an antioxidant that may originate from hydrolysis of oleuropein during the milling process (Amiot et al., 1986). The effect of oleuropein and hydroxytyrosol could be considered as additives in artificial larval diet of \( B. \text{oleae} \) during domestication and afterwards during mass rearing. Except for the evaluation of olive oil amounts (Manoukas, 1977) and the addition of other allelochemicals in larval diet (Manoukas, 1986), which both proved ineffective, this has not yet been done. Further studies of diet composition and essential olive compounds for fly production and their associated microbe composition are clearly warranted.

The third option for exploiting gut symbionts would be to add them to the diet as probiotic supplements. For probiotic applications, the target bacterial species should be easy to culture and to add to the diet. This makes \( Ca. \) E. dacicola currently not suitable, but there are several other potential cultivable candidates. Usually the transiently associated facultative bacteria have a higher chance to be cultivable (Estes et al., 2011). There are successful probiotic applications in other fruit flies such as medfly (Niyazi et al., 2004; Ben Ami et al., 2010; Yuval et al., 2010; Gavriel et al., 2011; Hamden et al., 2013; Augustinos et al., 2015) or Bactrocera species (Drew et al., 1983; Meats et al., 2009; Yao et al., 2017) which could give indications or useful species for \( B. \text{oleae} \) probiotic trials. Administering live \( Klebsiella \text{oxytoca} \) (Flügge) Lautrop to adult irradiated medflies improved sexual performance and starvation tolerance of sterile males and reduced the density of pathogenic \( Pseudomonas \) species (Ben Ami et al., 2010; Gavriel et al., 2011). Positive effects were also observed after mixing a cocktail of live bacteria [\( Klebsiella \text{pneumoniae} \) (Schröter) Trevisan, Enterobacter spp., and \( Citrobacter \text{freun dii} \) (Braak) Werkman & Gillen] into the larval food before
irradiation (Hamden et al., 2013). In the study of Augustinos et al. (2015), probiotics containing *Enterobacter* spec. provisioned to larvae resulted in higher pupal and adult recovery, as well as to enhanced protandry phenomena. However, no significant effect on mating competitiveness and longevity under starvation was found. The positive effects were more pronounced in the live bacteria applications, which also contributed to enhanced protandry phenomena. Yao et al. (2017) discovered that adding live as well as dead *Enterobacter* isolates from the medfly to the larval diet enhanced the fitness of a *Bactrocera cucurbiteae* (Coquillett) genetic sexing strain by increasing pupal weight and survival rate.

Thus far, few studies have considered the effect of adding probiotics to the diet in *B. oleae* (Ghiardi, 2009, cited in Estes et al., 2011; Sacchetti et al., 2014). Feeding adult *B. oleae* with the live bacterial *Pseudomonas putida* Trevisan as an additive had a positive effect on female productivity, but a negative effect on male lifespan (Ghiardi, 2009, cited in Estes et al., 2011; Sacchetti et al., 2014). *Bactrocera oleae* larval diet-based probiotic applications have not been investigated so far. Although bacteria are involved in nutrient uptake by *B. oleae* (Estes et al., 2011; Ben-Yosef et al., 2015), it is not known yet whether the nutrients are products of the bacteria or whether *B. oleae* consumes the bacteria themselves. This is important when considering the use of probiotics; if bacterial products are important, live bacteria could be used to inoculate the flies in order to aid in digestion, or their products could be added to the diet. However, if the flies consume the bacteria themselves, dead bacterial biomass could be provided as a diet component, replacing the protein source altogether. For practical reasons such as health safety and food storage it would be most pragmatic to add dead bacterial mass to the food in mass-rearing facilities or to keep the bacteria alive in the fly (Cohen, 2003).

**Potential of gut symbionts for parasitoid rearing**

In the same way that insect-associated bacterial species could be of importance in *B. oleae* mass rearing, microbiota might also be a determining factor for efficient – i.e., high-quality and cost-effective – parasitoid rearing. Effects could be direct, by the gut microbiota of the wasp, or indirect, by the host-associated bacteria. Interesting findings from other host–parasitoid systems may be relevant for *B. oleae* parasitoid mass rearing and applications. For instance, host finding is often influenced by chemicals from the host’s faeces as in *Halictoidea laevigata* (Thomson) wasps and their tephritid host, *Myoleja lucida* (Fallén) (Hoffmeister & Gienapp, 1999). In the case of the moth parasitoid *Diadromus pulchellus* (Wesmael) this interaction was proven to be mediated by microbes (Thibout et al., 1993). As suggested by Leroy et al. (2011) for aphids and their natural enemy hoverfly, attractant bacteria and their associated chemicals could be used in biological control to help guide the predators towards host-infested spots. Laboratory adaptation can change host preference towards hosts reared on artificial diet, as shown in a study on the parasitoid *D. longicaudata* and the host *Bactrocera dorsalis* (Hendel) (oriental fruit fly) (Bautista & Harris, 1997). The extent to which the *B. oleae*–parasitoid interactions in terms of host finding are affected by host-associated microbes remains to be determined.

After oviposition, once the egg starts developing in the host, there are several host-derived effects on the parasitoid, which may affect parasitoid quality (Salt 1968; Eben et al., 2000). Besides the host defence strategies that depend on humoral or cellular mechanisms (Godfray, 2016), symbiotic bacteria can also have an important role in host defence against parasites and pathogens, either directly or indirectly (Oliver et al., 2014). Several studies in aphids have found defensive symbiotic bacterial species against parasitoids, including *Candidatus Hamitonella* defensa (Oliver et al., 2003, 2005; Schmid et al., 2012), *Regiella insecticola* Moran et al. (Vorburger et al., 2010), and *Serratia symbiotica* Moran et al. (Oliver et al., 2003), whereas *Spiroplasma* has also been reported as protective symbiont in various *Drosophila* species (Xie et al., 2010, 2011, 2014, 2015; Mateos et al., 2016; Paredes et al., 2016). Whether such defensive symbionts also exist in *B. oleae*, or in the medfly used for parasitoid rearing, remains to be investigated.

When the parasitoid larva is growing in the host, consuming host cells and fluids, it can pick up bacteria from its host. For example, white fly parasitoids were shown to contain *Rickettsia* and *Hamiltonella* bacteria of their host (Chiel et al., 2009). The *Rickettsia*, but not the *Hamiltonella*, were retained in adults. This horizontal transmission mode was more persistent than transmission by host feeding. In none of the cases did the bacteria transfer vertically to the wasp offspring. These experiments show that some parasitoid species can obtain bacteria from their host, and certain bacteria are more easily transferred than others.

Besides providing immunity, host bacteria can influence the suitability of the host for parasitoid rearing. An effect of host diet on parasitoid quality parameters such as longevity, size, and fertility was shown for the parasitoid *D. longicaudata* with the tephritid host *Anastrepha ludens* (Loew) (Eben et al., 2000; Cicero et al., 2012). As seen from the *B. oleae* and medfly studies mentioned above, host quality can be influenced by microbial symbionts and these symbionts can be influenced by host diet. Therefore,
offering the rearing host probiotics could indirectly influence parasitoid quality. Both, the microbiome effects on the quality of *B. oleae* as a host, as well as the potential effects on the olive fruit fly parasitoids, remain unexplored to date.

There may be many other functions of microorganisms that affect the biology of parasitoids. It would, for instance, be interesting to explore whether bacteria may aid developing parasitoids in overcoming or avoiding the host immune system in the same way they use viruses (Lawrence, 2004), or whether (similar to fruit flies) certain bacteria could provide nitrogen to wasps to survive longer in the field on their low-nitrogen adult diet. Well known is the manipulation of reproduction by *Wolbachia* and other bacteria, but this falls beyond the scope of this review. More relevant examples are a *Wolbachia* strain that is needed for oogenesis in the *Drosophila* parasitoid *Asobara tabida* ( Förster) (Dedeine et al., 2001) and (unidentified) microorganisms that provide *Trichogramma bourarachae* (Pintureau & Babault) a higher infestation rate (Girin & Boulétreau, 1994). Now that there is an enormous research effort towards unravelling the role of the microbiome in organismal functioning, more functions of associated microbes in parasitoids may well be discovered in the near future.

**Conclusions and challenges**

The development and implementation of a SIT programme, as a component of an AW-IPM strategy to control populations of *B. oleae*, depends on a robust and cost-effective mass-rearing system for this insect pest species and its parasitoids. Being monophagous, the domestication and mass rearing of *B. oleae* on an artificial rearing system remain a challenge. This is most likely due to the genetic and symbiotic changes that occur during the domestication process. The current data suggest that the main symbiont *Ca. E. dacicola* is lost during this process. With modern genomic approaches it is now possible to determine exactly when this loss takes place, as well as any other changes in the microbiota. As the microbiome composition also depends on the host genotype and rearing medium, the genetic diversity of the established *B. oleae* population and the composition of the diet need to be revised.

If the loss of *Ca. E. dacicola* is unavoidable, an interesting area for future research would be the exploitation of *B. oleae* (or tephritid)-associated microbiota to identify cultivable bacterial species that could be used as probiotics and/or potential functional replacements of the major symbiont. This would require an extensive characterization of the *B. oleae*-associated microbiota from both laboratory and wild populations, including samples from different geographic areas, olive tree varieties, and developmental stages.

Regarding *B. oleae* parasitoids, there is little knowledge about their mass rearing and their associated microbiota. There are indications from other parasitoid–host systems that microbes can be beneficial to host finding, sex ratio, and infestation rate. On the other hand, the host could have defence bacteria that make it harder for the parasitoid to infest. Bacteria may also have additional unknown influences on other life-history traits in parasitoids. Interesting potential areas of research might be the digestion of adult food sources for the wasp, and the effect of host bacteria on host quality. Given the importance of symbiotic bacteria in the physiology, ecology, nutrition, reproduction, immunity, and evolution of insects, it is of paramount importance to characterize the microbiota associated with *B. oleae* parasitoids. This will yield crucial information on which microbes could be exploited to improve productivity and quality in parasitoid mass rearing.

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