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Relationships between the olive fly *Bactrocera oleae* and its endosymbiont *Candidatus* Erwinia dacicola: from basic knowledge to practical applications

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DECLARATION

I, the undersigned Gaia Bigiotti, declare that this thesis is the result of my personal work as Ph.D. student during the doctoral period 2015-2018 at University of Florence. Everyone that contributed to define experimental designs, data collection, data analysis, manuscripts review, funding provision or other information has been included as co-author, acknowledged or cited in accordance with the standard referencing rules. I also guarantee that this dissertation was not previously submitted to any University or other Institution for the award of any other degree.

Gaia Bigiotti

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Per aspera ad astra…

ABSTRACT

This research investigates the study, from basical knowledge to practical applications, of the endosymbiosis between *Bactrocera oleae* (Rossi), a key pest of the olive crops in the Mediterranean basin, and *Candidatus* Erwinia dacicola. This bacterium is considered essential for the olive fly. It is vertically transmitted through generations and it benefits both larvae and adults in field; whereas, it had been rarely found in lab colonies, probably because of preservatives in artificial diets. The aim of this Ph.D. thesis is to evaluate the possibility of controlling the olive fly through the management of this symbiosis, with its interruption in field by the use of several natural substances and favouring its maintenance in lab rearing for Sterile Insect Technique purposes. Regarding the first goal, several substances were evaluated in lab trials, as copper at 5%, copper at 20%, propolis and 3 different types of mono-floral honey (acacia, orange, chestnut). Regarding the second objective, a horizontal transfer was set out among wild and lab adults, exposing lab flies to different sources contaminated by wild flies with *Ca*. E. dacicola; for the same goal, the effects of lab procedures on the maintenance of the endosymbiosis were evaluated, exposing eggs laid by wild females to two different treatments: a propionic acid solution, a mixture of sodium hypochlorite plus Triton X. Also irradiation of wild males at two different doses was evaluated. Obtained results lead us to affirm that all the lab-tested substances in this research decreased the symbiosis rate, even if with different results. So that, the same substances should be further field-tested. For the symbiosis maintenance, we can affirm that preservatives in lab rearing significantly decreased the symbiont load on eggs interrupting the vertical transfer whereas it was demonstrated that a horizontal transfer could occur if a wild population cohabits with a lab strain. Moreover, this research contributed to set up a consistent molecular procedure for *Ca*. E. dacicola screening. Thus, these original results open further outlooks for the biological control of the olive fly and new opportunities to establish a permanent symbiotic colony, a strategic tool for future SIT applications, improving perspectives for a sustainable agriculture and low environmental impact control strategies.

RESEARCH UNITS

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INTRODUCTION

1. *Bactrocera oleae*: distribution, morphology and life cycle

1.1 Classification

Bactrocera oleae (Rossi, 1790), firstly described as *Musca oleae* Gmelin, *Daculus oleae* (Gmelin) and *Dacus oleae* (Gmelin), is a Tephritid more commonly known as the olive fruit fly (OLF). It belongs to the Dacinae subfamily (Diptera: Tephritidae) and, among the carpophagous species, it is considered one of the major insect pests for the olive tree *Olea europaea* (White and Elson-Harris, 1992).

1.2 Geographic distribution and host plants

B. oleae's distribution includes many countries all around the Mediterranean Sea (Figure 1) and most areas where plants of the genus *Olea* are grown extensively. It is widespread in our Mediterranean basin but it is present also in Africa, Canary Islands and from the Middle East to India. Recently, it invaded several areas like North America, California but also some parts of north-western Mexico (Rice *et al.*, 2003).

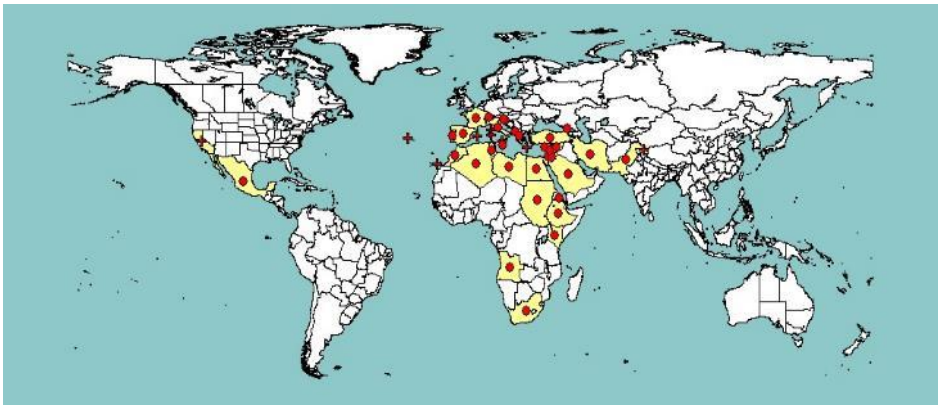

Figure 1 – Geographic distribution of the olive fruit fly *B. oleae*; source E.P.P.O, (September 24th, 2018).

1.3 Morphology

Adult - The adult is brownish and it is generally 4-5 mm long. The head is brownish-reddish, while the colour of the eyes varies between green and red according to the age. The head hosts a pair of antennae, with two black spots under them. The thorax is greyish, with three black longitudinal lines, while the humeri and the scutellum are yellowish. The wings are transparent, hyaline with a dark spot near the apex. Compared to the male (Figure 2) that has a tiny abdomen, the female (Figure 3) is characterized by a larger abdomen with a secondary ovipositor incompletely evaginated (8^{th} and 9^{th} segments remain in the 7^{th} urite).

Figure 2 – *B. oleae* male.

Figure 3 – *B. oleae* female.

Egg - The egg (Figure 4) is rod shaped, slightly curved, white and narrow, with a characteristic cup-like protrusion at one pole. with an average length of about 0.738 ± 0.01 mm and a mean diameter of around 0.21 ± 0.06 mm (Genc, 2014).

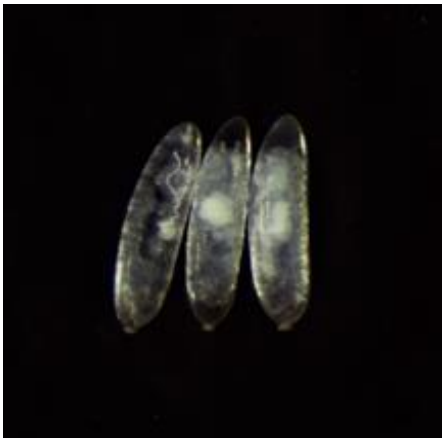

Figure 4 – *B. oleae* eggs at different stages of embryonic development.

Larva – *B. oleae* larva (Figure 5) is light yellow, it has three growth's instars and measures 7-8 mm long when fully grown. As all the tephritids of the Dacinae family, olive fly larvae are microcephalic with two pairs of spiracles: two of them thoracic and two abdominal. The head is involuted and the thorax is divided in three segments while the abdomen in eight (Fletcher, 1987).

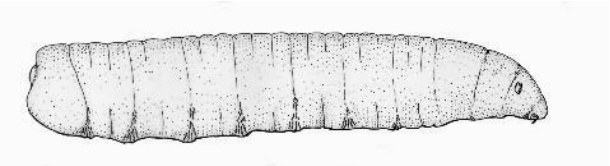

Figure 5 – Drawing of *B. oleae* 3rd instar larva. From Belcari, 1989.

Pupa – Pupae (Figure 6) are about 4-6 mm long (Tremblay, 1994), contained in a puparium coloured from yellowish when newly formed to brownish when adults are next to emerge.

Figure 6 – *B. oleae* puparium.

1.4 Life cycle

Oviposition generally starts two days after mating: females select a site on the olive surface and they drill a hole with their ovipositor, laying one egg just under the fruit skin. By this way, they create a small oblong chamber (Tzanakakis, 2003). In field, the oviposition period begins around of the olive tree phenological phase called "pit hardening", empirical parameter that indicates the achievement of the receptivity of the drupe to the oviposition (Spanedda and Terrosi, 2008).
Females generally lay one egg per fruit (Figure 7A), except cases of high fly population when they lay more than one egg per olive (Tzanakakis, 2006). The number of eggs laid by a female in a lifespan is generally a few hundred, even if in some cases they joined maxima of 700-1200, as reported by Tzanakakis (1989): flies reared on olives can lay 13-40 eggs/female/day, with a maximum of 45-70, while they can lay if reared on artificial diet 13-21 eggs/female/day.
When the larva ecloses, it starts to feed upon the olive fruit pulp. The larval mouth hooks permit to the newly hatched larva to create galleries into the fruit, macerating the mesocarp before ingesting it and causing a big pulp loss (Fletcher, 1987; Daane and Johnson, 2010). The amount of pulp that goes lost during the larval feeding is about 50 mg per larva (Neuenschwander and Michelakis, 1978).

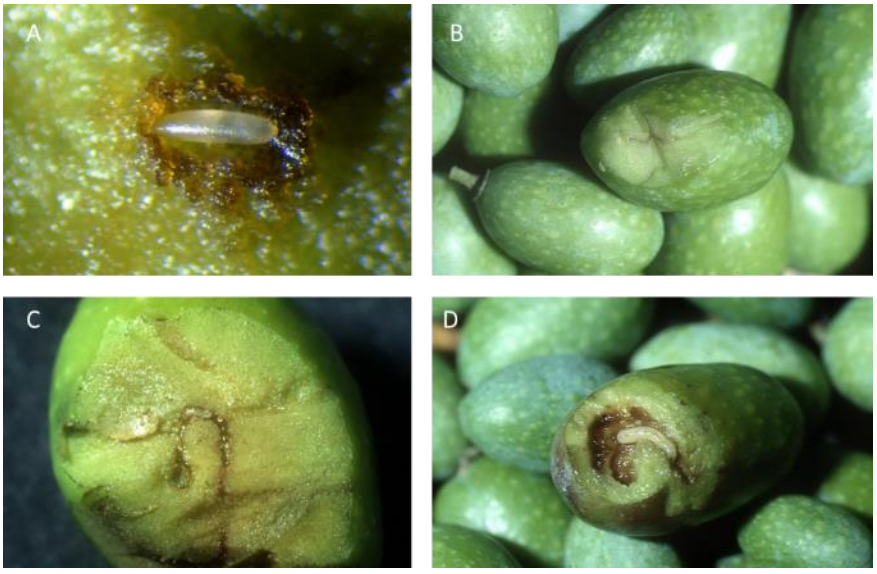

Figure 7 – *B. oleae* egg (A) and trophic tunnels of: 1st instar larva (B), 2nd instar larva (C) and fully grown larva (D).

At the end of the third instar the larva extends its tunnel to the epicarp and pupates inside the olive or into the soil. The duration of each stage depend on temperature, humidity and habitat conditions; many studies have been carried out in order to clarify the effect of temperature on *B. oleae* development. The optimum temperatures found by Tsitsipis (1977) were considered to be 27.5 °C for eggs, 25-27.5 °C for larvae and 22.5-25 °C for pupae. In a study carried out by Crovetti *et al*. (1983), the thermal constant and the thermal summation were defined to set up a development model for the olive fly. Moreover, according to Fletcher (1987), at optimal conditions (25 °C) eggs take 1-2 days to complete their development, as well as 7-8 days for the larval stage and 10-11 days for pupae. So that, the life cycle is completed in approximately 1 month. Meanwhile, the number of annual generations varies through regions and is influenced by temperature and environmental conditions, together with the availability of olives in the orchard (Daane and Johnson, 2010) going from 6-7 generations in southern Italy to 4 in northern regions (Delrio and Cavalloro, 1977).
Regarding its displacement, its habitat may be a single or group of host plants, so as the surrounding vegetation. Moreover, according to Fletcher (1989), *B. oleae* is a "between habitat" fruit fly. As a matter of fact, there are two types of fruit flies: "within habitat" and "between habitat". While the first type stays and lives in a stable olive orchard on a large quantity of

olive trees, the second one lacks of a stable place. This second type is also called the migratory or dispersive type. Flights of the dispersive type are characteristic of those flies that have failed in searching a good and appropriate area to be colonized (Fletcher, 1989).

1.5 Habits

Olive fly adults need to feed on various organic substances like honeydew, flower nectar, plant exudates, pollen, damaged fruits. Furthermore, they also feed on insect excreta, bird dung and bacteria spread on the phylloplane, (Sacchetti *et al*., 2008) as noticed in other fruit flies' species. In nature, the fly lays its egg only inside the mesocarp (pulp) of fruit of the genus *Olea*, including *O. europaea*, *O. verrucosa*, *O. chrysophylla* (Neuenschwander *et al.*, 1986; Tzanakanis, 2006) but also *O. europaea* ssp. *cuspidata* (Mkize *et al*., 2008). Thus, it is considered an oligophagous insect because its larvae feed and grow in this plant genus (Tzanakakis, 2006). On account of it, its larva macerates the mesocarp before ingesting it, causing a big pulp loss and modifying the organoleptic properties of olive oil (Tzanakakis and Tsitsipis, 1967).

The adult of both sexes are attracted to traps containing various ammonium salts, such as solutions in water, and they are attracted also to solutions or suspensions of protein hydrolysates, which act as feeding stimulants (Katsoyannos and Kouloussis, 2001). According to the colours that adults are attracted to, there are some differences between sexes. On account of a study carried out in 2001, males are more attracted by yellow and orange rather than females, that are well trapped with red and black (Katsoyannos and Kouloussis, 2001). According to shapes, the rounded or olive-shaped objects are the most attractive (Prokopy and Haniotakis, 1976).

Concerning mating, this activity occurs after few days from emergence, and this time is related to temperature, maturation rate, olives presence and ripening, females' follicles maturation. Mating usually takes place at dusk and at temperatures above 16 °C. Generally, there are two reproductive peaks, in spring and autumn, when the temperatures are around 23-26 °C and 75% of humidity. Recent research highlighted that *B. oleae* mating behaviour is very close to the performance showed by *Bactrocera dorsalis*: attraction, courtship and copulation (Benelli *et al.*, 2012). About light, mating success is related to low light intensities and it occurs the most during the latter part of the photophase (Loher and Zervas, 1979). During this phase, males vibrate wings against a set of hairs (pecten) located on the side of their abdomen, producing a typical sound hearable also by the

human ear (Féron, 1960). This wing vibration call seems to be very important for mating success (Benelli *et al.*, 2012). Along with this, males also produce a sexual pheromone that contains the same component of the sex pheromone produced by virgin females (Mazomenos 1989) and that would not negatively affect mating probabilities (Benelli *et al.*, 2013).
Furthermore, there are many others movement that males do in front of females and near other males too. For instance, they turn around during wing vibration, they clean their wings and their legs too. In contrast to males which are polygamous, females generally mate once in their life even if they can do it twice or three times (Tzanakakis *et al.*, 1968). On the other hand, a particular behaviour that concern females is what they do at the end of the oviposition process; females suck the juice that flows out slowly from the oviposition hole, when the ovipositor is still inside it. Secondly, they retract their ovipositor and regurgitate the juice around the same hole. According to Girolami (1981) and Cirio (1971), this juice acts as a deterrent to other oviposition.

2. Association with bacteria

The relationships between insects and microorganisms range from clear mutualism to relationships involving unbalanced benefits, or costs to one member, up to pathogenesis (Ishikawa, 2003; Dillon *et al*., 2004). Moreover, insect symbioses can vary from temporary associations to long-life obligate partnerships and from external, loose coalitions to very close alliances (Ishikawa, 2003). The microorganisms involved can be found in the environment, growing outside the insect's body, or harboured within the body cavity in specialized cells or organs (extracellular or intracellular endosymbionts). These microorganisms could be transmitted through successive generations, typically via vertical transmission from mother to progeny (maternal inheritance) (Moran, 2006). The manifold and intricate functions played by the wide assortment of microorganisms have not been deeply studied, and only some metabolic interactions are fully understood (Douglas, 2015). Regardless, the crucial roles played by symbionts in the survival and evolution of their insect partners have been repeatedly demonstrated, and different mechanisms of transmission through host populations and generations have evolved (Ishikawa, 2003).
The non-pathogenic bacterial symbionts of insects have been classified as ranging from primary, ancient obligate symbionts that are restricted to specialized cells (bacteriomes), necessary for the host, to secondary, recent facultative symbionts that are located in insect organs and non-essential for

insect survival (Ishikawa, 2003; Dale *et al*., 2006). The transmission of primary symbionts (P-symbiont) in plant-feeding insects has been investigated in detail in aphids (Douglas *et al*., 2003; Baumann, 2005), various sucking insects (Kaltenpoth *et al*., 2009; Prado *et al*., 2009; Szklarzewicz *et al*., 2017) and beetles (Nardon, 2006; Wang *et al*., 2017). P-symbionts are transferred vertically to offspring through contamination of the egg surface, deposition of bacterial capsules on eggs, consumption of the mother's excrement or through transovarial transmission (Szklarzewicz *et al*., 2017). Maternal inheritance is also the typical transmission route for secondary symbionts, although there is substantial evidence of horizontal transmission as well as rare paternal transmission (Moran *et al*., 2006; Peccoud *et al*., 2014).

Similar to sucking insects, Tephritid fruit flies display many types of symbiotic associations involving both intracellular (e.g. Wolbachia), and extracellular symbionts. Lauzon (2003) critically reviewed this topic, commenting on known features and highlighting important issues with possible practical consequences for insect pest control.

So that, the relationship between the olive fruit fly *B. oleae* and his associated bacteria represents one of the most questionable issues in this research area. Several studies referred in this chapter highlighted how bacteria seem to be necessary for this Tephritid's fitness, emphasizing that epiphytic bacteria and endosymbionts play a very relevant role in the whole *B. oleae*'s life, in both larvae and adults. Thus, this topic has been considered very important to understand its behaviour and its life cycle.

Regarding epiphytic bacteria, several ecological researches confirmed a very close relationship between them and the olive fly. As a matter of fact, this Tephritid seems to look for all these bacteria in the field to use them as a food source (Sacchetti *et al.,* 2008), as previously evidenced in *B. tryoni* (Drew *et al*., 1983); to reach this goal, the olive fruit fly follows bacterial volatile compounds, which could be used, for this reason, as natural attractants, as demonstrated for *Pseudomonas putida*, an epiphytic bacterium commonly associated to the olive fruit fly (Sacchetti *et al.,* 2007 and 2008; Liscia *et al.,* 2013).

However, in this Tephritid, it has long been known that there are many symbiotic bacteria associated with *B. oleae* living in special structures of its alimentary canal (Petri, 1909). These symbionts are particularly abundant in a special oesophageal diverticulum, called oesophageal bulb, but they have been found also inside the digestive tract, in the diverticula which communicate with the rectum lumen and, in females, near the ovipositor (Figure 8).

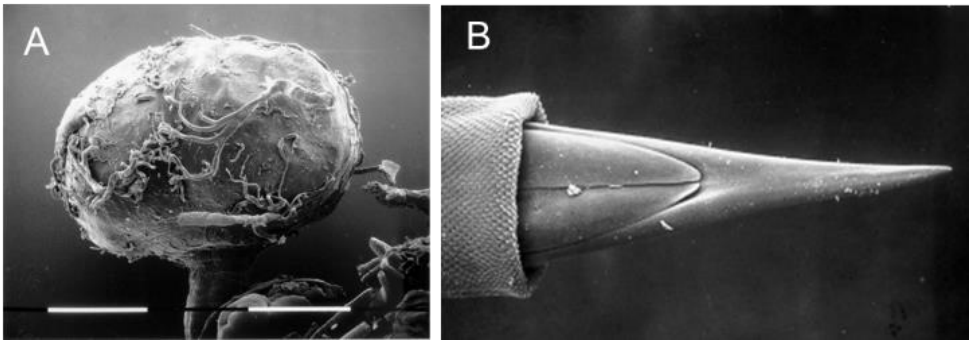

Figure 8 – Oesophageal bulb (A) and ovipositor (B) of *B. oleae* adults (Scanning Electron Microscope pictures taken by A. Belcari).

According to the literature, these bacteria are smeared on the egg surface during the oviposition (Figure 9), so that they can be transferred vertically to the young larva (Petri, 1909; Hagen, 1966; Yamvrias *et al.*, 1970; Girolami, 1973; Mazzini and Vita, 1981; Sacchetti *et al*., 2008; Estes *et al*., 2009).
Petri has been the first, at the beginning of the '900, who described many "long-shaped bodies" inside *B. oleae*'s midgut, both in larvae and adults. He highlighted that the bacterial mass wasn't lost during the metamorphosis and that these masses augmented during the olive fly life cycle. Besides, he also found bacteria inside some *B. oleae* adults' oesophageal bulbs, and he wrongly thought it was *Pseudomonas savastanoi*, the bacterium agent of the olive knot, known at that time as *Bacterium savastanoi*. He justified its presence inside the olive fly by saying that this bacterium should help the Tephritid during the digestion (Petri, 1909).
Later on, Mazzini and Vita (1981) investigated how symbionts could be transmitted in the olive fly. As shown in the previous studies, they found bacteria inside the midgut, the oesophageal bulb and the ovipositor. Moreover, thanks to the electronic microscopy, they described these organs and, on account of it, they could study also the associated bacteria. They stated that these bacteria were not damaged by gastric juices and that these masses were more abundant in the midgut instead of the last part of the digestive tract. However, they observed many bacterial masses (between 60 and 150) in many finger-like processes the fly has near the ovipositor, which are joined with the anal tract (Mazzini and Vita, 1981).
Besides, during the 20th century other studies investigated the different bacterial species that were present inside the olive fruit fly and their role as

symbionts; however, they were carried out with traditional methods and they could not clear so much (Hellmuth, 1956; Tsiropoulos, 1983).

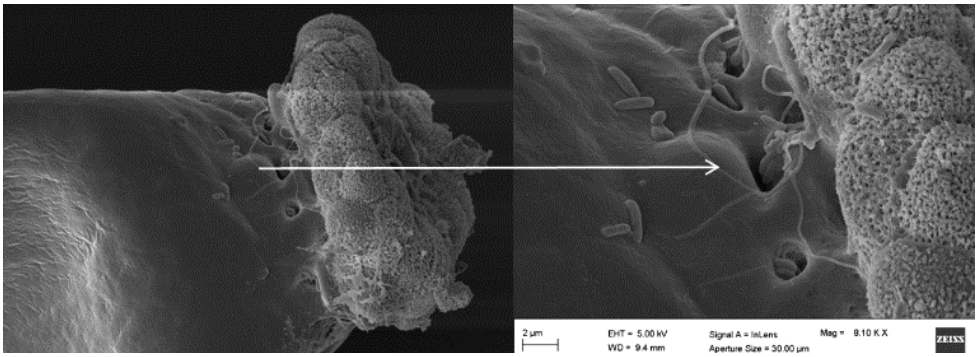

Figure 9 – *B. oleae* egg. The image shows how bacteria are smeared near the micropylar area (SEM pictures taken by S. Ruschioni).

2.1 *Candidatus* Erwinia dacicola

In 2005, thanks to new biological molecular techniques as PCR analysis and DNA extraction, a new bacterium was identified; this bacterium was called *Candidatus* Erwinia dacicola, and even if it couldn't have been cultivated at that time, his DNA was the most abundant among other bacterial DNAs and it was supposed to be the major *B. oleae* obliged endosymbiont (Capuzzo *et al.*, 2005). It has been recently sequenced and named *Erwinia dacicola* Oroville (Estes *et al*., 2018); however, in this thesis, it is mentioned with its previous name for convenience reasons. Its presence has been confirmed many times; it belongs to the Proteobacteria group, family *Enterobacteriaceae*, and it is considered a persistent and resident endosymbiont for *B. oleae* (Estes *et al.,* 2012). It is vertically transmitted, generation after generation, from the female to the egg, and it has been found in every flies' stage, mainly in the adult one (Estes *et al*., 2009; Estes *et al.,* 2012). It exists in two different haplotypes or "lineages", called htA and htB (Savio *et al.,* 2012); it lives and multiplies itself inside the olive fruit flies' oesophageal bulb, but we can found it in other organs of the adult, as the midgut and the last digestive tract near the ovipositor (Capuzzo *et al.,* 2005). Furthermore, it was shown that *Ca*. E. dacicola switches from an intracellular existence to an extracellular one during the host insect development, residing intracellularly within larval midgut cells and extracellularly in the adult foregut; in larvae, bacterial cells are found

within host cells, instead of inside the digestive system lumen, as in the adult oesophageal bulb (Estes *et al.*, 2009). Hence, this transition could be essential to endosymbiont survival in a holometabolous host. Moreover, the same study confirmed the presence of the symbiont in another digestive tract full of bacteria, the crop system, that was already generally described by Mazzini and Vita (1981) many years before.
However, the endosymbiont seems to remain strictly related to the olive tree, since its presence has never been confirmed in laboratory reared flies (Sacchetti *et al.*, 2008; Estes *et al*., 2009 and 2012).

2.2 The role of *Ca.* E. dacicola

Several studies report that bacteria could be useful for their host in many ways (Lauzon, 2003); for instance, they can be a source of different compounds such as amino acids (Ben-Yosef *et al.*, 2010), substances necessary for fertility and for flies' fitness in general, or vitamins and growth factors which are not common to be found in *B. oleae*'s diet (Estes *et al.*, 2009). What is more, bacteria can help flies in metabolize and stock nitrogen (Ben-Yosef *et al.*, 2010). Along with this, owing to the role played by bacteria in the *B. oleae* development, *Ca*. E. dacicola could represent an important source of proteins which help this Tephritid to improve its fitness; as a matter of fact, intestinal microbiota and endosymbionts can strongly sustain female fecundity, supplying essential amino acids and useful nutrients (Ben-Yosef *et al.*, 2010). *Ca*. E. dacicola positively affects *B. oleae* population fitness, enhancing female fertility, pheromones production and oviposition success (Estes *et al.*, 2012). Recent studies have shown some differences in the endosymbiont quantity presence, comparing olive-reared flies with artificial diet reared ones. In addition, owing to the chemical compounds presence in the artificial diet, this endosymbiont disappears, causing physiological and behavioural changes (Estes *et al.*, 2012).
The endosymbiont keeps the olive fruit fly disease-free and, sometimes, it can detox some defence plant compounds (Lauzon, 2003). More recent studies highlighted that *B. oleae* larvae can survive easier in unripe olives, owing to the *Ca*. E. dacicola presence; in fact, larvae strictly depend on their gut bacterial population in order to develop in unripe olives, even if they are able to mature on their own without bacteria in ripe fruits (Ben-Yosef *et al.*, 2015). According to this, the endosymbiont would be able to deactivate some compounds like oleuropein, which has never been confirmed to be toxic to *B. oleae* but which acts indirectly as an anti-nutrient, permitting the

larval development and letting flies take a higher nitrogen level. In fact, oleuropein may inactivate enzymes or reduce the digestibility of dietary protein, impeding larvae from acquiring nutrients (Ben-Yosef *et al.,* 2015).
More recent researches highlighted the possibility of restoring the endosymbiont presence in reared flies. As showed in a study carried out to evaluate *Ca*. E. dacicola effects on mating success by crossing wild adults with laboratory reared ones, it was hypothesized that the symbiont could be horizontally transmitted, from wild flies to lab strains, even if in a little percentage (Estes *et al.,* 2014). Providing that, this hypothesis let scientists believe that horizontal bacterial transmission was possible, in order to create a stronger and more competitive olive fly strain so as to improve the Sterile Insect Technique, aiming to give another frontier for *B. oleae* biological control.

3. Economic importance and control strategies

As mentioned above, the olive fly is considered one of the major pest among the carpophagous species for olive crops. Recently in 2014, it was recorded a production loss of 37% in Italy, compared to 2013, with damage peaks of 45% in two regions of excellence such as Umbria and Tuscany (ISMEA), since newspapers called it that "the black year for olive oil production: one olive oil bottle out of three has been drank by an awful fly" (Anonymous, 2018).
In cases of heavy infestation, in addition to the product loss (due to the trophic activity of larvae, of about 10-12%), in oil cultivars could happen a reduction of resistance to fruit detachment, together with alterations of the physical-chemical parameters of the olives and a drastic worsening of the qualitative and organoleptic characteristics of the final product, mainly caused by oxidation of the fat part present in the mesocarp; this usually lead to obtain low quantity and quality of olive oils (Caravaca *et al.,* 2008).
On the basis of these losses, both empirical and mathematical-statistical thresholds have been defined, taking into account various parameters, among which the average quantity of olive / ha production, the cultivar, the production value per unit, the production costs per unit and the costs of phytoiatric measures are considered at first place.
In any case, the intervention threshold for cover sprays against eggs and young larvae is still today around values of 10-15% of infestation. Meanwhile, in the table olive varieties there is an obvious downgrading of the commercial value of the drupes. In addition, the olive fly infestation generally causes an early fall of the infested fruits (Crovetti *et al.,* 1996).

Organophosphates and neonicotinoids are the only chemical insecticides that are currently allowed to control *B. oleae* eggs and larvae in Italy. The first products have translaminar action and among them the two most used active ingredients are dimethoate and fosmet, the former widespread used since its considerable effectiveness, accompanied by low fat solubility and therefore low residual content in olive oil; among the systemic neonicotinoids the most used and the only allowed is imidacloprid, to be used according to the current regulations of integrated pest management production (Anonymous, 2018).
However, since the large use of these products in the past, the related arise of resistance problems (Skouras *et al.*, 2007) and the recent need to use low impact control strategies and integrated pest management regulated by the "DM 22/01/2014" law (Anonymous, 2014), new techniques based on the reduction of adult population density have been recently introduced (Margaritopoulos *et al.*, 2008). More specifically, several technical measures have been introduced to increase and improve biological control of the olive fly: yellow coloured traps (Katsoyannos, 1989) for monitoring, baits with protein hydrolysates (Montiel-Bueno and Jones, 2002), ammonia salts (Katsoyannos and Koloussis, 2001), and pheromones (Daane and Johnson, 2010) to increase mass trapping and lure-and-kill strategies. Moreover, the efficacy of copper products has been also highlighted in several studies. As a matter of fact, copper is supposed to have an antimicrobial effect and a symbionticide activity against useful bacteria impeding larval growth (Tzanakakis, 1985; Belcari and Bobbio, 1999; Caleca and Rizzo, 2006) causing the lack of those bacterial compounds that make the fruit attractive to the fly for the oviposition. In addition, it also acts as a deterrent for oviposition (Prophetou–Athanasiadou *et al.*, 1991). Although the large availability of different control strategies, in some year characterised by high humidity and mild summer temperature, olive fly populations can quickly increase causing severe damage. Therefore, other integrated pest management methods still need to be investigated or enhanced, as the Sterile Insect Technique.

4. The Sterile Insect Technique

The Sterile Insect Technique or SIT represents a relevant component of area-wide integrated pest management control methods. According to the International Plant Protection Convention (IPPC) this technique's definition is: "a method of pest control using area-wide inundative releases of sterile insects to reduce fertility of a field population of the same species"

(Klassen, 2005). As a matter of fact, it consists in field releasing of gamma-irradiated sterile males which are able to compete for females with wild males; in this way, females lay unfertilized eggs (birth control method). SIT is currently used in many countries to control several pests, including many fruit flies (Mediterranean fruit fly, Mexican fruit fly, oriental fruit fly, melon fly), tsetse fly, screwworm, moths (codling moth, pink bollworm, false codling moth, cactus moth, and the Australian painted apple moth) and mosquitoes (IAEA website). Nowadays SIT is considered as a very effective and cost-convenient technique for the mentioned species. As a matter of fact, many studies had been carried out to evaluate the effectiveness of this control method against fruit flies as *Ceratitis capitata* (Hamden *et al.,* 2013) *Anastrepha ludens* (Sanchez-Rosario *et al.,* 2017) *Anastrepha obliqua* (Aceituno-Medina *et al.,* 2017) *Bactrocera tryoni* (Khan *et al.,* 2017). Regarding *B. oleae*, pioneer trials have been carried out also against this fly, even if without consistent results (Economopoulos and Zervas, 1982). More recently, SIT applications for the olive fly has been re-considered and, more specifically, two main issues have been pointed out. At first, the possibility of creating a Genetic Sexing Strain using molecular techniques was evaluated (Koukidou *et al*., 2006; Ant *et al.,* 2012); moreover, the importance of *Ca*. E. dacicola lead scientists to go in depth with the manipulation of the endosymbiosis for SIT applications (Estes *et al.,* 2012); as a matter of fact, since *Ca*. E dacicola positively affect this Tephritid physiology and his mating success, it would be useful to guarantee the endosymbiont presence, also in laboratory-reared adults. Regarding *B. oleae* these arguments are crucial to be pointed out and represent critical points for a successful SIT intervention, since mass rearing is strictly related to antibiotics (Estes *et al.,* 2012; Ben-Yosef *et al.,* 2014) causing the endosymbiont loss and, as a consequence, lower longevity and efficiency of reared males. Unfortunately, research seems to be still sceptical about this issue and further studies should be carried out to investigate better these purposes.

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Sitography

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AIMS

This thesis focuses on the relationships between the olive fly (OLF) *B. oleae* and symbiotic bacteria, especially the endosymbiont *Ca.* E. dacicola, in order to improve the basic knowledge about OLF biology and behaviour, as they relate to bacterial associations. Furthermore, this dissertation wanted to investigate the development of new biological control methods through OLF-bacteria study, exploring the possibility of SIT applications. Specifically, during the last three years, my research has aimed at the following goals:

1. To determine if *Ca.* E. dacicola could be transferred horizontally from adults of a wild population to those of a lab reared strain;
2. To evaluate the antibacterial effects of copper, at two different concentrations (5% and 20%), and propolis on the presence of the symbiont *Ca.* E. dacicola in a wild population;
3. To evaluate the effect of small-scale rearing procedures, such as the use of antimicrobials as propionic acid solution and a sodium hypochlorite + Triton X mixture (1:1) on the presence of bacteria on eggs laid by wild flies;
4. To evaluate the natural antibacterial action of three different types of honey (chestnut, orange, and acacia honey) on the presence of the symbiont *Ca.* E. dacicola in a wild population;
5. To evaluate the effect of irradiation on wild adult males, both in terms of endosymbiont presence and mating success;
6. To cultivate in vitro *Ca.* E. dacicola.

Data are reported as in preparation or accepted papers. My contribution to each paper is specified. Moreover, a section “Other data” will follows, containing those experiments that totally concerned the Ph.D. program and objectives but that did not lead to noteworthy results.

Chapter One

Horizontal transfer and finalization of a reliable detection method for the olive fruit fly endosymbiont, *Ca.* E. dacicola

	Idea	Data collection	Molecular analyses	Data analysis	Manuscript preparation
My contribution	*	*	*	*	*

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Horizontal transfer and finalization of a reliable detection method for the olive fruit fly endosymbiont, *Candidatus* Erwinia dacicola

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Abstract

Background: The olive fly, *Bactrocera oleae,* is the most important insect pest in olive production, causing economic damage to olive crops worldwide. In addition to extensive research on *B. oleae* control methods, scientists have devoted much effort in the last century to understanding olive fly endosymbiosis with a bacterium eventually identified as *Candidatus* Erwinia dacicola. This bacterium plays a relevant role in olive fly fitness. It is vertically transmitted, and it benefits both larvae and adults in wild populations; however, the endosymbiont is not present in lab colonies, probably due to the antibiotics and preservatives required for the preparation of artificial diets. Endosymbiont transfer from wild *B. oleae* populations to laboratory-reared ones allows olive fly mass-rearing, thus producing more competitive flies for future Sterile Insect Technique (SIT) applications.

Results: We tested the hypothesis that *Ca*. E. dacicola might be transmitted from wild, naturally symbiotic adults to laboratory-reared flies. Several trials have been performed with different contamination sources of *Ca*. E. dacicola, such as ripe olives and gelled water contaminated by wild flies, wax domes containing eggs laid by wild females, cages dirtied by faeces dropped by wild flies and matings between lab and wild adults. PCR-DGGE, performed with the primer set 63F-GC/518R, demonstrated that the transfer of the endosymbiont from wild flies to lab-reared ones occurred only in the case of cohabitation.

Conclusions: Cohabitation of symbiotic wild flies and non-symbiotic lab flies allows the transfer of *Ca*. E. dacicola through adults. Moreover, PCR-DGGE performed with the primer set 63F-GC/518R was shown to be a consistent method for screening *Ca*. E. dacicola, also showing the potential to distinguish between the two haplotypes (htA and htB). This study represents the first successful attempt at horizontal transfer of *Ca*. E. dacicola and the first step in acquiring a better understanding of the endosymbiont physiology and its relationship with the olive fly. Our research also represents a starting point for the development of a laboratory symbiotic olive fly colony, improving perspectives for future applications of the Sterile Insect Technique.

Keywords: *Bactrocera oleae*, endosymbiont, oesophageal bulb, ARDRA, DGGE, SIT

Background

Relationships between fruit flies (Diptera: Tephritidae) and microorganisms, especially bacteria, have been studied for a long time. Much research has focused on the biology and behaviour of many of these flies, but their symbiotic associations have been less investigated. In particular, the role that these microorganisms could play in fly biology, physiology and behaviour has not been well studied [1, 2]. One of the most questionable issues in this research area, which scientists are still working on, is the relationship between the olive fruit fly *Bactrocera oleae* (Rossi) and its associated bacteria [3, 4, 5, 6]. In particular, symbiotic bacteria seem to be necessary for this Tephritid's fitness [7, 8]. Furthermore, recent studies have shown that symbiosis plays a very relevant role in the *B. oleae*'s lifespan [9, 10]. Thus, symbiosis in the olive fruit fly is considered to be very important to understand its behaviour and its life cycle.

At the beginning of the 20th century, Petri [11] was the first scientist who described the bacteria inside the *B. oleae* gut, both in larvae and adults; later, other scientists tried to better define this endosymbiosis [5, 8, 12, 13]. More recently, thanks to the advent of biological molecular techniques, such as PCR amplification and sequencing, the *B. oleae* endosymbiont was identified as *Candidatus* Erwinia dacicola [14]. It was found only in wild *B. oleae* flies, and even if it could not be cultivated, it was supposed to be more abundant than other bacteria. It was therefore assumed to be a tightly associated endosymbiont of the olive fruit fly [15]. *Ca*. E. dacicola lives and multiplies inside a small organ of olive fruit flies, which Petri first described as a "cephalic vesicle" or "pharyngeal gland" [11]. In more recent studies, this organ is referred to as the "oesophageal bulb" [6, 14, 16]. Despite this, the symbiont has been detected in other adult organs, including the gut and the last digestive tract near the ovipositor [14, 15].

Ca. E. dacicola has been assigned to the Enterobacteriaceae family within the γ-Proteobacteria group [14] and is considered a P-symbiont (persistent) for *B. oleae.* It is vertically transmitted through generations, from the female to the egg, and it has been found in every stage of the fly lifespan, particularly in the adult one. In addition, it was shown that *Ca*. E. dacicola seems to switch from an intracellular existence to an extracellular one during the host insect development, since it lives intracellularly within cells of the larval midgut caeca and extracellularly in the adult gut [15]. Recent studies have highlighted the fact that the larvae can develop in unripe olives, owing to the presence of *Ca*. E. dacicola presence [17]. According to this, the endosymbiont strictly affects the larval survival of unripe olives. Larvae,

thanks to *Ca.* E. dacicola, are able to overcome the effects of some compounds such as oleuropein, which seems to be detrimental, acting as an anti-nutrient and allowing both larval development and a higher nitrogen level assumption. Along with this, oleuropein may inactivate enzymes or reduce the digestibility of dietary proteins, preventing larvae from assuming nutrients [17]. The symbiont seems to be strictly related to the olive tree agro-ecosystem, since its presence has never been confirmed in laboratory-reared flies [6, 10] with the exception of a recent research in which the bacterium was found in few specimens of a lab hybrid population [18]. *B. oleae* is a fruit fly that is difficult to rear artificially; however, long lasting research has demonstrated that there are still several mass rearing difficulties, including high costs and labour-intensive procedures [19]. Lab colonies are usually obtained from lab-adapted wild populations. Flies often will not easily oviposit in artificial rearing devices such as wax domes and tend not to develop well on a cellulose-based artificial diet, two essential aspects of the mass rearing technique [20]. Previously, when *B. oleae* was reared through these procedures for a long time, several genetic and biological changes appeared [21] as well as behavioural modifications [22]. This suggests that an endosymbiont lacking in lab-reared flies could be involved in all these rearing issues. The absence of *Ca*. E. dacicola in lab-reared colonies could also be caused by the widespread use of antibiotics in the artificial diet; importantly, recent studies have demonstrated that *B. oleae* can be reared without antibiotics [23]. In this way, the endosymbiont might not be lost. To improve mass-rearing and to produce more competitive flies, it would be favourable to transfer the endosymbiont from wild *B. oleae* populations into lab reared flies in order to start up Sterile Insect Technique (SIT) field applications. This would allow for the release of sterile and more competitive males due to the endosymbiont *Ca*. E. dacicola. This would likely be a more effective and highly sustainable method to reduce *B. oleae* field populations. Moreover, recent research has highlighted the endosymbiont presence in reared flies, demonstrating that the endosymbiont may have entered the lab colony during cohabitation with wild flies [18]. Along with horizontal transfer, it is important to determine the precision and reliability of the *Ca*. E. dacicola DNA detection procedure. Since 2005, endosymbiont presence has been detected many times in wild flies, both in larvae and adults. However, its DNA has never been confirmed using the same set of primers [6, 14, 15, 24, 25]. Based on these findings, we tested the hypothesis that *Ca*. E. dacicola horizontal transfer can occur from a wild *B. oleae* population to adults of an artificially reared non-symbiotic colony. A second aim of this work was to find the easiest, fastest

and most reliable method to detect *Ca*. E. dacicola DNA in *B. oleae* oesophageal bulb samples.

Methods

Insects – Wild flies were obtained from infested olives harvested in several Tuscan olive orchards, during October - December 2015. Olives were kept in open boxes to maintain their freshness and to avoid fungi or mildew growth. A few days after harvesting, pupae were collected and transferred into plastic cages (BugDorm®, MegaView Science, Taiwan). Adults were supplied with sugar and water and kept at room temperature (18-20 °C). Artificially reared *B. oleae* adults were obtained from a laboratory-adapted colony (Israel hybrid, IAEA, Seibersdorf, Vienna, Austria). Larvae were reared on a cellulose-based diet [26], while adults were reared in plastic cages (BugDorm®) and kept in a conditioned rearing room at 25±2 °C, RH 60±10%, and a 16:8 L:D photoperiod. Flies were supplied with water in a 30 mL plastic container with a sterile sponge strip acting as a wick and with a standard diet consisting of sugar, hydrolysed enzymatic yeast (ICN Biomedicals) and egg yolk (40:10:3).

Experimental design – Trials were started in February 2016. Since the goal was to transfer *Ca*. E. dacicola from a wild *B. oleae* population to a lab-reared one, the experiment was divided in two phases: a "contamination phase," during which wild flies had time to contaminate different substrates, and an "acquisition phase," in which lab flies were allowed to contact the substrates that had putatively been contaminated by *Ca*. E. dacicola. Before starting the experiment, the presence of *Ca*. E. dacicola in wild flies was confirmed by sequencing, as described below.

Contamination phase – Six treatments were tested as contamination sources: olives, gelled water, wax domes, wild faeces and cohabitation (lab females and wild males; lab males and wild females). The contamination sources are described below:

i) Olives – Freshly harvested ripe olives were given to 2-month-old wild adult flies to allow contamination with *Ca*. E. dacicola. Three Petri dishes with 30 olives each were put into a cage with more than 500 wild adults one week before the acquisition phase.
ii) Gelled water – Gelled water was given to 2-month-old wild adult flies to be contaminated by *Ca*. E. dacicola. Three Petri dishes with gelled water (8.35 g/L Gelcarin®, Duchefa Biochemie, The Netherlands) were put into

a cage with more than 500 wild adults three days before the acquisition phase.

iii) Wax domes – Wax domes were used to collect eggs laid by wild flies; the domes were washed with a 2% sodium hypochlorite solution, rinsed twice in distilled sterile water and offered to 2-month-old wild adult flies to allow the females to oviposit. The resulting eggs were expected to be contaminated by *Ca*. E. dacicola based on previous research [27], and this was confirmed by sequencing. Three oviposition wax domes were placed into a cage with more than 500 adults two days before the acquisition phase.

iv) Wild faeces – Wild faeces were the fourth substrate used as a possible *Ca*. E. dacicola contamination source. One month before starting the acquisition phase, 100 wild flies ca. were put inside the cages assigned for the next phase (as described below) in order to contaminate the cage with their faeces.

v) Cohabitation between lab females x wild males – Cohabitation was used as a horizontal transfer method for *Ca*. E. dacicola, as described by Estes et al. [23]. The setup is described below.

vi) Cohabitation between lab males x wild females – The setup for this cohabitation method is described below.

Acquisition phase – Except for the faeces treatment, the next phase was started up in different cages (plastic boxes 2 L volume with a side closed by a nylon fine net, supplied with water and sugar) and set up as described below.

i) Olives - Three Petri dishes with olives putatively contaminated by *Ca*. E. dacicola were inserted into the plastic boxes (3 boxes, one dish each box) containing 25 males and 25 females newly emerged lab flies (younger than 24 h).

ii) Gelled water - Three Petri dishes with gelled water putatively contaminated by *Ca*. E. dacicola were inserted into plastic boxes (3 boxes, one dish each box) containing 25 males and 25 females newly emerged lab flies (younger than 24 h).

iii) Wax domes - Wax domes were opened and inserted on the bottom of the box (one each box) to let the lab flies get directly in contact with the eggs laid by the wild flies. The plastic boxes contained 25 males and 25 females newly emerged lab flies (younger than 24 h).

iv) Faeces – The 100 wild adults were removed from the dirty plastic boxes and 25 males and 25 females of newly emerged flies (younger than 24 h) were transferred to each.

v) Cohabitation between lab females x wild males (labF x wildM) – Twenty-five newly emerged female flies (younger than 24 h) and 25 wild male flies of the same age were transferred into the plastic boxes.
vi) Cohabitation between lab males x wild females (labM x wildF) – Twenty-five newly emerged male lab flies (younger than 24 h) + 25 wild female flies of the same age were transferred into plastic boxes.

For each treatment, the acquisition phase lasted 15 days. Each treatment was replicated 3 times (6 trials with olives, gelled water, wax domes, faeces, labF x wildM, labM x wildF = 18 boxes, with a total of 900 tested flies). Boxes were arranged randomly on 4 shelves and moved daily to avoid any lighting bias. The setup of the overall experiment is summarized in Table 1.

Insect dissections – After the acquisition phase, 30 flies were taken from each treatment (5 males and 5 females per cage for all three replicates), killed by freezing at -20 °C for 15 min and dissected. The dissection procedure was performed entirely under a laminar flow hood. Flies were first washed with a 2% sodium hypochlorite solution and then rinsed twice in distilled sterile water. Second, each adult's head was cut and opened under a stereoscopic microscope with sterile tools, and each oesophageal bulb was extracted. Sex, sample number and bulb aspect (transparent or milky) were noted. Finally, each bulb was put inside a 1.5 mL tube for DNA extraction.

Culture-independent microbiological analyses – Bacterial DNA from the oesophageal bulbs, faeces or sponge samples was extracted using 50 µL of InstaGene Matrix (BioRad Laboratories, Hertfordshire, UK) according to the manufacturer's instructions. Bacterial DNA extracted from flies was obtained only from the oesophageal bulb and not from any other parts of the fly. Faeces were collected from the inner side of the cage top by rubbing sterile cotton on approximately 30 cm length. For the bacterial DNA extraction, the sterile cotton was treated as the oesophageal bulbs. Sponges were removed from the cages and transferred under laminar flow hood. Then, a small piece was removed with a scalpel and treated like the bulbs and faeces for the bacterial DNA extraction. The extracted DNA was stored at -20 °C until PCR amplification. A preliminary PCR analysis was completed with EdF1 [15] and EdEnRev [10] primers designed to selectively amplify the 16S rRNA gene of *Ca*. E. dacicola. PCR-reactions were carried out using a T100 Thermal Cycler (Bio-Rad Laboratories, Hertfordshire, UK) in 25 µl volumes containing 1X Flexi PCR buffer

(Promega, Madison, WI), 1.5 mM $MgCl_2$, 250 μM deoxynucleotide triphosphates (dNTPs), 400 nM of each primer, and 1U GoTaq®Flexi DNA polymerase (Promega).

Table 1 - Setup of the horizontal transfer experiment

Substrates and other contamination sources of *Ca*. E. dacicola	Contamination phase	Acquisition phase
Olives	Exposed to wild flies for 7 days	25 lab males + 25 lab females per cage exposed for 15 days to 30 putatively contaminated olives
Gelled water	Exposed to wild flies for 3 days	25 lab males + 25 lab females per cage exposed for 15 days to putatively contaminated gelled water
Wax domes	Exposed to wild flies for 24 hours	25 lab males + 25 lab females per cage exposed for 15 days to a wax dome bearing eggs laid by wild females
Faeces	Approximately 100 wild symbiotic flies per cage for 30 days	25 lab males + 25 lab females per cage exposed for 15 days to faeces dropped by wild flies
labF x wildM	Wild naturally symbiotic males	25 wild males + 25 lab females in cohabitation for 15 days
labM x wildF	Wild naturally symbiotic females	25 lab males + 25 wild females in cohabitation for 15 days

Amplifications were performed under the following conditions: an initial denaturation of 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 45 s, and a final extension of 72 °C for 10 min. After PCR, the amplified products were verified by agarose gel electrophoresis (1.5% w/v), and the presumed presence/absence of *Ca*. E. dacicola in the specimens was scored based on the presence/absence of the targeted amplicon.
Additional primer sets were used in order to clarify the obtained results. For each primer set, the PCR reaction was carried out as described above. Ed1F was also paired with 1507R [28] to generate a nearly complete (1,300 bp) 16S rDNA fragment used for the subsequent screening of flies by ribosomal DNA restriction analysis (ARDRA). The 16S rDNA PCR products were digested separately with the restriction enzymes *Pst*I and *Cfo*I (Roche Diagnostics Ltd., Basel, Switzerland) as recommended by the manufacturer. The restriction fragments were separated by agarose gel electrophoresis (2% w/v), creating a specific restriction pattern for *Ca*. E. dacicola that distinguishes it from the other Enterobacteriaceae. The primer sets 986F-GC and 1401R [29] and 63F-GC and 518R [30] were used for the denaturing gradient gel electrophoresis (DGGE) analysis. PCR products were first verified by agarose gel electrophoresis (1.2% w/v) and successively loaded onto a polyacrylamide gel (40% acrylamide/bis 37.5:1; Serva Electrophoresis GmbH, Germany) containing a linear chemical denaturant gradient obtained with a 100% denaturant solution consisting of 40% v/v deionized formamide and 7 M urea. DGGE gels were run for 17 h at 60 °C and a constant voltage (75 V), using the Dcode DGGE System (Bio-Rad). After the electrophoresis gels were stained with SYBR®Gold (Molecular Probes, Eugene, OR) diluted 1:1,000 in 1X TAE buffer, the images were digitally captured under UV light (λ = 302 nm) using the ChemiDoc XRS apparatus (Bio-Rad). DGGE rDNA fragments from *Ca*. E. dacicola showed a distinct migration behaviour and could be easily distinguished from fragments derived from other oesophageal bulb-associated bacteria. PCR amplification and DGGE were also performed on DNA extracted from wild fly faeces and from sponges used as water wicks in each cage.

Sequence analysis - The middle portion of several DGGE-bands was aseptically excised and placed in 30 µL of distilled water. The PCR products were eluted from the gel through freezing and thawing and were subsequently re-amplified as described above and subjected to direct sequencing by Genechron (Ylichron, ENEA, Italy; http://www.genechron.it). Another subset of PCR products, obtained with

the Ed1F and 1507R primers, was sequenced in both directions to verify the identity of *Ca*. E. dacicola in the oesophageal bulb specimens. The 16S rDNA sequence chromatograms were edited using Chromas Lite software (v2.1.1; Technelysium Pty, Ltd. http://www.technelysium.com.au/chromas-lite.htm) to verify the absence of ambiguous peaks and convert them to a FASTA format. The DECIPHER's Find Chimera web tool (http://decipher.cec.wisc.edu) was used to uncover chimaeras hidden in the 16S rDNA sequences. The web-based BLAST tool available at the NCBI website (http://www.ncbi.nlm.nih.gov) was used to find taxonomically closely related nucleotide sequences. The nucleotide sequences identified in this study were deposited in the GenBank database under accession numbers MF095700 to MF095734.

Results

Screening - As a result, PCR amplifications carried out with the primers EdF1 and EdEnRev highlighted a product with an expected size. A total of 17 of the 30 samples of wax domes, 26 of the 30 olive samples, 0 of the 30 gelled water samples, 16 of the 30 faeces treatment conditions, 16 of the 30 samples of labF x wildM, and 13 of the 30 samples of labM x wildF were found to be positive through PCR. As a double check, samples that were positive for the EdF1/EdEnRev amplification were screened by ARDRA. PCR products from both the wild flies and the cohabitation flies showed no recognition for the restriction enzyme *Pst*I; nevertheless, samples from lab reared flies and from those of other horizontal transfer crosses revealed the presence of one site for this enzyme (Figure 1), as previously described by Estes et al. [15]. ARDRA carried out with restriction enzyme *Cfo*I (Figure 2) revealed two unique patterns. One pattern corresponded to the wild fly samples and to those from the cohabitations, while the other pattern corresponded to the lab reared fly samples and those from the horizontal transfer cross. Bacterial DNA samples from oesophageal bulbs showing these two different patterns were re-amplified with EdF1/1507R primers and sequenced in both directions to obtain a nearly complete 16S rRNA gene sequence.

Then, samples from wild flies, lab flies and from the horizontal transfer experiment crosses were tested by DGGE analysis, performed with the 986F-GC and 1401R primers. Visual inspection of DGGE revealed the presence of a single dominant band in all samples; in addition, some samples also showed other less prominent bands (data not shown).

Meanwhile, samples from wild flies and from most of the flies from cohabitations (n = 30) showed a similar migration pattern (data not shown). Despite this, the rest of the samples were found to have different fragment motilities. Successively, DGGE carried out with the 63F-GC and 518R primers was used to characterize the wild fly samples and compare them to those of the cohabitation fly samples. The DGGE profiles were comprised of a single dominant reoccurring band, as well as other less noticeable bands. All the profiles obtained from wild flies and most obtained from the cohabitation flies corresponded to one of the two main migration behaviours (Figure 3).

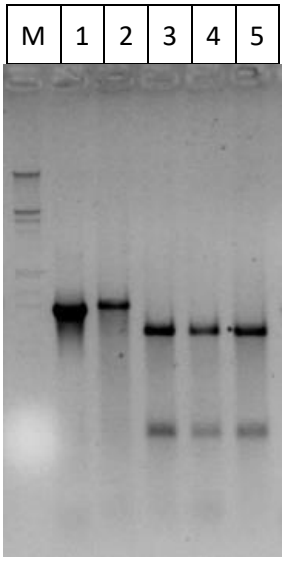

Figure 1 - ARDRA patterns generated after the digestion of the amplified 16S rRNA gene with *Pst*I. Lane M corresponds to DNA Molecular Weight Marker III (Roche Diagnostics Ltd.), lane 1 corresponds to a non-digested 16S rDNA amplicon from a wild fly oesophageal bulb, lane 2 corresponds to the ARDRA pattern from a lab fly oesophageal bulb bacterial content, lane 3 corresponds to the ARDRA pattern from a wild fly oesophageal bulb bacterial content, and lanes 4 and 5 correspond to the ARDRA patterns from two lab fly oesophageal bulbs of the cohabitation treatment.

Six unique bands separated by DGGE were selected according to their relative mobility, excised from the gel, and sequenced.

Sequencing – The presence of *Ca*. E. dacicola in the oesophageal bulb samples of wild flies was confirmed before starting the horizontal transfer experiment by sequencing the PCR products (n = 6) obtained using EdF1 and 1507R primers. In all cases, we obtained species-level identity ascribed to the sequence of *Ca*. E. dacicola (100% similarity to GenBank accession number HQ667589 or HQ667588). PCR products (n = 3) amplified from the oesophageal bulbs of lab-reared flies were also sequenced to obtain species-level identity with the sequence of *Morganella morganii* (99% similarity to GenBank accession number NR_113580).

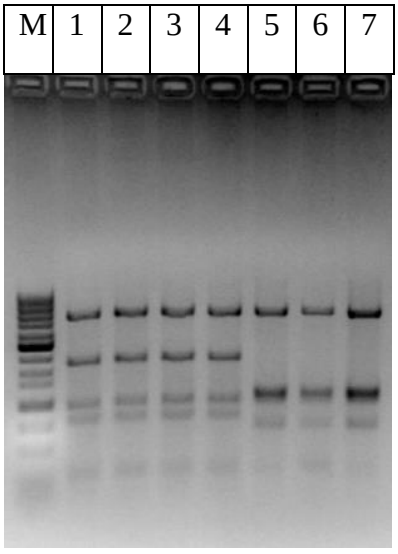

Figure 2 - ARDRA patterns generated after digestion of the amplified 16S rRNA gene with *Cfo*I. Lane M corresponds to a 100 Base-Pair Ladder (GE Healthcare), lane 1 corresponds to the ARDRA pattern from a lab fly oesophageal bulb, lanes 2, 3 and 4 correspond to the ARDRA patterns of three lab fly oesophageal bulbs, lane 5 corresponds to the ARDRA pattern from a wild fly oesophageal bulb, and lanes 6 and 7 correspond to the ARDRA pattern from two lab fly oesophageal bulbs from the cohabitation treatment.

By sequencing, the DGGE isolate (n = 2) bands of the wild fly specimens were confirmed to correspond to the sequence of *Ca*. E. dacicola (>99% similarity). In particular, the lower band (Figure 3) was assigned to *Ca*. E. dacicola haplotype A (GenBank accession number HQ667588) and the upper band (Figure 3) to *Ca*. E. dacicola haplotype B (GenBank accession number HQ667589), as already distinguished by Savio et al. [24]. The

exclusive incidence of *Ca*. E. dacicola was additionally confirmed in 4 isolated DGGE bands of fly specimens from the cohabitation experiments, which demonstrated similar migration behaviours to the wild fly samples.

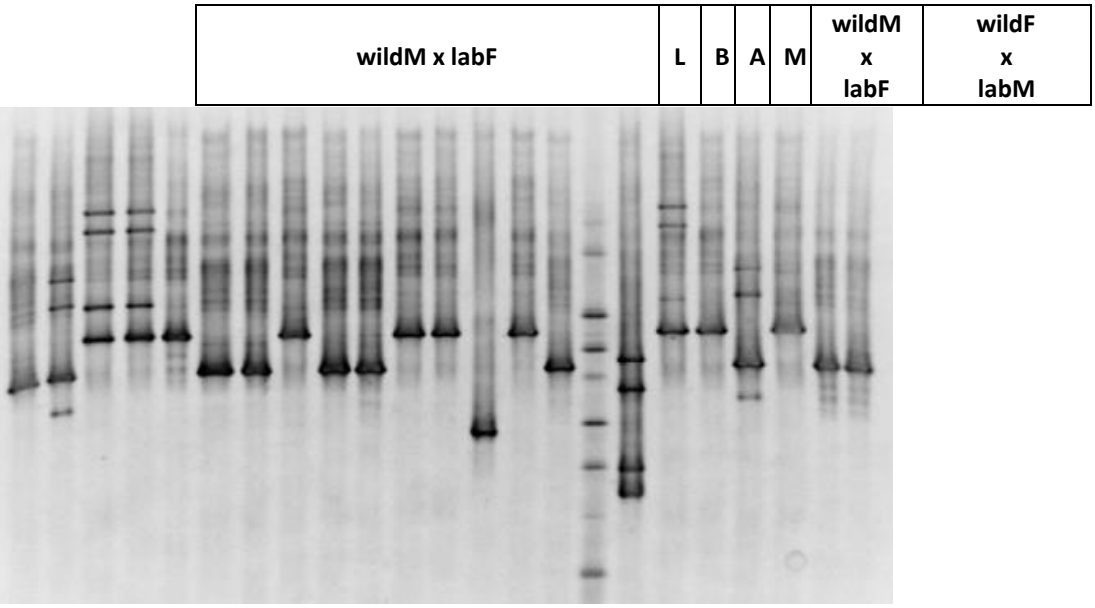

Figure 3 - Analysis of the bacterial communities within the oesophageal bulbs of *B. oleae* after the cohabitation experiments: The DGGE profiles of 16S rDNA fragments obtained by amplification with the 63FGC/518R primer set. The letter M on the gel image indicates the marker used for the normalization of the bands in the profiles. L refers to a lab sample, while B and A correspond to the two different *Ca*. E. dacicola lineages from wild flies (htB and htA, respectively). The other headings refer to the two different cohabitation treatments.

On the other hand, the DGGE isolate (Figure 3) bands of the lab-reared flies were found to share sequence identity with *M. morganii* (99% similarity to GenBank accession number NR_043751). Other bands showing different migration behaviours from those of the wild or lab flies were not sequenced.

Faeces and Sponges - PCR-DGGE analyses of wild flies' faeces (Figure 4) and the subsequent sequencing of the excised DGGE bands provided evidence of the presence of taxa mainly related to the γ-Proteobacteria phylum and, in particular, to the Enterobacteriales order (Table 2). The nucleotide-sequence identities ranged from 91% to 100%, and most matches

showed identities greater than 99%. *Ca*. E. dacicola was also found (with 100% similarity to GenBank accession number HQ667589), although it was detected as a less pronounced band and a narrow denaturing gradient needed to be applied to highlight its presence in the faeces samples (Figure 4B).

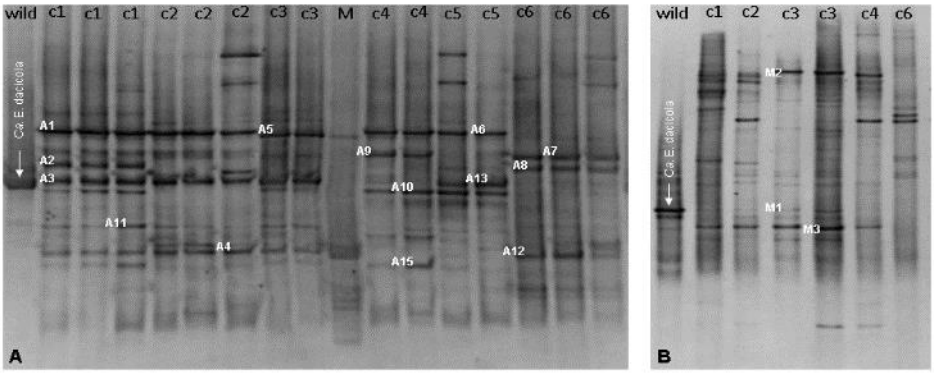

Figure 4 - Analysis of the bacterial communities within the faeces of *B. oleae*: DGGE profiles of the 16S rDNA fragments obtained by amplification with the 986FGC/1401R primer set. DGGE denaturing gradients of 45-68% (A) and 50-65% (B). The arrowed bands indicate the PCR products obtained by the amplification of DNA extracted from the wild fly oesophageal bulbs used as species markers of *Ca*. E. dacicola. Numbered bands (A1-A15; M1-M3) were selected for sequencing. The faeces were deposited by wild fly samples in cages 1-5 (c1-c5) and by lab flies in cage 6 (c6), with 2 or 3 replicates for each cage. M, marker.

Furthermore, PCR-DGGE analyses performed on the sponges highlighted the presence of *Ca*. E. dacicola on those taken from the replicates of the faeces treatment (data not shown). Analyses on the sponges from different treatments (olives, wax domes, cohabitation and gelled water cages) did not show any match with the *B. oleae* endosymbiont.

Discussion

The goal of these investigations was to attempt to observe the horizontal transfer of the endosymbiont *Ca*. E. dacicola from a wild *B. oleae* population to a laboratory colony. A secondary goal was to determine the best and most efficient method to reliably screen for this endosymbiont in *B. oleae* samples. It was predicted that horizontal transfer could occur via both oral contamination (wild flies' regurgitation on gelled water and olives) and

via anus-genital contamination (eggs laid inside oviposition domes by wild flies, wild faeces, and cohabitation with wild flies).
Concerning the oral contamination transmission route and more specifically regurgitation, we tested the hypothesis that flies could regurgitate saliva with bacteria on two different substrates, olives and gelled water. Petri first described this behaviour in 1907 [32], and he reported a peculiar behaviour.

Table 2 - Identification of 16S rDNA fragments selected from PCR-DGGE of the *B. oleae* faeces. Taxonomic identification was achieved using different sequence similarity thresholds: a similarity ≥97% was used for species level identification, while similarities of 95%, 90%, 85%, 80% and 75% were used for assignment at the genus, family, order, class and phylum levels, respectively [31].

Isolate PCR-DGGE band	Nearest match (GenBank accession no.; % sequence similarity)	Taxonomic classification
FA1	*Ewingella americana* CIP81.94 (NR_104925; 99%)	*Ewingella americana*
FA2	*Rosenbergiella collisarenosi* 8.8A (NR_126304; 99%)	*Rosenbergiella collisarenosi*
FA3	*Erwinia aphidicola* Och2N7 (NR_104724; 99%)	*Erwinia aphidicola*
FA4	*Enterobacter muelleri* JM-458 (NR_104647; 100%)	*Enterobacter muelleri*
FA5	*Serratia marcescens* NBRC102204 (NR_114043; 99%)	*Serratia marcescens*
FA6	*Rahnella woolbedingensis* FRB227 (NR_146848; 99%)	*Rahnella woolbedingensis*
FA7	*Morganella morganii* DSM14850 (NR_043751; 99%)	*Morganella morganii*
FA8	*Leclercia adecarboxylata* CIP82.92 (NR_104933; 91%)	unclassified *Enterobacteriaceae*
FA9	*Morganella morganii* DSM14850 (NR_043751; 99%)	*Morganella morganii*
FA10	*Lactococcus taiwanensis* 0905C15 (NR_114327; 95%)	unclassified *Lactococcus*
FA11	*Cedecea lapagei* DSM4587	*Cedecea lapagei*

	(NR_126318; 99%)	
FA12	*Enterobacter muelleri* JM-458 (NR_104647; 100%)	*Enterobacter muelleri*
FA13	*Erwinia aphidicola* Och2N7 (NR_104724; 99%)	*Erwinia aphidicola*
FA15	*Acidibacter ferrireducens* MCF85 (NR_126260; 95%)	unclassified *Acidibacter*
FM1	*Ca*. Erwinia dacicola clone htB (NR_667589; 100%)	*Ca*. Erwinia dacicola
FM2	*Serratia marcescens* NBRC102204 (NR_114043; 99%)	*Serratia marcescens*
FM3	*Acinetobacter septicus* AK001 (NR_116071; 100%)	*Acinetobacter septicus*

of *B. oleae* in which the fly sucked and regurgitated olive juice during the oviposition process, commonly known as "the kiss" [33]. Tzanakakis [34] also described this action in *B. oleae*, assuming that, at the end of the oviposition process, the female retracts the ovipositor and regurgitates the juice sucked from the hole to deter subsequent oviposition. Drew and Lloyd [35] also described strict relationships between tropical Dacinae and the bacteria of host plants. They showed that the bacteria present in the alimentary tract of flies were also found on the surface of host fruit from plants in which flies had been collected, suggesting that regurgitation was involved in this bacterial presence. However, in our experiment, even if the substrates had been contaminated through bacterial regurgitation by the wild olive fruit fly, the transfer of *Ca*. E. dacicola to lab flies did not occur, either through the olives or the gelled water. However, no attempts to detect *Ca*. E. dacicola on these two substrates were carried out, since the transfer did not occur we presume that the symbiont was not present on them or, if present, it was probably not available for the horizontal transfer.

Regarding the possible anus-genital transfer, wax domes containing eggs laid by wild females were tested as a contamination source. The presence of *Ca*. E. dacicola was found on the eggs, not only by biological molecular techniques [10] but also by morphological observations dealing with the presence of bacterial colonies around the ano-genital opening and in the micropylar area [6]. Furthermore, previous observations had highlighted the presence of bacterial masses on *B. oleae* eggs [36]. Since several previous studies demonstrated that *Ca*. E. dacicola is vertically transmitted from the

female to the egg [9, 10, 15, 17, 25]; we predicted that a horizontal transfer mechanism could occur after the lab flies have direct contact with the eggs lad by wild females. However, our attempt was not successful. In terms of vertical transmission, there are many ways to "pass" symbiotically useful bacteria via the egg, from the mother to the progeny. For instance, symbiotic bacteria can be maternally transmitted by "capsule transmission" or by "egg smearing," as observed in stinkbugs [37]. It could also be transferred to the egg as it passes through the micropyles, as is believed to occur in fruit flies [38]. For the vertical transfer of *Ca*. E. dacicola in *B. oleae*, the bacterium seems to be maternally transmitted by "egg smearing" [6]. Thus, even if the endosymbiont is smeared on the egg's surface, its passage to the young larva is probably strictly related to the micro environment inside the olive. Given these assumptions, we predict that in the present work, this horizontal transfer via egg using wax domes did not occur, perhaps because *Ca*. E. dacicola on the egg surfaces was exposed to air for too long, instead of remaining in the "small oblong chamber" inside the olive [34] with low oxygen levels, thus limiting the possibility of horizontal transfer. Another hypothesis could be that after oviposition inside the fruit, the endosymbiont needs some olive compounds that enable it to stay viable until larval assumption.

Because the symbiont passes through and colonizes the digestive tract during the entire adult lifespan [15], and especially given its role in nitrogen metabolism [25], we tested the hypothesis that it could be partially released in the faeces after digestion. The endosymbiont was indeed detected on faeces and on sponges taken from the replicates of the faeces treatment. These sponges stayed in contact with the wild flies for a long time (they were inserted during the contamination phase along with wild adults, and they were not exchanged with new sterile sponges for the acquisition phase, as in other theses). We therefore believe that they were contaminated by faeces. However, no horizontal transfer was observed after using this substrate as a contamination source. Based on this, we presume that even if *Ca*. E. dacicola DNA was detected both on the faeces and sponges, the bacterium may not be viable or may on these substrates and may not be horizontally transferred in this way. These findings further suggest that *Ca*. E. dacicola may be a bacterium that needs low levels of oxygen to maintain its vitality and grow.

Consistent with our hypotheses and the results of Estes et al. [23], horizontal transfer via cohabitation with wild flies was the only treatment in which transfer occurred. To our knowledge, the transmission of *Ca*. E. dacicola could have occurred through different methods, including mating,

coprophagy or trophallaxis. Copulation between males and females was not directly verified; there is a high probability that the flies did mate, but we cannot be sure that this was the way through which the transfer occurred. Further trials assessing cohabitation between wildM x labM or wildF x labF could be set out in order to better clarify this finding. The flies in the cohabitation scenario also had ample opportunities to regurgitate and defecate in the same cage. This observation allowed us to make a second hypothesis: perhaps not only the mating, but also the coprophagy and/or the trophallaxis behaviour between wild and lab flies during their cohabitation accounted for the horizontal transfer. The only thing we know is that the wild and lab flies stayed together for 15 days and they had time to perform other behaviours and to be in contact frequently in different ways. Trophallaxis represents an "exchange of alimentary liquid among colony members and guest organisms," and it can occur before, during, or after mating. It can also be direct or indirect, stomodeal or proctodaeal, and it has been described in approximately 20 species of Tephritidae, representing a behaviour that involves the transfer of substances [39]. Several studies described the mating trophallaxis in Tephritidae [40, 41, 42] but did not demonstrate the transfer of any substance during the contact between the mouthparts of the mates. Our results lead us to suppose that this behaviour could be involved in endosymbiont transfer, as predicted by Estes et al. [23]. They hypothesized that bacterial transfer occurs through coprophagy, presumably thanks to pre/in direct proctodaeal trophallaxis. Moreover, it must be noted that we found *Ca*. E. dacicola DNA inside the oesophageal bulb of lab flies that cohabited with wild flies; as a consequence, trophallaxis appears to be more likely to be responsible for transfer than *Ca*. E. dacicola matings. Further research, such as the analysis of the proctodaeal diverticula and/or the crop system of lab flies after cohabitation with wild adults, together with behavioural studies, would better clarify this aspect. Moreover, cohabitation was the only treatment in which the endosymbiont was not as exposed to oxygen. In contrast, the other treatment conditions, such as the olives, gelled water, eggs laid by wild females and faeces likely exposed to *Ca*. E. dacicola, were all exposed to oxygen for a longer period. We can therefore presume that *Ca*. E. dacicola prefers microaerophilic conditions for its vitality and transfer. In addition, we can affirm that transfer via cohabitation is not related to the sex of the wild symbiotic fly, since it occurred both when the *Ca*. E. dacicola contamination sources were wild females or wild males.

Hence, a symbiotic wild fly (male or female) in cohabitation with a non-symbiotic lab fly (male or female) is all that is required for the successful

horizontal transfer of *Ca*. E. dacicola. Thus, this could be the first step in obtaining a permanently symbiotic laboratory olive fruit fly colony, likely reared on different substrates than the cellulose-based one, which allow for the avoidance of genetic modifications possibly caused by symbiont absence [19, 20].

The aim of the present study was to provide a reliable and consistent tool for implementing the detection of the endosymbiont in a large number of *B. oleae* specimens and/or environmental samples. According to the obtained results, it seems that the primers EdF1 and EdEnRev are not sufficiently specific for *Ca*. E. dacicola, as previously described by Estes et al. [15]. Indeed, samples that were positive to *Ca*. E. dacicola with these primers did not show the same results after DGGE analysis. Moreover, an *in silico* analysis conducted using the Probe Match function within the RDP-II database (http://rdp.cme.msu.edu) showed a higher number of exact matches to the 16S rRNA gene sequences from members of Enterobacteriaceae family (3% respect to the total Enterobacteriaceae sequences in RDP database) belonging to *Erwinia*, *Serratia*, *Proteus*, *Buttiauxella*, *Enterobacter* and other genera. Thus, we suggest that to confirm the presence of *Ca*. E. dacicola, the screening of oesophageal bulbs or other specimens by PCR with EdF1/EdEnRev primer has to be combined with subsequent analyses [27]. Sequencing is a time consuming and expensive method, and this does not seem to be the most convenient system, especially when a large number of samples must be analysed. ARDRA has been previously and successfully performed to compare profiles from uncultivable bulk bacteria residing in the oesophageal bulb with those from cultivable bacteria occasionally arising on plates in an attempt at endosymbiont isolation (Capuzzo et al., 2005) and, more recently, to distinguish the two different bacterial haplotypes (htA and htB) [24]. Furthermore, Ben-Yosef et al. [25] used DGGE performed with 986F-1401R primers and succeeded in detecting *Ca*. E. dacicola in *B. oleae* adult oesophageal bulbs and larvae. In this study, both ARDRA and DGGE techniques were applied. ARDRA demonstrated that it was possible to identify a specific profile corresponding to *Ca*. E. dacicola that was clearly distinguishable from that of other Enterobacteriaceae, such as *M. morganii*. Moreover, DGGE appears to be the best molecular fingerprinting method, since different bacterial taxa may be associated with oesophageal bulbs, both as individual dominant bacterium and in the bacterial consortium. The PCR-DGGE fingerprint was widely used to compare the microbial community structure in a variety of environments [43, 44, 45, 46]. Furthermore, it supports the identification of bands, because PCR products

can be recovered and sequenced [47]. As an alternative to sequencing, the identification of bacteria may be achieved by the comparison of the PCR amplicon DGGE migration behaviour with that of a reference strain, used as species marker [48]. Thus, the choice of which target hypervariable regions of the 16S rRNA gene are to be amplified may strongly affect the quality of information obtained by DGGE [47]. This study demonstrated that PCR-DGGE performed with the primer set 63F-GC/518R and targeting the V1-V3 hypervariable regions, provides the best procedure for the rapid and straightforward screening of the presence of *Ca*. E. dacicola in a high number of fly specimens. This also reflects the two different *Ca*. E. dacicola haplotypes (htA and htB).

Considering the ARDRA profiles and the migration behaviour of PCR products on DGGE and nucleotide-sequence identity by BLAST, approximately 50% of the oesophageal bulbs of lab flies after cohabitation highlighted the presence of *Ca*. E. dacicola as a prominent associated species, and in particular, 13 corresponded to *Ca*. E. dacicola haplotype A and 13 to *Ca*. E. dacicola haplotype B, confirming previous findings from fly samples collected in Tuscany [24]. Conversely, all the oesophageal bulbs of the lab-reared flies of the other crosses in the horizontal transfer experiment did not demonstrate the acquisition of *Ca*. E. dacicola. Furthermore, the other associated bacteria were supposed to be related to different taxa within the Enterobacteriaceae family.

The fact that *M. morganii* was detected in lab flies shows that the lab strain has been exposed to many bacteria and that *M. morganii* could have competed with *Ca*. E. dacicola, thus preventing horizontal transfer. This does not mean that *M. morganii* could represent a pathogen for *B. oleae*, as shown in recent studies on *Anastrepha* spp. [49, 50]. Furthermore, this bacterium has already been found in the oesophageal bulb of lab-reared *B. oleae*'s flies [13] and does not seem to represent a threat for the olive fruit fly. Along with this, supplementary observations would be appropriate to better evaluate the effects of this bacterium on *B. oleae* fitness and other parameters such as adult mortality or egg production.

Conclusions

This research demonstrates that the cohabitation of wild and lab reared flies is the only way through which the horizontal transfer can occur. Thanks to these investigations, it has been possible to find a viable way to transfer the endosymbiont *Ca*. E. dacicola from an adult wild *B. oleae* population to a laboratory colony. As a result, this study represents the first step in better

understanding *Ca*. E. dacicola behaviour, physiology and culturing requirements.
DGGE was the most reliable detection method, although it has some inherent associated limitations; DGGE proved to be a consistent method for screening the endosymbiont *Ca*. E. dacicola in *B. oleae*, further distinguishing between the two *Ca*. E. dacicola haplotypes.
Further investigations should be completed in order to improve these findings, and other horizontal transfer experiments should be completed during different periods of the year and/or in different conditions. Moreover, the resulting endosymbiotic laboratory-reared flies should be evaluated in terms of different parameters, such as egg production, egg hatching, larval development and pupal recovery for the pre-imaginal stages and mortality, lek behaviour and mating success for the adult stages. Nevertheless, the trials in which the transfer did not occur (olives, gelled water, wax domes, faeces) may be tested again using a different approach to better understand how to solve the problems that hindered the transfer. In this way, different strategies could be identified in order to improve the success of the horizontal transfer. Thus, laboratory-reared flies could compete with the wild ones, improving the Sterile Insect Technique as a possible tool for the sustainable control strategies within the olive system.

Author contributions

All authors conceived of and designed the experiments. GB and RG reared the insects and performed the experiments. GB and PS performed insect dissection and DNA extraction. RP designed the molecular biology procedures. GB and RP performed the PCR analyses, DGGE, ARDRA and other molecular techniques applied to the samples. GB and RP drafted the manuscript; AB and PS discussed and revised the initial draft of the manuscript.
All authors read and approved the final version of the manuscript.

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Competing interests

The authors declare that they have no competing interests.

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Chapter Two

Evaluation of the antibacterial effects of copper, at two different concentrations (5% and 20%), and propolis on the presence of the symbiont *Ca.* E. dacicola in a wild population

	Idea	Data collection	Molecular analyses	Data analysis	Manuscript preparation
My contribution	*	*	*	*	*

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Symbiosis interruption in the olive fly: effect of copper and propolis on *Candidatus* Erwinia dacicola

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Abstract: The relationship between *Bactrocera oleae* (Rossi 1790) and its endosymbiont *Candidatus* Erwinia dacicola is important to achieving effective control of the olive fly population in the field. This bacterium plays a crucial role in the life of *B. oleae* and is necessary for its fitness. Thus, in the absence of the endosymbiont, B. oleae wild populations in the field might decrease considerably. Copper is one of the most used antimicrobials for horticultural crops worldwide, and its efficacy against *Ca*. E. dacicola has been demonstrated in field trials. Propolis is another natural antimicrobial compound largely used for its activity in several fields. If propolis and copper prove to be efficient against wild populations of the endosymbiont *B. olea*e in the field, such a biological restraint might improve sustainable agriculture. We evaluated, under laboratory conditions, the effect of two different copper products (at two different concentrations, 5% and 20%) and propolis on the content of *Ca*. E. dacicola in the eggs and in the adult esophageal bulbs of *B. oleae*. Bulbs were extracted twice, after 2 and 5 weeks of exposure. Real-time PCR on the bulbs showed a reduction in *Ca*. E. dacicola content in flies treated with copper (at both 5% and 20%), and from the first to the second extraction, both while flies treated within propolis showed an increment of the relative abundance of *Ca*. E. dacicola(35%) and copper (40.5% and 61% reduction for copper at 5% and 20%, respectively) treatments, compared with the content of the control. Both copper products (5% and 20%)treatments reduced the egg production after 2 and 5 weeks in comparison with the control and propolis treatments; furthermore, both copper products significantly reduced the egg production after 5 weeks relative to the propolis. Moreover, adult mortality was significantly higher with propolis compared with the other treatments. Thus, our preliminary results encourage further research in order to develop new tools for the control of the olive fly in the framework of an integrated pest management strategy.

Key words: natural compounds, adult mortality, antimicrobial activity, Real-time PCR, *Bactrocera oleae*, endosymbiont.

Introduction

Until recently, synthetic chemical products such as organophosphates and pyrethroids have been considered the most useful insecticides against the olive fly *Bactrocera oleae* (Rossi 1790), one of the most important pests of olive crops. This species is widespread across the Mediterranean basin, but it is also present in South Africa and from the Middle East to India, and it has invaded new olive growing areas in California and Mexico (Rice *et al*., 2003; Daane and Johnson, 2010). With the advent of alternative antimicrobial compounds and the movement toward low-environmental-impact strategies, together with the development of integrated pest management control programs, other products, such as copper compounds and/or kaolin, have been recently field tested against this tephritid, not only for their direct action against the olive fly but also for the antibacterial activity that they play against associated bacteria (Belcari and Bobbio, 1999; Belcari *et al*., 2005; Caleca and Rizzo, 2006 and 2007; Rosi *et al.,* 2007; Tsolakis *et al.,* 2011).

To date, several kinds of relationships and associations between bacteria and the different tephritids have been described (Lauzon, 2003, Ben-Yosef *et al.,* 2008; Lauzon *et al.,* 2009; Hadapad *et al.,* 2015; Liu *et al.,* 2016; Naaz *et al.,* 2016), and many studies have been conducted to investigate the bacterial interactions involving *B. oleae* (Stamopoulos and Tzanetakis, 1988; Manousis and Ellar, 1988; Capuzzo *et al.,* 2005; Robacker, 2007; Sacchetti *et al.,* 2008; Estes *et al.,* 2009; Kounatidis *et al.,* 2009; Savio *et al.,* 2012).

An understanding of the main symbiont of the olive fly, first discovered by Petri (1906) and later named as *Ca*. E. dacicola (Capuzzo *et al.,* 2005), relative to the olive fruit fly behavior and life cycle is important. *Candidatus* E. dacicola belongs to the Enterobacteriaceae family within the gammaproteobacteria group. It is not culturable, and it is considered a P-symbiont (persistent) for *B. oleae*. At oviposition, it is smeared over the eggs (Sacchetti *et al*., 2008) and is transferred vertically to the progeny; it has been found in all phases of this fly's life cycle, particularly in the adult stage (Estes *et al*., 2012). *Candidatus* E. dacicola plays a very relevant role in the entire *B. oleae* life cycle (Tzanakakis, 1985; Ben-Yosef *et al.,* 2010). As a matter of fact, *Ca*. E. dacicola is thought to be necessary for *B. oleae* adult fitness and larval survival (Ben Yosef *et al.,* 2014; 2015). The symbiont could represent an important source of proteins for this tephritid; as a matter of fact, intestinal microbiota and endosymbionts can strongly sustain female fecundity, supplying essential amino acids and useful nutrients (Ben-Yosef *et al*., 2010), thereby enhancing pheromone production

and oviposition success (Estes *et al*., 2012). In addition, *Ca*. E. dacicola seems to switch from an intracellular existence to an extracellular one during the host insect development, as it lives intracellularly within the larval midgut cells and extracellularly in the adult gut (Estes *et al.*, 2009). The symbiont seems to be strictly related to the olive tree agroecosystem, since its presence has never been confirmed in laboratory-reared flies (Sacchetti *et al.*, 2008; Estes *et al.*, 2012), although a recent study did demonstrate that it could be horizontally transmitted from a wild to a laboratory-reared population by cohabitation among adults (Bigiotti *et al.*, in press). One of the main roles of *Ca*. E. dacicola is that it helps larvae to survive and develop in unripe olives, allowing them to overcome the effects of some compounds such as oleuropein, a phenolic compound considered detrimental to the larval stage (Ben-Yosef *et al.*, 2015). Thus, in the absence of the endosymbiont, *B. oleae* wild populations in the field might decrease considerably, as previously shown in the field trials and laboratory experiments using antibiotics as symbionticides (Fytizas and Tzanakakis, 1966; Tzanakakis, 1985).

As previously noted, copper is one of the most used antimicrobials (antifungal and antibacterial) for horticultural crops worldwide, even though its use has always been under review because of the environmental issue of its possible build-up in the soil (Pennino *et al.*, 2006; Lamichhane *et al*., 2018) and its accumulation in animal tissues, resulting toxic to non-target species, such as earthworms, as highlighted for *Lumbricus rubellus* Hoffmeister, 1843 in previous studies (Marinussen *et al.*, 1997). Its efficacy against *Ca*. E. dacicola already has been demonstrated, not only for impeding larval growth (Tzanakakis, 1985; Belcari and Bobbio, 1999) but also for acting as a deterrent to oviposition (Prophetou–Athanasiadou *et al.*, 1991). In fact, copper is supposed to kill bacteria, causing the lack of those bacterial compounds that make the fruit attractive to the fly for oviposition (Belcari *et al.*, 2003; Caleca and Rizzo, 2006 and 2007).

Traditional copper products are thought to be helpful in inhibiting bacterial growth; however, other natural compounds could have the same action. Propolis, for instance, the resinous substance produced by honeybees that is involved in the beehive maintenance and construction (Burdock, 1998), is known since ancient times to have a wide range of biological activities. It was first described in the Latin essay *Naturalis historia* written by Pliny the Elder in 77 AD, where he described the properties of this substance, writing that propolis was “remarkable for its utility in medicine” (Bostock and Riley, 1855). From the second half of the 20th century, with the advent of natural and alternative medicine, scientists started to study its properties,

highlighting the many different skills, not only in hindering bacterial, fungal and viral propagation but also acting as cytotoxic, antioxidant, and anti-inflammatory agents (Burdock, 1998; Bankova *et al.,* 2014). Furthermore, the antimicrobial activity of propolis has been demonstrated also on Gram-negative bacteria (Rahman *et al.,* 2010; Kubiliene *et al.,* 2015). Therefore, propolis also might be effective against *Ca*. E. dacicola, thereby interrupting the symbiosis with *B. oleae*. In this way, young larvae deprived of bacterial assistance would be unable to feed on green olives and would die.

Based on these previous studies, we evaluated the effect of two different active ingredients— propolis and copper at two different concentrations, 5% and 20%—not only on the presence of *Ca*. E. dacicola on *B. oleae* eggs and adult esophageal bulbs but also on adult mortality and egg production.

Materials and Methods

Insects

Wild pupae were obtained from infested olives collected in Follonica (Grosseto, Italy) in November 2017 at the experimental orchards of Santa Paolina (CNR – IVALSA). Olives were kept in open boxes to maintain their freshness and to avoid fungi or mildew growth. Five to six days after the harvest, larvae started exiting the olives; pupae were collected and maintained at 12°C in order to synchronize adult emergence. Newly emerged adults < 24 h old were then transferred to the designed experimental cages (25 males and 25 females), consisting of plastic cylindrical containers (approximately 2 L). Flies were kept in the laboratory at room temperature of 16±2°C, relative humidity (RH) of 40±10%, and under a photoperiod of 10 h:14 h (L:D).

Experimental design

Experiments began on January 10th, 2018, and ended in February. The goal of the experiment was to investigate the effect of three different antibacterial products, via oral administration, on *Ca*. E. dacicola presence in the esophageal bulb of wild *B. oleae* adults and on eggs. Three treatments, plus the control, were set up: propolis (Propoli®, flavonoid-resinous extract from propolis in glycol, Serbios s.r.l., Rovigo, Italy), 20% copper (Poltiglia Caffaro 20 DF NEW, water-dispersible copper granules from copper sulfate neutralized with slaked lime, Isagro and Sumitomo Chemicals, Italy), and

5% copper (Manisol, wettable powder with 5% copper from copper hydroxide and calcium hydroxide - CAS number 1305-62-0 - Manica S.p.A, Rovereto, Italy). All products were diluted with tap water according to each mean dose as recommended by the manufacturer's instructions for field applications and were given to flies through watering places and diet. Diluted products were supplied to flies as the water source; moreover, 2 mL of the diluted antimicrobials were added to the powdered diet (7 g in a Petri dish) consisting of sugar, hydrolyzed enzymatic yeast (ICN Bio medicals) and egg yolk (40:10:3). To prevent flies from getting stuck in the liquid food, the mixture was dried in the stove at 56°C overnight; after being dehydrated, it was given to the flies. Flies of the control treatment were supplied only with water and diet without any addition.
Each treatment and control had 3 replicates containing 50 flies each, for a total amount of 600 tested flies. All replicates were positioned randomly on a shelf unit and moved daily. Mortality was noted day by day until the last fly of one treatment died.

Egg collection

After 2 and 5 weeks, a wax dome was inserted in each cage for the oviposition and the consequent egg collection; once the dome was provided, flies were placed in a conditioned rearing room with temperature of 18±2°C and RH 60±10% to enhance both mating and egg production. Domes were inserted into tissue culture dishes containing approximately 3 mL of water to prevent egg dehydration and then exposed to flies for 24 h; laid eggs were removed by washing the domes with sterilized water, under a laminar flow hood, and then sieved with a sterilized cloth and gathered in a sterile beaker. Finally, the total number of eggs for each cage was counted to estimate the egg production. A certain number of eggs for each treatment and control was collected under the stereomicroscope and then transferred in a 1.5 mL Eppendorf tube for DNA extraction.

Adult dissections

Flies were dissected after 2 and 5 weeks from the start of the experiment to evaluate *Ca*. E. dacicola content inside the esophageal bulb. Flies were dissected as follows: 2 males and 2 females were taken from all the replicates of each treatment (12 flies/treatment), killed by holding at -20°C for 5 min and then dissected. The dissection procedure was performed under a laminar flow hood in all its steps. First, each specimen was washed with a

2% sodium hypochlorite solution and then rinsed twice in sterilized water. Second, each esophageal bulb was extracted by dissecting fly heads under a stereoscopic microscope, using sterilized steel tools. Sex, sample number and bulb aspect (transparent or milky) were noticed. Finally, each bulb was transferred to a 1.5 mL Eppendorf tube for the bacterial DNA extraction.

Bacterial DNA extraction

DNA extraction from eggs and esophageal bulbs was performed by using 30 µL of InstaGene Matrix (Bio-Rad Laboratories, Hertfordshire, UK) according to the manufacturer's instructions, with the exception that a small quantity (approximately 8 mg) of sterile silica powder was added to the eggs to ease grinding. Samples were incubated for 10 min in a 56°C thermostatic bath, with a pause after 5 minutes to shake the vial contents. Successively, samples were incubated for 6 minutes at 100°C. Finally, samples were centrifuged at 10,000 rpm for 8 minutes, and the supernatant was transferred to a new Eppendorf. DNA samples were stored at -20°C until the microbiological analyses.

Culture-independent microbiological analyses

PCR and DGGE analyses

A preliminary Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) analysis was performed using the 63F-GC and 518R (El Fantroussi *et al.*, 1999) primers to determine the presence/absence of *Ca*. E. dacicola in sampled flies. PCR reactions were carried out using a T100 Thermal Cycler (Bio-Rad Laboratories, Hertfordshire, UK) in 25 µl volumes containing 1X Flexi PCR buffer (Promega, Madison, WI), 1.5 mM-MgCl2, 250 µM-deoxynucleotide triphosphates (dNTPs), 400 nM each primer, and 1U GoTaq®Flexi DNA polymerase (Promega). Amplifications were performed under the following conditions: an initial denaturation at 94°C for 5 min; 35 cycles at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s; and a final extension at 72°C for 10 min. After PCR, the amplified products were verified by agarose gel electrophoresis (1.5% w/v). DGGE electrophoresis was performed by loading amplicons onto a polyacrylamide gel (40% acrylamide/bis 37.5:1; Serva Electrophoresis GmbH, Germany) with a linear denaturing gradient (from 49 to 57%) obtained with a 100% denaturing solution consisting of 40% v/v deionized formamide and 7 M

urea. DGGE gels were run for 17 h at 60°C and a constant voltage (75 V) using the Dcode DGGE System (Bio-Rad). After the electrophoresis gels were stained with SYBR®Gold (Molecular Probes, Eugene, OR) diluted 1:1,000 in 1X TAE buffer, the images were digitally captured under UV light (λ = 302 nm) using the ChemiDoc XRS apparatus (Bio-Rad).

Real-time PCR analysis

Real-time PCR analysis was used with the aim of quantifying the *Ca*. E. dacicola on the eggs and esophageal bulbs. Amplifications were carried out in a PTC-200 Peltier Thermal Cycler with a Chromo 4 Detector (Bio-Rad) with 10 μL reaction volumes containing 2X Sso Advanced Universal SYBR®Green Supermix (Bio-Rad), 400 nmol/L of each primer and 1 μL of template DNA. The primers used in this study were EdF1 (Estes *et al.,* 2009) and EdEnRev (Munson *et al.,* 1991), which are specific for the *Ca*. E. dacicola 16S rRNA gene and 341F and 515R (López-Gutiérrez *et al.,* 2004) for the bacterial 16S rRNA gene. The amplification conditions involved denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. DNA extracted from an esophageal bulb of a wild *B. oleae* female with *Ca*. E. dacicola was serially diluted, with "10" as the fold dilution, and used to measure the efficiency of each primer pair (E) by applying the equation: E = -1/slope (Pfaffl, 2001). Amplicon specificity was tested with a dissociation curve analysis by increasing the temperature of 0.5°C every 30 s from 65 to 95°C. Negative controls were run on each plate. Fluorescence data were collected at the end of the hybridization step, and data output were generated by Opticon Monitor software version 2.03 (Bio-Rad). Each sample was run in triplicate, and the threshold cycle (C_T) of these technical replicates were averaged for each individual sampled. Since the efficiency of each primer set did not differ by more than 10%, the relative abundance of *Ca*. E. dacicola was calculated according to the delta-delta C_T method (Pfaffl, 2001), according to the following formula:

$R = 2^{-\Delta\Delta C_T}$

For each sample, the ΔC_T value was calculated by subtracting the bacterial 16S C_T from the *Ca*. E. dacicola 16S C_T, and the number of copies of *Ca*. E. dacicola 16S rDNA in the esophageal bulbs of flies treated with copper (5% and 20%) and propolis was normalized relative to the number of copies of the *Ca*. E. dacicola 16S rDNA found in the esophageal bulbs of the flies treated with water (control) and sampled after 2 weeks. Thus, the CT values obtained from the control samples at 2 weeks were averaged and used as a

calibrator. Hence, bulbs with a relative abundance of *Ca*. E. dacicola value below 1 have fewer copies of 16S than the control bulbs, while bulbs with a relative abundance of *Ca*. E. dacicola value greater than 1 have more copies of 16S than the control bulbs.

Data analysis

Statistica software (Palo Alto, CA, USA) was used to check data for normality assumptions by using the Kolmogorov-Smirnov test and to analyze the data by one-wayfactorial analysis of variance (ANOVA), followed by the Fisher least-significance difference (LSD) post hoc test, and to assess the significance of differences between mean values ($p < 0.05$). Adult mortality was evaluated with Kaplan-Meier survival analysis, followed by the log rank (Mantel-Cox) test (95% confidence interval). Survival analyses were performed with IBM SPSS Statistics software, rel. 25.0.0 (Armonk, NY, USA).

Results

Candidatus *E. dacicola content in eggs and esophageal bulbs*

DNA from eggs and esophageal bulbs was first tested by PCR-DGGE for the presence of *Ca*. E. dacicola. Visual inspection of the DGGE revealed the presence of a single dominant band in all samples with a similar migration pattern of the wild flies used as the marker (data not shown); moreover, all positive samples tested by PCR-DGGE corresponded to milky bulb specimens during dissections.

As a result, the real-time PCR on the esophageal bulb samples showed a reduction of the relative abundance of *Ca*. E. dacicola in flies treated with copper (at both 5% and 20%), and from the first to the second extraction (relative abundance of *Ca*. E. dacicola after 5 2 weeks vs that one after 2 5 weeks) whereas flies treated with propolis showed an increment of the relative abundance of *Ca*. E. dacicola both in flies treated with propolis (35%) and copper (40.5% and 61% after 2 weeks for 5% and 20% copper, respectively) (Fig. 1A). ANOVA analysis of the data revealed that the main factors, treatment and sampling time, did not have a significant effect on the relative abundance of *Ca*. E. dacicola in the oesophageal bulbs. The only significant difference was found between flies treated with propolis after 2 weeks and flies treated with copper (5%) after 5 weeks (Fig. 1B).

In contrast, an analysis of the presence of *Ca*. E. dacicola on the B. oleae eggs laid by wild females did not show any significant reduction in the relative abundance of Ca. E. dacicola after both the propolis or copper treatments (data not shown).

Egg production

ANOVA analysis showed a significant effect of all tested products on the egg production ($F9 = 5.282$; $p < 0.001$). As a result, the copper treatments (5% and 20%) significantly reduced the egg production compared to the control and propolis treatments in both cases, after 2 and 5 weeks (Fig. 2A). Propolis did not show a significant reduction in comparison with the control. Moreover, the number of eggs per female produced after 5 weeks of copper treatments (5% and 20%) was significantly reduced in comparison to the number of eggs laid by females treated with propolis, at 2 and 5 weeks (Fig. 2B).

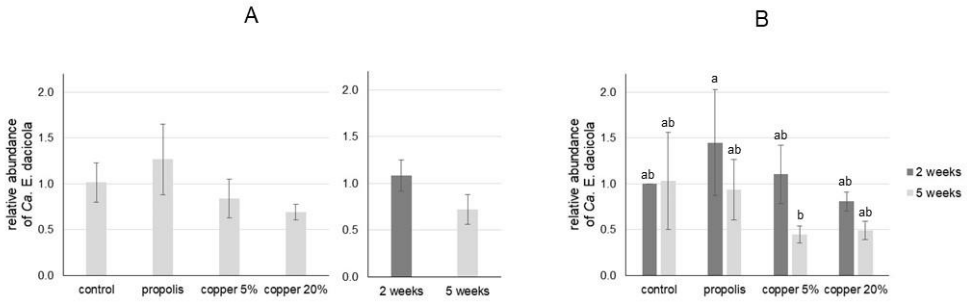

Figure 1 - Relative abundance of *Ca*. E. dacicola in the esophageal bulbs of *B. oleae* flies treated with propolis, copper 205% and copper 520% in comparison to the control after 2 weeks (relative abundance considered equal to 1). Real-time PCR was performed on 12 flies from each treatment at two times (2 and 5 weeks). Data were analyzed by one-wayfactorial analysis of variance (ANOVA): main effects (A) and interaction (B) followed by Fisher's Least Significant Difference (LSD) post hoc test to assess the significance of differences between mean values ($p < 0.05$). Bars show standard error. Different letters above the bars indicate significant differences between among treatments at $p < 0.05$ (Fisher's Least Significant Difference, LSD).

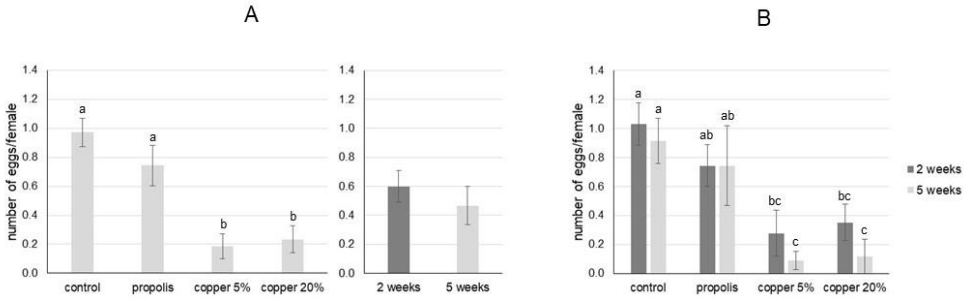

Figure 2 - Number of eggs per female laid in 24 h at two different times after each treatment (2 and 5 weeks). Data were analyzed by a one-wayfactorial analysis of variance (ANOVA): main effects (A) and interaction (B) followed by Fisher's least significance difference (LSD) post hoc test to assess the significance of differences between mean values ($p < 0.05$). Bars show standard error. Different letters above the bars indicate significant differences between among treatments at $p < 0.05$ (Fisher's Least Significance Difference, LSD).

Adult mortality

Mortality rates indicated a significantly different survival probability for flies of the propolis from the other treatments (Log-rank $\chi 2 = 238.967$; d.f. = 3; $P < 0.000$). While insects exposed to copper treatments (5% and 20%) showed a survival similar to flies from the control treatment along the entire experiment, *B. oleae* adults treated with propolis showed higher mortality after approximately 15 days (Fig. 3). No difference was recorded between the females and males for the different treatments or among replicates (data not shown).

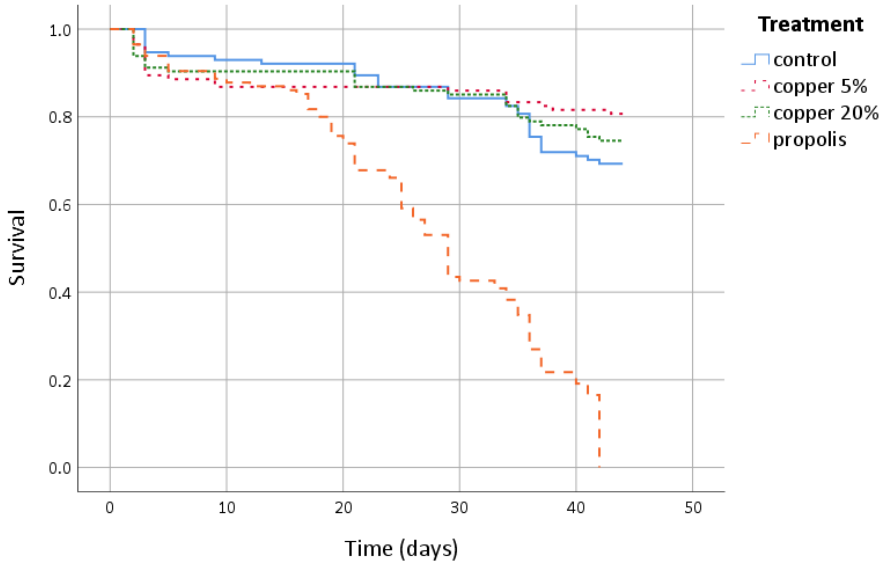

Figure 3 - Survival curves of wild *B. oleae* populations exposed to control, propolis, copper 20% and copper 5% treatments (three replicates, 25 males and 25 females each). Survival curves were analyzed using Kaplan-Meier, Mantel-Cox log-rank test, $p < 0.05$; each treatment was compared to the control. Observations finalized at 43 days.

Discussion

Our results suggest that propolis and copper have several effects both on the fly fitness and on the presence of the endosymbiont *Ca*. E. dacicola in the esophageal bulbs. More specifically, both products displayed an antibacterial action on the symbiont inside the esophageal bulb, as well as on adult mortality and fecundity. Anyway, the presence of the symbiont on eggs in this experiment suggests that vertical bacterial transfer, from the female to the progeny, occurred. This highlights the fact that with *Ca*. E. dacicola, even if its load inside the esophageal bulb is reduced by the tested products, the remaining symbiont colonies might reproduce themselves, and/or be stored inside the last tract of the hindgut, where approximately 25 finger-like processes indicate that plenty of bacteria are present, as evidenced by Mazzini and Vita (1981). Therefore, we could hypothesize that the bactericidal activity of the tested products occurred only in the first tract of the alimentary canal, still allowing and not influencing the endosymbiont transfer.

Moreover, the fact that the copper products significantly reduced the production of eggs per female during the tested time leads us to hypothesize that copper also may act negatively on fly physiology, including egg production. However, the efficacy of the copper products was evident after only 2 weeks. Interestingly, the 5% copper treatment (Manisol) showed a decrease in egg production similar to that observed with the 20% copper (Poltiglia Caffaro), probably due to the specific composition of the former. As a matter of fact, Manisol is generally used as a foliar fertilizer in the field and contains 5% copper and 80-82% calcium hydroxide as inert material. This latter compound, which is usually applied in organic agriculture as slaked lime or clay to help distribute the active ingredients, prevents insect damage and acts as a deterrent for oviposition. The deterring effect of such compounds has been demonstrated for the olive fly (Caleca *et al*., 2010) and Medfly (Lo Verde *et al*., 2011). These findings suggest that the presence of calcium hydroxide possibly produced a synergic effect with the low copper percentage, decreasing the fecundity. These assumptions are supported by previous studies. In fact, the antimicrobial effect of calcium hydroxide is commonly known in endodontics and oral microbiology, and several studies have highlighted its bactericidal properties (Ulusoy *et al.*, 2016; Kousedghi *et al.*, 2012), describing this alkaline substance as "one of the most effective antimicrobial dressings during endodontic therapy" (Siqueira and Lopes, 1999).

Nevertheless, a reduction in egg production, although not significant, was also noticed in the control treatment, when the two egg collection times (2 and 5 weeks) were compared. This result suggests that *B. oleae* fecundity could also be negatively affected by laboratory rearing procedures, such as the artificial diet, conditioned rearing room, plastic cage and fly physiology (Sacchetti *et al.*, 2014). When reared in the laboratory, wild olive flies may lack the presence of naturally associated bacteria and those bacterial compounds that are supposed to enhance the olive fly fitness in the field, as previously stated by many authors (Belcari *et al.*, 2003; Granchietti *et al.*, 2007; Ben-Yosef *et al.*, 2014). Moreover, we observed a further decrease in egg production after 5 weeks that might be related to the increase in adult mortality, strengthening the hypothesis that the tested products could negatively affect *B. oleae* fitness.

In the current study, we fed flies with the above-cited antimicrobials instead of spraying them on the oviposition site (wax domes, in our case). In doing so, we could suppose that the copper and propolis acted through ingestion and not as oviposition deterrents. In fact, previous studies investigated the

deterring action of copper products, highlighting how copper (Prophetou-Athanasiadou *et al.*, 1991; Belcari and Bobbio, 1999; Belcari *et al.*, 2003) and also kaolin (Saour and Makee, 2004; Caleca and Rizzo, 2006 and 2007; Caleca *et al*., 2010) make the oviposition site less attractive because of residues or because of the lack of some bacterial compounds on the olive surface.

Afterward, the adult mortality significantly increased only in the case of the propolis treatment; so far, to our knowledge, no literature reports a positive correlation between the insecticidal effect of propolis and increasing mortality in *B. oleae*. Our results might suggest that, although bee glue might be used as an insecticide, in addition to the use of other compounds, as it proved promising in this laboratory assay, it should also be field-tested, both alone or in combination with natural substances that could display similar activity as shown with kaolin and propolis in a previous study conducted in the field (Iannotta *et al.*, 2006). Notably, the propolis composition is highly variable; as a matter of fact, propolis may contain different chemicals in different proportions depending on the local flora present at the collection site (Bankova *et al*., 2014). However, its antimicrobial activity has been widely demonstrated, even when collected from different geographic locations and notwithstanding its variable chemical composition (Kujumgiev *et al*., 1999).

Thus, further trials should be established to evaluate whether the lab-tested products in this experiment may have the same effect in the olive ecosystem. Concerning the possible use of propolis, further investigations should be done to better evaluate the effect and the possible application of this natural substance. In addition, the effect of products tested herein also should be evaluated on the population density of beneficial organisms and useful insects to verify their selectivity in the field and their compatibility with biological control programs. Previous studies investigated the possibility of symbiosis interruption instead of the use of insecticides to reduce *B. oleae* population in the field, such as the use of antibiotics (Tzanakakis, 1985), but many issues arose against the effectiveness of this method: the problem of their wide-spectrum action and the increase of resistant strains of microorganisms harmful to humans and, consequently, human public health issues. The movement toward low-impact products would help to reduce all negative effects related to the use of chemicals.

Therefore, this study contributed to the investigation of the possible usage of two natural products against *B. oleae* populations in the field, increasing perspectives for organic agriculture and low-environmental-impact control strategies, opening new possibilities for integrated pest management programs.

Authors contribution

Gaia Bigiotti, Patrizia Sacchetti, Roberta Pastorelli and Antonio Belcari conceived the research. Gaia Bigiotti conducted the experiments. Antonio Belcari, Patrizia Sacchetti, and Roberta Pastorelli contributed material. Gaia Bigiotti, Roberta Pastorelli, and Patrizia Sacchetti analyzed data and conducted statistical analyses. Gaia Bigiotti and Roberta Pastorelli wrote the manuscript. Antonio Belcari, Patrizia Sacchetti and Roberta Pastorelli secured funding. All authors read and approved the manuscript.

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Chapter Three

Evaluation of the effect of a propionic acid solution and a sodium hypochlorite + Triton X mixture (1:1) on the presence of bacteria on eggs laid by wild flies

	Idea	Data collection	Molecular analyses	Data analysis	Manuscript preparation
My contribution		*	*	*	*

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Olive fruit fly rearing procedures affect the vertical transmission of the bacterial symbiont *Candidatus* Erwinia dacicola

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Abstract

Background: The symbiosis between the olive fruit fly, *Bactrocera oleae*, and *Candidatus* Erwinia dacicola has been demonstrated as essential for the fly's larval development and adult physiology. The mass rearing of the olive fruit fly has been hindered by several issues, including problems which could be related to the lack of the symbiont, presumably due to preservatives and antibiotics currently used during rearing under laboratory conditions. To better understand the mechanisms underlying symbiont removal or loss during the rearing of lab colonies of the olive fruit fly, we performed experiments that focused on bacterial transfer from wild female flies to their eggs. In this research, eggs laid by wild females were treated with propionic acid solution, which is often used as an antifungal agent, a mixture of sodium hypochlorite and Triton X, or water (as a control). The presence of the bacterial symbiont on eggs was evaluated by real-time PCR and scanning electron microscopy.

Results: DGGE analysis showed a clear band with the same migration behavior present in all DGGE profiles but with a decreasing intensity. Molecular analyses performed by real-time PCR showed a significant reduction in *Ca*. E. dacicola abundance in eggs treated with propionic acid solution or a mixture of sodium hypochlorite and Triton X compared to those treated with water. In addition, the removal of bacteria from the surfaces of treated eggs was highlighted by scanning electron microscopy.

Conclusions: The results clearly indicate how the first phases of the colony-establishment process are important in maintaining the symbiont load in laboratory populations and suggest that the use of products with antimicrobial activity should be avoided. The results also suggest that alternative rearing procedures for the olive fruit fly should be investigated.

Keywords: *Bactrocera oleae*, disinfectant, propionic acid, qPCR, DGGE, egg morphology, insect rearing.

Background

Insects display a great variety of symbiotic relationships with microorganisms that allow them to exploit almost every substrate as food source and to colonize any habitat on earth. Such microorganisms comprise viruses as well as bacteria, fungi, protozoa and multicellular symbionts [1]. In insects, nonpathogenic bacterial symbionts can range from primary, obligate symbionts restricted to bacteriomes and necessary for the host, to secondary, facultative symbionts located in various organs and non-essential for insect survival [1, 2]. The transmission of primary symbionts (P-symbiont) in plant-feeding insects has been investigated in detail in aphids [3, 4], various sucking insects [5, 6, 7] and beetles [8, 9]. P-symbionts are transferred vertically to offspring through contamination of the egg surface, deposition of bacterial capsules on eggs, or consumption of the mother's excrement or through transovarial transmission [7]. Maternal inheritance is the typical transmission route for secondary symbionts, although there is substantial evidence of horizontal transmission as well as rare paternal transmission [10, 11].

Similarly to sucking insects, Tephritid fruit flies display many types of symbiotic associations involving both intracellular (e.g. *Wolbachia*), and extracellular symbionts. Lauzon [12] critically reviewed this topic, commenting on known features and highlighting important issues with possible practical consequences for insect pest control. Many Tephritid fruit flies species are insect pests of economic importance, causing damage to agricultural crops in tropical, subtropical and temperate areas [13]. By studying the relationships of fruit fly species with symbiotic bacteria, new control strategies might be developed [14]. During the last decade, research on the symbiotic relationships of fruit flies has often focused on potential pest control applications. Moreover, following Lauzon's review [12], research on this topic was greatly increased by the advent of molecular techniques, improving microorganism identification.

An example of a symbiotic relationship that was clarified via molecular techniques is that one between the olive fruit fly, *Bactrocera oleae* (Rossi), which is the major insect pest of olive crops in countries where it occurs, and the bacterium *Candidatus* Erwinia dacicola, which was named in 2005 [15]. This symbiosis was the first one involving Tephritids to be described, discovered at the beginning of the twentieth century, although the bacterium was erroneously identified as *Pseudomonas savastanoi*, the agent of olive knot disease. Relying only on microscopic observations, Petri [16, 17] carefully described a specialized foregut organ that harbored the symbiont (a

cephalic evagination later named “oesophageal bulb”) as well as female hindgut pockets from which bacteria were released to be deposited on the egg surfaces and transmitted to the next generation. Since Petri's investigations, several authors have increased knowledge on the olive fruit fly and bacterium symbiosis, providing indirect evidence of the essential role of the symbiont for the insect's survival (see the reviews by Drew and Lloyd, [18], and Lauzon, [12]). However, there were no major findings until the discovery of PCR amplification and 16S rRNA gene sequencing techniques which have substantially improved our knowledge on olive fruit fly symbiotic associations.

By summarizing the recent findings, it appeared that *Ca*. E. dacicola is an unculturable bacterium that belongs to the Enterobacteriaceae family of gammaproteobacteria [15]. This bacterium is considered an obligate symbiont (P-symbiont) that coevolved with its host *B. oleae* wherein it dwells extracellularly inside the adult gut (in the oesophageal bulb, crop, midgut and female rectal pockets) and the larval midgut (gastric caeca) [15, 19]; it also lives intracellularly inside epithelial cells of the larval midgut [19]. *Ca*. E. dacicola forms bacteriomes in the larval gut, whereas in adults, it typically develops biofilms that line the inner surfaces of organs or fills the lumen of different organs with abundant free bacterial masses [19, 20]. The species occurs as two different haplotypes in Italian populations of *B. oleae* [21, 22]. Regarding its roles in host physiology, the symbiont is essential for larvae, allowing them to feed on olives, mainly when they are unripe, and neutralizing the negative effects of the phenolic compound oleuropein [23]. Moreover, *Ca*. E. dacicola is necessary for adults of the olive fruit fly as it metabolizes complex nitrogen compounds and supplies growth factors that can promote fly survival and reproduction in food-inadequate habitats such as olive orchards [24, 25].

According to the observations by Petri [17], the symbiont is vertically transmitted to the progeny: When eggs exit the oviduct, they pass through the terminal rectal tract, where the rectal sacs open and bacterial masses are deposited onto the eggs’ surfaces. Then, the larvae emerge by breaking the eggshell in the micropylar area and ingest the bacteria. This hypothesized mechanism of transmission was supported by ultrastructural investigations using SEM and TEM [19, 26], that showed the presence of abundant bacteria stored in rectal evaginations in association with the genital and anal openings.

Having established the importance of *Ca*. E. dacicola for the regular development and adult fitness of the olive fruit fly, we can understand how the symbiotic relationship might be manipulated to improve the strategies

for managing this pest. A few years ago, Estes and colleagues [27] reviewed knowledge on the possible application of the Sterile Insect Technique (SIT) for the olive fruit fly, highlighting critical issues, possible improvements and future directions. In nature, *B. oleae* larvae develop only in olives, however, several attempts have been made for developing the artificial diet for its mass rearing [27, 28].

The symbiont *Ca*. E. dacicola has never been retrieved from lab-reared olive flies [19, 29, 30], this could be due to the usage of preservatives and antibiotics that are typically added to larval and/or adult diets [28]. Moreover, the yield and quality of mass-reared olive fruit flies, in term of fitness and behavior, have yet to reach satisfactory levels [31, 32]. So that, paying particular attention to the maintenance of this symbiosis in lab strain would lead to an effective mass-rearing, developing feasible SIT programs. We believe that two approaches should be pursued: a) supply lab flies with diet-enriched transient bacteria to potentially replace the role played by the natural symbiont *Ca*. E. dacicola and b) begin the colonization process anew from wild symbiotic olive fruit flies while avoiding symbiont-removing or symbiont-suppressing procedures in the rearing protocol.

The first approach was recently initiated with promising results [33], while the second approach has to be initiated, although the rearing of wild olive fruit flies on an antibiotic-free diet for eight generations has been attempted [34].

The present study is part of a long-term research program addressing the multiple relationships between *B. oleae* and bacteria and aimed at identifying target points that might be used to develop new control strategies. To evaluate the effects of commonly used procedures to rear olive fruit flies in the laboratory on *Ca*. E. dacicola, we assessed the effects of disinfectants that are used for handling eggs, which is the first step in both small-scale and large-scale rearing efforts, through PCR amplification-denaturing gradient gel electrophoresis (PCR-DGGE), quantitative real-time PCR and Scanning Electron Microscopy (SEM). In addition, by evaluating the impacts of germicides, we ascertained the transmission mechanism of *Ca*. E. dacicola from wild olive fruit fly females to their progeny reared in laboratory.

Methods

Insects - The adults of wild olive flies used in this study developed from pupae that had been collected from infested fruits in several olive orchards in Vaccarizzo Albanese (Cosenza; Italy). Flies (approximately 800 per cage)

were housed in plastic cages (BugDorm-1, MegaView Science, Taiwan). Flies were supplied with sugar and water *ad libitum*, and kept at room temperature (18-20 °C), in order to maintain longer the stock colony. At the beginning of the experiments, to enhance egg production, flies were transferred into a conditioned rearing room with conditions of 25±2 °C, 60±10% RH and a 16:8 (L:D) photoperiod and supplied a diet of sugar, hydrolyzed enzymatic yeast (ICN Biomedicals) and egg yolk (40:10:3).

Egg collection - The eggs of wild flies were collected using wax domes that had been washed previously with 2% hypochlorite solution and then rinsed twice with deionized water. The domes were inserted into the bottom of tissue culture dishes (35/10 mm) containing approximately 3 mL of deionized water. These measures were taken to minimize the bacterial contamination and prevent egg dehydration and subsequent shrinkage. The domes were placed inside the adults' cage and left there for 24 hours. Eggs were then collected by washing the internal surface of the domes with sterilized deionized water under a laminar flow hood and sieving with a sterilized cloth, the eggs were then placed in a sterilized beaker. Finally, the eggs were collected with a sterilized micropipette and transferred to three different sterilized crucibles.

The three cruciblescontained the following treatments, respectively: a) 0.3% propionic acid solution (PA) (pH=2.82±0.03) commonly used as disinfectant in rearing procedures of the olive fruit fly [28], b) a mixture (1:1) of 1% sodium hypochlorite + 0.1% Triton X (SHTX) previously used to externally sterilize all of the developmental stages of the olive fruit fly by Estes *et al*. [35], and c) sterilized water as a control. All the eggs were vortexed for 30s, and then the eggs of the treatments PA and SHTX were rinsed twice in deionized sterilized water (in order to remove treatment residues which would have hampered DNA extraction). Eggs of each group (eggs treated with propionic acid; PAE, eggs treated with sodium hypochlorite + 0.1% Triton X; SHTXE, and eggs washed with water as a control treatment; CE) were designated for microbiological analyses as well as for morphological observations or larval development. Egg collection was performed four times during the experiment, each time from a different cage.

In addition, and in order to evaluate the bacterial titer of the water or rinse water where eggs were taken from, liquid samples were also collected for further molecular analysis: egg collection water of the control treatment (CW), the second rinse water after 0.3% propionic acid treatment (PAW) and the second rinse water after SHTX treatment (SHTXW).

An explanatory list of the samples analyzed in the experiment is summarized in Table 1.

Table 1 Explanatory legend of samples analyzed in the egg treatment experiment

Sample description	Sample name
Eggs washed with water (control)	CE
Eggs treated with 0.3% propionic acid	PAE
Eggs treated with a mixture (1:1) of 1% sodium hypochlorite + 0.1% TritonX	SHTXE
Water from control eggs	CW
Second rinse water after treatment with PA	PAW
Second rinse water after treatment with SHTX	SHTXW

Progeny development - This experiment was carried out in the same conditioned rearing room described above. Eggs intended for larval development were spread over a black fabric disk soaked in water and positioned in a Petri dish. After 48 hours, the hatched and unhatched eggs were counted. Each group of larvae from the different egg treatments (CE, PAE, SHTXE) was transferred to a cellulose-based artificial diet [32] until pupation. Then, the pupae were collected and placed in vials for adult emergence. Newly emerged adults were singly placed in small cages and fed with water and sugar until they were 15 days old, when they were dissected for bacterial DNA extraction.

DNA extraction from eggs and DGGE analysis - Ten eggs per treatment were sampled under the stereomicroscope and transferred into a 1.5 mL tube containing 50 µL of InstaGene Matrix (BioRad Laboratories, Hertfordshire, UK) plus a small quantity (approximately 8 mg) of sterile silica powder to ease egg tissue and cell disruption. Then, the content of each tube was mashed with a sterile pestle and processed for DNA extraction following the manufacturer's instructions. DNA extraction was also performed from liquid samples of the water or rinse water from treated eggs: 1.5 mL of CW, 1.5 mL of PAW and 1.5 mL of SHTW, were transferred in Eppendorf tubes and centrifuged at 13,000 rpm for 8 min. The supernatant of each sample was

replaced by 25 µL of InstaGene Matrix and processed for DNA extraction following the manufacturer's instructions. Finally, the supernatant of each vial (containing DNA from eggs or liquids) was transferred into another 1.5 mL tube and preserved at -20 °C until the molecular analyses. According to the DNA extraction, a DGGE analysis was performed to determine the presence of *Ca*. E. dacicola in the DGGE bacterial profiles before performing real-time PCR. Amplification of the V6-V8 region of the 16S rRNA gene was carried out with the universal primer pair 986F-GC and 1401R [43] in a 25-µL mixture containing 2 µL of template DNA, 1.5 mmol L^{-1} $MgCl_2$, 200 mmol L^{-1} of each deoxynucleotide triphosphate (dNTP) (Promega Corporation), 10 pmol of each primer (TIB MolBiol), 1x green GoTaq® flexi buffer (Promega), and 1 U of GoTaq® polymerase (Promega). The reaction conditions were as follows: 94 °C for 4 min, followed by 35 cycles of denaturation at 95 °C for 45 s, annealing at 55 °C for 45 s, and extension at 72 °C for 45 s; and final extension at 72 °C for 7 min. Three independent PCR amplifications were performed for each sample, and the triplicate amplification products were pooled to minimize the effect of PCR biases. The amplification products were loaded onto a polyacrylamide gel (acrylamide/bis 37.5:1; Euroclone), with a linear denaturing gradient obtained with a 100% denaturing solution containing 40% formamide (Euroclone) and 7 M Urea (Euroclone). The gels were run for 17 hours in 1X TAE buffer at constant voltage (80 V) and temperature (60 °C) using the INGENY phorU-2 System (Ingeny International BV). Then, gels were stained with SYBR®GOLD (Molecular Probes) diluted 1:1,000 in 1X TAE, and the gel images were digitized using a Chemidoc XRS apparatus (Bio-Rad).

DNA extraction from flies - *B. oleae* flies were killed by freezing at -20 °C for 15 min, washed with a 2% sodium hypochlorite solution and then rinsed twice in deionized sterile water in a laminar flow hood. Each adult's head was dissected under a stereoscopic microscope with sterile tools, and the oesophageal bulb was extracted. DNA extraction of each bulb was carried out as described above for eggs. DNA extracted from the oesophageal bulbs of wild *B. oleae* flies was amplified as described above and used as a *Ca*. E. dacicola positive control in end-point PCR and as a marker in DGGE analysis, and it was used to construct the standard curve for the real-time PCR. DNA was also extracted from the oesophageal bulbs of *B. oleae* flies developed from eggs than had been externally treated with the SHTX mixture. Amplification followed by DGGE was performed as described above.

Real-time PCR - Quantitative real-time PCR analysis was performed with primers EdF1 [23] and EdEnRev [44] was used to determine the relative abundance of *Ca*. E. dacicola varied across eggs surface treatments. Amplifications were carried out using a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hertfordshire, UK) in a 20-µL mixture containing 2X SsoAdvanced Universal SYBR® Green Supermix (Bio-Rad), 400 nmol/L of each primer and 2 µL of template DNA. The amplification conditions involved denaturation at 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. Fluorescence data were collected at the end of the hybridization step. Amplicon specificity was tested with a dissociation curve analysis by increasing the temperature by 0.5 °C every 30 s from 65 to 95 °C. Negative controls and standard curves were run on each plate. The standard curve was prepared with a sample of DNA extracted from the bulb of a wild *B. oleae* female with *Ca.* E. dacicola and 5-fold serially diluted. The efficiency of the primer pair (E) was determined by calculating the slope of the log-scale standard curve and applying the following equation: $E=10^{(-1/slope)}$ [45]. Each standard dilution and unknown sample was run in triplicate, and the threshold cycle (Ct) of these technical replicates were averaged for each individual sampled. The relative abundance of *Ca.* E. dacicola (R) was calculated according to Estes et al. [42]. The number of copies of *Ca.* E. dacicola 16S rRNA gene in egg samples treated with sodium hypochlorite (SHTXE) or propionic acid (PAE) or in water samples where eggs had been taken (CW, PAW, SHTXW) (E_{sample}) was normalized relative to the number of copies of *Ca.* E. dacicola 16S rRNA gene found in egg samples washed with water (E_{CE}) according to the formula:

$$R = E_{CE}^{(Ct\ CE)} / E_{sample}^{(Ct\ sample)}$$

Four separate real-time PCR amplifications were performed using egg samples from four experimental replicates conducted over time, and the data from each treatment were averaged over the four replicates. Quantitative real-time PCR analysis was also performed with universal primers 338F-518R [46], as described above, to determine the relative abundance of bacteria on eggs surface and rinse water as well as.

Sequence analysis - The middle portions of several DGGE bands were aseptically excised from the gel and directly sequenced by Macrogen

Service (Macrogen LTD, The Netherlands). The sequence chromatograms were edited using Chromas Lite software (v.2.1.1; Technelysium Pty Ltd.; http://www.technelysium.com.au/chromas-lite.htm) to verify the absence of ambiguous peaks and to convert them to FASTA format, DECIPHER's Find Chimeras web tool (http://decipher.cee.wisc.edu) was used to uncover chimeras in the 16S rRNA gene sequences. The sequences were analyzed via the web-based BLASTN tool (NCBI; http://www.ncbi.nlm.nih.gov/BLAST) of GenBank to identify bacterial species of highest similarity. The nucleotide sequences were deposited in the GenBank database under accession numbers MG800838 to MG800842.

Scanning electron microscopy - Fifty eggs of each treatment were dehydrated in a series of graded ethanol from 50% to 99%, with 15 min at each grade. After dehydration, the eggs were allowed to dry under a hood at room conditions. On each aluminum stub, at least 5 eggs were mounted, taking care to arrange them horizontally to obtain a clear view of the area underlying the micropylar cup, which corresponds to the base of the egg anterior pole. Mounted eggs were gold-sputtered using a Balzers Union® SCD 040 unit (Balzers, Vaduz, Liechtenstein). For the observations carried out at the Electronic Microscopy Labs at SIMAU, Polytechnic University of Marche, a FE-SEM Zeiss® SUPRA 40 scanning electron microscope (Carl Zeiss NTS GmbH, Oberkochen, Germany) and a Philips® XL 30 scanning electron microscope (Eindhoven, The Netherlands) were used. Additional investigations were conducted at the Department of Agricultural, Food and Agro-Environmental Sciences, University of Pisa, using a FEI Quanta 200 high-vacuum scanning electron microscope. The densities of the bacterial colonies present on the eggs from the three treatments were determined by counting the number of visible rods in a sample area enclosed by an electronic rectangular frame (approximately 800 μm^2) applied to the SEM screen where the base of the egg anterior pole was visible.

Statistical analyses - Quantitative data from real-time PCR and data on the bacterial colonies on the egg surface (after square-root transformation to satisfy normality requirements) were analyzed through one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) test for means separation ($P \leq 0.05$) [47]. All of the analyses were performed using Statistica 6.0 (Statsoft, Italy).

Results

DGGE analysis - The first experiment was conducted to detect the presence of *Ca*. E. dacicola on the surface of *B. oleae* eggs. The PCR-DGGE profiles of egg samples washed with water (CE) showed more complex band patterns than did those obtained from egg samples treated with propionic acid (PAE) and the mixture hypochlorite + TritonX (SHTXE) or samples of water CW, PAW and SHTXW (Figure 1).

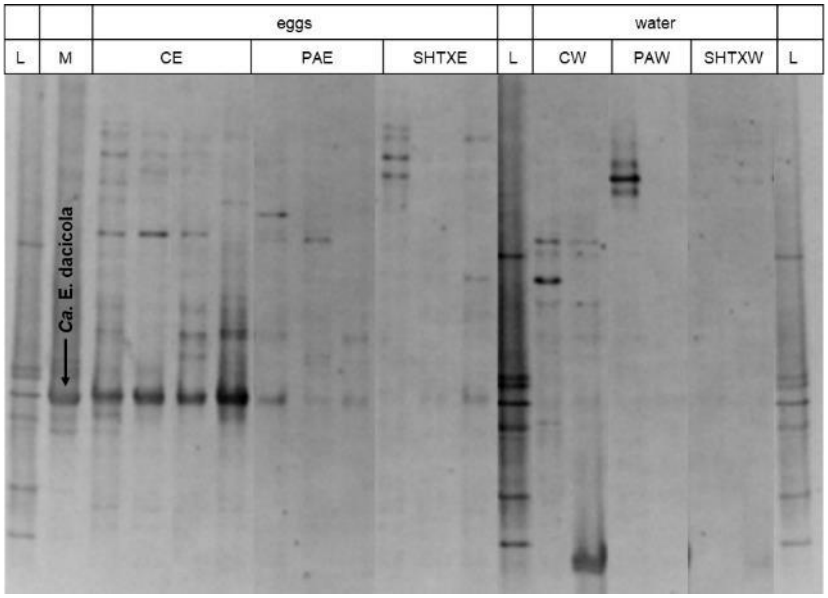

Figure 1 - PCR-DGGE profiles of the 16S rRNA gene fragments obtained by amplification of DNA extracted from egg samples and rinse water. DGGE denaturing gradient 42-68%. Arrowed band indicates a DNA fragment obtained by amplification of DNA extracted from wild fly oesophageal bulbs and used as species marker of *Ca*. E. dacicola. L, ladder; M, 16S rRNA gene fragment obtained by amplification of DNA extracted from the oesophageal bulb of a wild fly and used as marker of *Ca*. Erwinia dacicola; CE, eggs washed with water (control eggs); PAE, eggs treated with 0.3% propionic acid; SHTXE, eggs treated with sodium hypochlorite + Triton X mixture; CW, water from control eggs; PAW, second rinse water after treatment with PA; SHTXW, second rinse water after treatment with SHTX.

In each DGGE profile of eggs treated with water, a clear band was consistently present that showed the same migration behavior as the band formed by the sample of the oesophageal bulb of *B. oleae* used as marker of

Ca. E. dacicola (M). This band was also present in the other DGGE profiles and showed a decreasing intensity from CE > PAE > SHTXE and rinse water samples.

Relative abundance of *Ca*. E. dacicola in *B. oleae* eggs - The analysis of the presence of *Ca*. E. dacicola on *B. oleae* eggs laid by wild females and treated with disinfectants showed that the amount of the symbiont was decreased in the eggs of the various treatments relative to eggs of the control treatment (Figure 2).

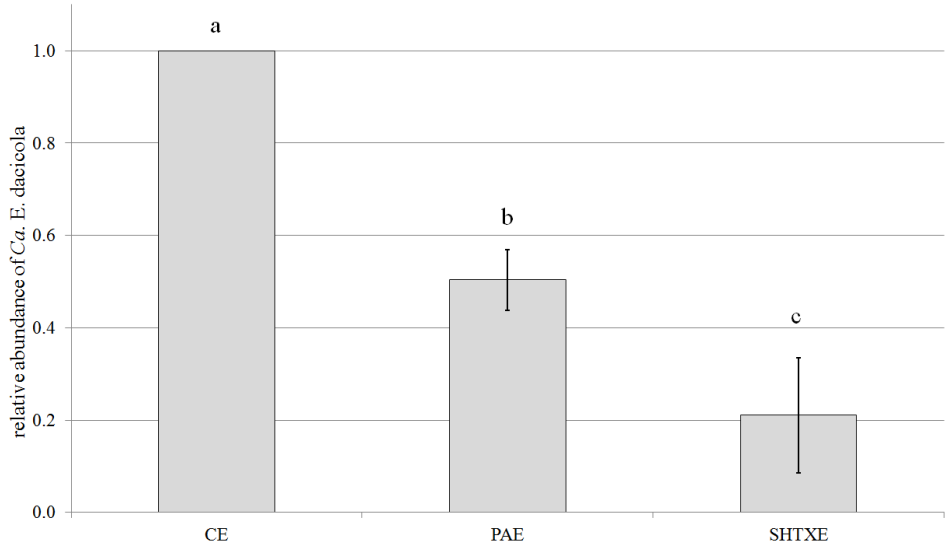

Figure 2 - Relative abundance of *Ca*. E. dacicola (mean ± SD) in eggs washed with water (CE, control eggs) considered equal to 1 in comparison with eggs treated with 0.3% propionic acid solution (PAE), or with sodium hypochlorite + Triton X (SHTXE). One-way ANOVA followed by Tukey's test at $P \leq 0.05$ (n=4) was performed; different letters above bars indicate significant differences between treatments.

Specifically, the quantity of the symbiont was reduced nearly by 2 times in eggs handled with the propionic acid solution (0.503±0.066 relative abundance of *Ca*. E. dacicola in PAE *vs Ca*. E. dacicola in CE), whereas in SHTXE, the bacterial load was decreased by approximately 5 times (0.211±0.125 relative abundance of *Ca*. E. dacicola in SHTXE *vs Ca*. E. dacicola in CE) relative to the quantity in the CE. One-way ANOVA revealed significant differences among the treatments ($F_{2,9} = 95$, $p<0.001$),

and post hoc HSD tests revealed significant differences between the various treatments and the control treatment.
Real-time PCR was performed on the rinse water of the three treatments to evaluate *Ca*. E. dacicola presence (Figure 3).

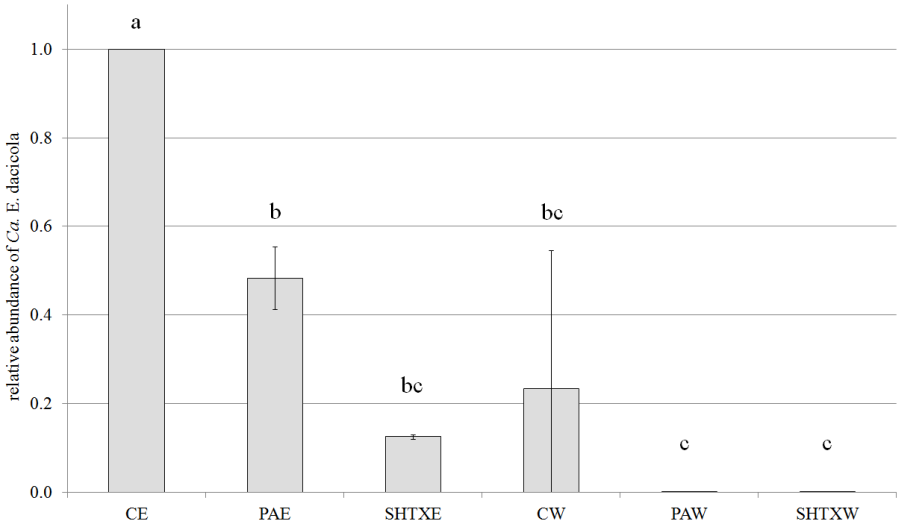

Figure 3 - Relative abundance of *Ca*. E. dacicola (mean ± SD) in eggs washed with water (CE, control eggs) considered equal to 1 in comparison with eggs treated with 0.3% propionic acid solution (PAE), sodium hypochlorite + Triton X (SHTXE) and the respective rinse water CW, PAW, SHTXW. One-way ANOVA followed by Tukey's test at $P \leq 0.05$ (n=3) was performed; different letters above bars indicate significant differences between treatments.

As expected, the relative abundance of the symbiont in the two rinse waters PAW and SHTXW was very low (0.00109±0.00017 and 0.0003±0.00021 relative abundance of *Ca*. E. dacicola in PAW and SHTXW, respectively, *vs Ca*. E. dacicola in CE). The water CW contained a greater quantity of *Ca*. E. dacicola (0.2349±0.31225 relative abundance of *Ca*. E. dacicola in CW *vs Ca*. E. dacicola in CE). Statistically significant differences were detected among treatments, with the bacterial content of the control rinse water comparable to the bacterial load on the eggs treated with both disinfectants ($F_{2,15}$ = 59 M, p<0.001). However, considerable amounts of the *B. oleae* symbiont are lost even when eggs are washed with water; the load was assessed via real-time PCR analysis as representing approximately 20% of the original load.

Morphological observations - Eggs treated with the two disinfectants (PAE and SHTXE) or washed only with water (CE) were observed via SEM. The egg of *B. oleae* is elongated and slightly curved (whole egg not shown); it is characterized by a well-developed anterior pole with an overturned cup-like protrusion that is supported by a short peduncle, forming the micropylar apparatus (Figures 4A and 4C).

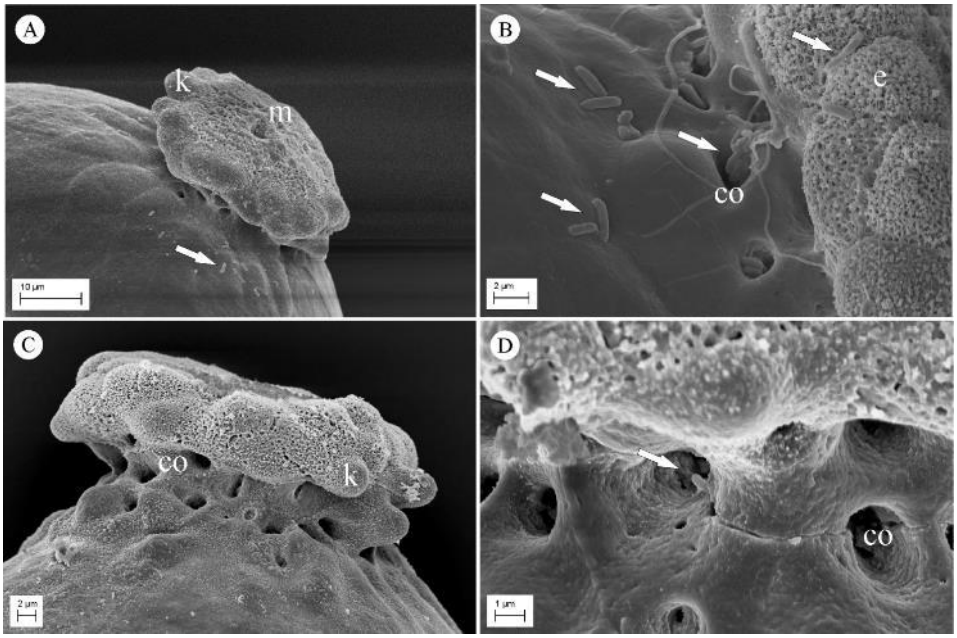

Figure 4 - Scanning electron micrographs of the anterior pole of *B. oleae* eggs. (A) Anterior pole of an egg treated with 0.3% propionic acid showing the reduction in the number of bacterial cells on the egg surface. (B) Magnification of an egg washed with water (control) showing the bacterial cells scattered on the micropylar apparatus and around the openings of the internal cavities. (C) Anterior pole of an egg treated with sodium hypochlorite + Triton X mixture (SHTX) showing the absence of bacteria on the egg surface. (D) Magnification of the base of the micropylar apparatus of an egg treated with sodium hypochlorite + Triton X mixture (SHTX) displaying a single bacterial cell (arrow) in an internal cavity opening. Arrows indicate rod-shaped bacteria; (co) cavity opening; (e) exochorionic layer with characteristic sponge-like feature; (k) knobs on protrusion margins; (m) micropylar opening.

The protrusion margins display several knobs forming a festooned rim, which give the micropylar apparatus the overall appearance of a balloon

tuft. The micropylar aperture is located in the center of the protrusion, and the peduncle shows several large openings connected with internal chambers (Figure 4). Eggs washed with water showed many rod-shaped bacterial colonies scattered on the micropylar apparatus as well as on its base, around the openings of the internal cavities (Figure 4B). In contrast, all the samples of eggs treated with SHTX or PA showed a total lack or negligible quantity of bacterial masses on the chorionic surface of the anterior pole (Figures 4A, 4C, 4D). Counts of the number of bacterial colonies within an electronic frame confirmed that treatment with the disinfectants greatly affected the presence of bacteria ($F_{2,12}$ = 23.57, p<0.001). PAE and SHTXE showed significant reductions of bacterial colonies relative to the colonies on CE (Figure 5).

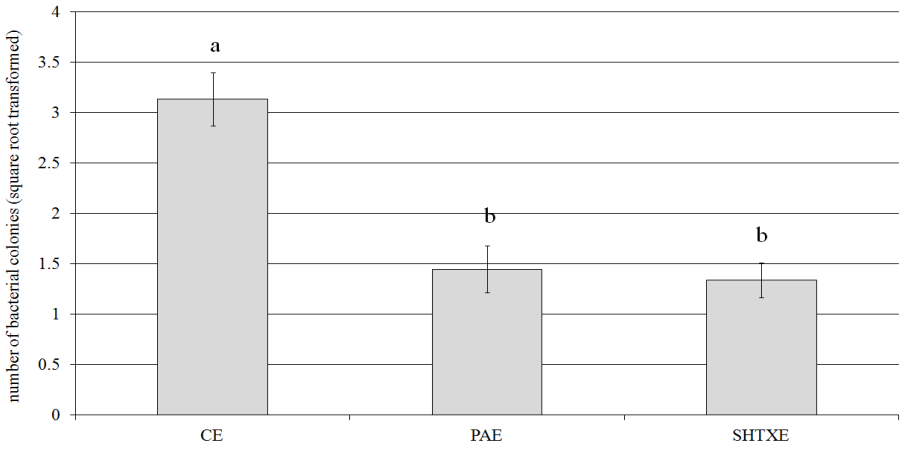

Figure 5 - Number of bacteria (mean ± SD) counted within an electronic frame in the area close to the cup-like protrusion of *B. oleae* eggs washed with water (CE) or after treatment with 0.3% propionic acid solution (PAE) or sodium hypochlorite + Triton X mixture (SHTXE). One-way ANOVA followed by Tukey's test at P≤0.05 (n=5) was performed; different letters above bars indicate significant differences between treatments.

Progeny development - Egg hatchability was low and did not differ among the treatments: on average, it was 35.99±8.01% for CE, 34.29±7.13% for PAE and 36.64±21.11% for SHTXE (4 replications; the number of eggs per treatment varied from approximately 30 to 100). Moreover, the pupal recovery was very low and variable among treatments: 6.43% (from 184 eggs) for CE, 3.42% (from 147 eggs) for PAE and 13.56% (from 189 eggs)

for SHTXE (percentages from the pooled data of 3 replications). Ultimately, only a few adults per treatment emerged from pupae reared on artificial diet: 11 from CE, 5 from PAE and 11 from SHTXE. A positive amplification product was obtained only from four oesophageal bulbs of flies that developed from SHTXE and their PCR-DGGE profiles are reported in Figure 6.

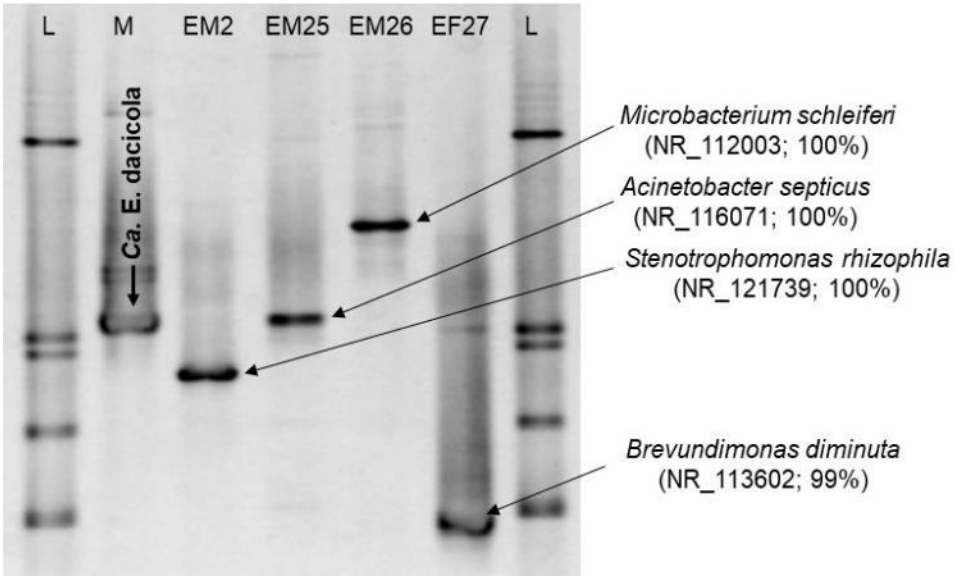

Figure 6 - PCR-DGGE profiles of the 16S rRNA gene fragments obtained by amplification of DNA extracted from the oesophageal bulb of wild *B. oleae* flies and *B. oleae* flies developed from eggs externally treated with SHTX (1% sodium hypochlorite + 0.1% Triton X mixture). DGGE denaturing gradient 48-65%. Arrowed bands indicate band excised; GenBank accession number and % sequence similarity of the nearest BLAST match are also reported. L, ladder; M, 16S rRNA gene fragment obtained by amplification of DNA extracted from the oesophageal bulb of a wild fly and used as marker of *Ca*. Erwinia dacicola; EM2, EM25-27, sample codes.

Each amplicon showed a characteristic migration pattern that differed from that produced by the *Ca*. E. dacicola marker. Bands were removed from the DGGE gels and sequenced, revealing their similarities to *Stenotrophomonas rhizophila* (100% similarity to GenBank accession number NR_121739), *Microbacterium schleiferi* (100% similarity to GenBank accession number NR_112003), *Brevundimonas diminuta* (99% similarity to GenBank

accession number NR_113602) and *Acinetobacter septicus* (100% similarity to GenBank accession number NR_116071).

Discussion

The main objective of this research was to evaluate the impact of disinfectants on the presence of *Ca*. E. dacicola on *B. oleae* eggs that had been laid by wild females. Our findings showed that only those eggs washed with water (CE) maintained most of the bacterial load delivered by the mother to the egg surface during oviposition. The bacterial symbiont on the collected eggs was *Ca*. E. dacicola, as evidenced by PCR-DGGE analysis, confirming previous studies [35].

According to our real-time PCR and SEM observations, eggs treated with PA, the antifungal agent recommended as part of standard olive fruit fly rearing procedures [28, 41], can lose up to half of the content of the symbiont transferred by the mother. Propionic acid was first evaluated and selected from among several disinfectants for its non-negative effects on egg hatching in the 1970s, when rearing procedures of the olive fruit fly were first established [42]. Propionic acid and propionates are considered as "Generally Recognized As Safe" (GRAS) food preservatives for humans. They are used as mold inhibitors and disrupt proton exchange across membranes, thereby negatively affecting amino acid transport [43]. In insect rearing protocols, propionic acid solutions are commonly recommended and used as antifungal agents, but they are considered ineffective against bacteria [44, 45]. It is likely that in our experiments, PA treatment significantly reduced the symbiont presence by facilitating the mechanical removal of bacteria from the egg surface during egg washing. Regardless of the mechanism, it appeared that its usage eliminates most of the *Ca*. E. dacicola cells transferred from the mothers to their eggs.

The second washing treatment used in our experiment was a mixture containing sodium hypochlorite and Triton X (SHTX). This mixture was used to obtain results that can be compared to those obtained by Estes et al. [35]. Sodium hypochlorite is widely used at mild concentrations to surface-sterilize insect adults before dissection, but it is also recommended for the surface sterilization of eggs for insect rearing [46]. Since bleach is a very effective bactericide, we expected a severe reduction of *Ca*. E. dacicola following the treatment of *B. oleae* eggs with the treatment mixture. Moreover, some of the bacteria present on the egg surfaces were likely to be removed by the combined surfactant action of Triton X. A detectable quantity of other bacteria, as evidenced by amplification with universal

primers, was observed only for the control water (CW) (data not shown). Exposure of DNA to sodium hypochlorite causes cleavages in DNA strands, breaking the DNA into small fragment or individual bases that precluded its amplification [47]. Therefore, we hypothesize that both PA and SHTX destroyed bacterial DNA, precluding the 16S rRNA gene amplification in rinse water.

These findings along with those of Estes et al. [35], provided better understanding about the importance of avoiding the loss of the symbiont from eggs. The relative abundance of *Ca.* E. dacicola in eggs laid by wild females had been estimated as being approximately 5,000 times lower than that in the larval stage [35]. Furthermore, the symbiont can grow and colonize the gastric caeca in the larval midgut. Thus, we speculate that common lab rearing procedures may reduce or remove the bacterial load under a minimum threshold symbiont egg load necessary to maintain the symbiotic relationship. These results clearly showed that in order to prevent reductions in bacterial transmission, efforts should be made to avoid the usage of disinfectants in egg collection. As a consequence, egg collection procedures should be improved, for instance testing different oviposition substrates where females can directly oviposit, as it has been attempted with various fruits [48, 49].

It is generally known that common procedures used in lab rearing can affect the presence of microorganisms that are associated with insects in complex symbioses. The importance of the gut microbiota in the mass rearing of the olive fruit fly has been recently noted, and new rearing methods and diets have been recommended [27, 50].

When insects are reared in a laboratory, small-scale insectary or large-scale facility, they are exposed to several sources of contamination, which are enhanced by diverse factors such as the artificial and constrained environment, the non-natural diet, and the high population density in mass rearing cages [46, 51]. For this reason, various antimicrobials are used to prevent the growth of potentially harmful microorganisms (pathogenic or non-pathogenic contaminants) in different phases of the rearing process [45, 51]. The current procedure used to rear the olive fruit fly [41] was established after numerous experimental tests to evaluate several technical conditions as well as all diet ingredients, however, the maintenance of the bacterial symbiont in the insect colony was not considered. Moreover, lab populations of the olive fruit fly, reared for successive generations under artificial conditions, have shown deleterious biological, genetic and behavioral changes [52, 53, 54]. Such alterations might be due to different causes, and antimicrobials and antibiotics are likely to be important

modifying agents. Streptomycin has been shown to negatively affect *B. oleae* larval growth [55], and nipagin has been shown to change the fly's microflora composition, causing variations in Adh allele frequencies [56]. Fitness reductions caused by antimicrobial agents have been documented in other insects, such as members of Hemiptera [57] and Lepidoptera [58]. Taking into consideration, recent findings on the olive fruit fly endosymbiont, *Ca*. E. dacicola, the indirect effects of piperacillin on adult fitness in *B. oleae* have been evaluated [24]. In addition, the toxicity of the different disinfectants used in artificial larval diets should be tested for potential destructive effects on the symbiont.

It is believed that bacterial symbionts are transmitted from olive fruit fly females to the progeny via eggs. This process was hypothesized by Petri [16, 17] and well documented by Mazzini and Vita [26]. Through SEM and TEM observations, these latter authors described the ovarian eggs and female reproductive organs as being devoid of bacteria, whereas the rectal, finger-like diverticula that converge into the ovipositor base harbor many bacterial masses. However, bacterial colonies have since been found close to the anogenital opening of the olive fruit fly female [20]. The absence of bacteria in ovarian eggs was also confirmed [59] in a study of the structure and morphogenesis of the *B. oleae* egg shell and micropylar apparatus. Moreover, submicroscopic observations have confirmed the absence of bacteria inside the vitelline membrane and the occasional occurrences of bacteria in the micropylar canal [26]. Based on these previous investigations, we can state that newly hatched larvae acquire bacterial symbionts from the cavities that underlie the micropylar apparatus, where bacteria likely grow during olive fruit fly embryogenesis and where the larva mouthparts burst at egg eclosion [60]. Our observations revealed the presence of bacterial cells over and around the micropylar apparatus, with some cells occurring inside the cavity opening.

Further insight into the symbiont's transfer can be drawn from the egg morphology of *B. oleae*. Based on previous studies [26, 59] and our SEM observations, we hypothesize that the peculiar morphology of the micropylar apparatus might be related to the transmission of the symbiont. The balloon tuft-like protrusion of the anterior pole appears to be a potentially advantageous structure for scraping bacteria from the lumen of the rectal tract, where the diverticula release their bacterial content. According to earlier studies [61] and our investigations, *B. oleae* eggs exit from ovaries with the posterior pole directed toward the ovipositor. In this way, eggs entering the ovipositor cross throughout the poky passage and are covered with bacteria that occur mainly around and below the protrusion of

the micropylar apparatus. Eggs are then laid inside the olive, oblique to the surface and with the anterior pole close to the pierced fruit skin [62, unpublished observations of the authors). The egg morphology of different species belonging to or closely related to the *Bactrocera* genus has not received much attention. Apart from some notes on *Zeugodacus cucurbitae* (Coquillet) and *B. dorsalis* (Hendel) [63], only one research, carried out using SEM, investigated the eggs of *B. carambolae* Drew and Hancock and *B. papayae* Drew and Hancock [64], the latter, recently synonymized to *B. dorsalis* [65]. None of these species display the characteristic shape of the anterior pole of *B. oleae* egg. Furthermore, eggs of *Anastrepha* species, which have been thoroughly studied, have a different micropylar shape [66]. Thus, it would be interesting to analyze and compare the micropylar structures of different species with reference to symbiont transmission.

Our initial findings on the development of eggs treated with antimicrobials appear to suggest that different bacteria may settle in the oesophageal bulb after the removal of most of the bacterial load from the eggs, including the symbiont load, as occurred after washing the eggs with SHTX. The four bacterial species recovered from flies are very different: *Stenotrophomonas*, *Brevundimonas* and *Acinetobacter* are genera of gammaproteobacteria belonging to the Pseudomonadales order, whereas *Microbacterium* is a genus of Actinobacteria. These species may be considered ubiquitous. *M. schleiferi* and *S. rhizophila* have been isolated from air, soil, water, and plants as well as from larval and insect guts [67]. *B. diminuta* is considered a major actor in the process of tissue decomposition as one of the most common organisms in the soil and other moist environments [68]. Isolates of *Brevundimonas vesicularis* were retrieved from the oesophageal bulb of wild olive flies using culture-dependent techniques in a survey aimed at studying the microbial ecology of *B. oleae* in Tuscany [29]. Although ubiquitous, *A. septicus* has mainly been isolated from animal and insect specimens (for example, *Anopheles gambiae*) and nosocomial infections [69].

Finally, considering that 1) we demonstrated a negative effect of disinfectants on the olive fruit fly symbiont, 2) olive flies can be reared on artificial diet without antibiotics for eight generations [34], 3) genetic changes can be avoided by refreshing lab colonies every five to eight generations with wild flies [32], and 4) *Ca*. E. dacicola can be transferred horizontally among adults through cohabitation, as recently shown [22], it appeared that a stable symbiotic strain of the olive fruit fly can be established and maintained under lab conditions.

Conclusions

As previously reported, wild populations of the olive fruit fly benefit from the symbiont *Ca*. E. dacicola in the larval and adult stages, while lab colonies, which lack the symbiont, display reduced fitness. However, SIT applications rely on the availability of high-quality, mass-reared insects. To establish a symbiotic laboratory strain of the olive fruit fly, *Ca*. E. dacicola must be maintained in all of the fly's developmental stages to produce high performing males and females. This research demonstrated that common disinfectants and antimicrobials used in egg collection strongly affect symbiont transmission from mother to progeny, with severe consequences, especially considering the bacterial "bottleneck" that naturally occurs in the transfer from female to larvae via the eggs. This study demonstrated a direct detrimental effect of disinfectants commonly used in olive fruit fly rearing on *Ca*. E. dacicola. To maintain the bacterial-insect symbiotic relationship in lab strains, "it is crucial to provide rearing conditions that allow the normal maintenance of the interaction", as Cohen stated [52]. Future research is needed to test different compounds and conditions for compatibility with symbiont presence in olive fruit fly lab colonies, especially during larval rearing using artificial diets, in which molds must be prevented. The findings of this research can be considered as a starting point for a general review of the entire rearing process for *B. oleae*.

Author contributions

All of the authors conceived of and designed the experiments. PS, GB and RG reared the insects and performed the experiments. PS, GB and RG carried out insect dissection and DNA extraction. RP, GB and CV designed and performed the molecular biology techniques. SR and AB conducted the SEM observations. PS and RP analyzed the data. PS, RP, GB and AB wrote the manuscript. All of the authors participated in the revision of the initial draft and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Chapter Four

Effect of different monofloral honeys on *Bactrocera oleae* endosymbiont *Ca*. E. dacicola

	Idea	Data collection	Molecular analyses	Data analysis	Manuscript preparation
My contribution	*	*	*	*	*

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Gaia Bigiotti, Roberta Pastorelli, Patrizia Sacchetti, Carol R. Lauzon Antonio Belcari (in prep.). Effect of different monofloral honeys on *Bactrocera oleae* endosymbiont *Candidatus* Erwinia dacicola.

Introduction

The olive fly and its endosymbiont - The importance of the endosymbiont *Candidatus* Erwinia dacicola for the olive fly *Bactrocera oleae* (Rossi) is widely known. The role of this bacterium in the whole *B. oleae*'s life has been investigated for a long time, even before its identification (Tzanakakis, 1985; Ben-Yosef *et al.,* 2010; Estes *et al.,* 2012). According to literature, it is part of the Enterobacteriaceae family, being included within the γ-Proteobacteria group (Capuzzo *et al.,* 2005), and it is considered a P-symbiont (persistent) for the olive fly. Even if it has never been cultivated, it can be transferred vertically to the progeny: as a matter of fact, it is smeared over the eggs through oviposition, passing from the mother to the progeny; it has been found in every flies' stage, particularly in the adult one (Estes *et al.,* 2012). *Ca*. E. dacicola is meant to be necessary for *B. oleae* fitness (Hagen, 1966; Stamopoulos and Tzanetakis, 1988) and to be strictly related to the olive tree agro-ecosystem, since its presence has never been confirmed in laboratory reared flies (Sacchetti *et al.,* 2008; Estes *et al.,* 2012). However, a recent study demonstrated that it could be horizontally transmitted from a wild to a lab reared population by cohabitation of wild and lab reared adults (Bigiotti *et al.,* in press).
The main account for its role in the olive fly's life is that it helps larvae to develop and survive in unripe olives, overcoming the effects of some compounds like oleuropein, hypothesised to be detrimental for the larval stage (Ben-Yosef *et al.,* 2015). Thus, if this essential symbiosis with *Ca*. E. dacicola was interrupted, we would assist to *B. oleae* populations reductions in field.

Honey - Honey could be defined as a "viscid, aromatic liquid obtained from plant nectars, collected by the honeybees, composed of different complex substances such as sugars, amino acids, enzymes, minerals, and vitamins with multiple beneficial health benefits" (Khan *et al.*, 2017). As a matter of fact, honey is a yellow-brownish, sticky, gluey, sweet-smelling syrup produced by the honeybee (*Apis mellifera*); it represents a natural mixture of carbohydrates (82,4%), water (17,1%), amino acids (0,5%) and other mineral and organic components in a non-relevant percentage (Garcia *et al.,* 1986; Cortes *et al.,* 2011; Khan *et al.,* 2017). On account of the presence of different types of floral nectars, dipending on diverse geographic and climatic areas, honey flavour could differ, together with its chemical composition and other characteristics (Umesh Hebbar and Rastogi 2008).

Honey in history: not only an ancient sweetener - Honey, in human history, is probably the first discovered natural sweetener ever. Even Pliny the Elder, in 77 d.C., described it as “the sweet of the heavens” in his Naturalis Historia (Bostock *et al.*, 1855). However, it was not only known as sweetener. As a matter of fact, the antimicrobial capacities and the therapeutic usage of this natural product have been known since 2000 years before the discovery that bacteria could be the cause of infections (Olaitan *et al.*, 2007). Nowadays, honey is used in many different ways, from food to medicine. Actually, it is now seen both as a very good beneficial food supplement and a therapeutic agent (Cortes *et al.*, 2011). Several aspects regarding honey composition could be involved in its antimicrobial properties. First of all, the high sugar content (84% solution of fructose and glucose) decrease the number of available water molecules for microorganisms (Olaitan *et al.*, 2007); secondly, the pH range, variable from 3.2 to 4.5, does not allow the growth of the most common pathogenic species (Olaitan *et al.*, 2007); moreover, the presence of hydrogen peroxide, produced enzymically by glucose oxidase secreted in the hypopharyngeal gland of bees for its preservation, would enhance its antibacterial activity (Mundo *et al.*, 2004; Olaitan *et al.*, 2007). Whereas, the antimicrobial activity of honey varies among its different floral origins, as documented here below.

Antibacterial properties of honey - According to the literature, the antibacterial effect of honey against pathogenic bacteria is widely documented; in spite of this, different honey types would have different antimicrobial properties (Olawuyi *et al.*, 2010; Sayadi *et al.*, 2015). Monofloral honeys seem to have a higher efficacy than multifloral ones (Mundo *et al.*, 2004; Rodriguez *et al.*, 2012). Several researches had been carried out analysing and comparing the different properties of different monofloral honeys. In a study carried out on some Mexican honeys, it has been shown that bell flower, orange blossom and eucalyptus honey (diluted at 10%) have a high antimicrobial activity against four of the most ubiquitous pathogens: *Bacillus cereus*, *Listeria monocytogenes*, *Salmonella typhimurium* and *Staphylococcus aureus* (Rodriguez *et al.*, 2012). Moreover, in a study carried out on several Malaysian honeys, the antibacterial properties of acacia honey were highlighted against *Shigella flexneri*, *Shigella sonnei*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Sayadi *et al.*, 2015). In addition, acacia honey was also tested on other Gram-negative bacterial strains as *Klebsiella pneumonia* and *Escherichia coli* (Zahoor *et al.*, 2014).

Our goal - Since these previous studies were carried out on a wide range of different bacteria, including Gram-negative ones, and since some of them belonged to the Enterobacteriaceae family, we hypothesized that the same honey types would have an antibacterial effect on *Candidatus* Erwinia dacicola - a Gram-negative bacterium that belongs to the Enterobacteriaceae family. As a consequence, the honey administrated to adult flies would change the bacterial pool contained in the oesophageal bulb, the specific organ where the symbiont is housed in the olive fly.
Thus, in this research we evaluated the effects of different types of organic honeys, *Robinia pseudoacacia* honey (acacia honey), *Castanea sativa* honey (chestnut honey) and *Citrus sinensis* honey (orange honey) on *Ca*. E. dacicola presence in the oesophageal bulb of wild *B. oleae* adults, so as to evaluate the antibacterial properties of this natural product against the endosymbiont.

Materials e Methods

Insects

Wild pupae were obtained from infested olives collected in Follonica (Grosseto, Italy) in November 2017, at the experimental orchards of Santa Paolina (CNR – INVALSA). Olives were kept in open boxes to maintain their freshness and to avoid fungi or mildew growth. After few days from the harvest, larvae started exiting olives; pupae were collected and maintained at 4°C for few days to let adults emerge at the same time. Newly emerged adults were transferred in the designed experimental cages (BugDorm®, MegaView Science, Taiwan) in a sex ratio 1:1; each cage contained 75 wild males and 75 females and was kept in an unconditioned rearing room with approximately 20±2 °C, RH 60±10%; photoperiod (L: D 16:8).

Experimental design

Four theses were set up. Cages corresponded to four different treatments: *Robinia pseudoacacia* honey (acacia honey), *Castanea sativa* honey (chestnut honey), *Citrus sinensis* honey (orange honey) and sugar as control treatment. While the control cage flies were provided water and a petri dish with white powdered sugar, flies of other treatments were fed with honey diluted with water in a solution 1:4 (v/v). Each dispenser (plastic container

and sponge) was microwaved for 2 minutes at 1000 Watt before usage for sterilization. This procedure on dispensers was replicated every three days, together with replacing the content with new water and/or new honey solution in order to avoid moulds growth. All types of honey were organic products bought at local grocery stores (Fior Fiore Coop) so as to avoid the presence of chemicals that could interfere with the expected results.

Each treatment had 1 replicate (= one cage) containing 150 flies (600 tested flies in total). Treatments were disposed randomly on a shelf unit and moved daily. Mortality was noticed day by day until the last dissection. Since the life expectance of wild flies is high and since we wanted to evaluate the effect of the designed treatments in a long-term, oesophageal bulb extractions were scheduled every 33 days; flies were dissected as follows.

Dissections

Dissections were performed on 5 males and 5 females from each treatment at dissection no. 1 (1^{st} month), and on 6 males and 6 females at dissection no. 2 (2^{nd} month), for a total amount of 88 flies). Flies were killed at -20 °C and dissected. The dissection procedure was performed under laminar flow hood in all its steps. At first, each specimen was washed with a 2% sodium hypochlorite solution and then rinsed twice in distilled sterile water. Secondly, adult's heads were cut and opened under a binocular and every oesophageal bulb was extracted, using steel tools that were sterilized in alcohol and passed through a flame step by step. Sex, sample's number and bulb aspect (transparent or milky) were noticed. Finally, each bulb was put inside a 1.5 mL Eppendorf for bacterial DNA extraction. Bacterial extracted DNA was addressed to molecular analyses.

Culture-independent microbiological analyses: PCR and DGGE analyses

Preliminary Polymerase Chain Reaction - Denaturing Gradient Gel Electrophoresis (PCR-DGGE) analysis was set out by using the 63F-GC and 518R (El Fantroussi *et al.*,1999) primers in order to clarify the presence/absence of *Ca*. E. dacicola in sampled flies. PCR-reactions were carried out using a T100 Thermal Cycler (Bio-Rad Laboratories, Hertfordshire, UK) in 25 µl volumes containing 1X Flexi PCR buffer (Promega, Madison, WI), 1.5 mM-MgCl2, 250 µM-deoxynucleotide triphosphates (dNTPs), 400 nM each primer, and 1U GoTaq®Flexi DNA

polymerase (Promega). Amplifications were performed under the following conditions: an initial denaturation of 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 45 s, and a final extension of 72 °C for 10 min. After PCR, amplified products were verified by agarose gel electrophoresis (1.5% w/v). DGGE electrophoresis was performed by loading amplicons onto a polyacrylamide gel (40% acrylamide/bis 37.5:1; Serva Electrophoresis GmbH, Germany) with a linear denaturing gradient (from 49 to 57%) obtained with a 100% denaturing solution consisting of 40% v/v deionized formamide and 7 M urea. DGGE gels were run for 17 h at 60 °C and a constant voltage (75 V), using the Dcode DGGE System (Bio-Rad). After the electrophoresis gels were stained with SYBR®Gold (Molecular Probes, Eugene, OR) diluted 1:1,000 in 1X TAE buffer, the images were digitally captured under UV light (λ = 302 nm) using the ChemiDoc XRS apparatus (Bio-Rad).

Culture-independent microbiological analyses: Real-time PCR analysis

A Real-time PCR analysis was set out aimed at quantifying *Ca*. E. dacicola in oesophageal bulbs. Amplifications were carried out in a PTC-200 Peltier Thermal Cycler with a Chromo 4 Detector (Bio-Rad) in a volume of 10μL containing 2X Sso Advanced Universal SYBR®Green Supermix (Bio-Rad), 400 nmol/L of each primer and 1 μL of template DNA. The primers used in this study were EdF1 (Estes *et al.*, 2009) and EdEnRev (Munson *et al.*, 1991) specific for *Ca*. E. dacicola 16S rRNA gene. The amplification conditions involved denaturation at 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. DNA extracted from an oesophageal bulb of a wild *B. oleae* female with *Ca*. E. dacicola was serially diluted, where "10" is the fold dilution, and used to measure the efficiency of primer pair (E) by applying the equation: $E= 10^{-1/slope}$ (Pfaffl, 2001). Amplicon specificity was tested with a dissociation curve analysis by increasing the temperature of 0.5 °C every 30 s from 65 to 95 °C. Negative controls were run on each plate. Fluorescence data were collected at the end of the hybridization step and data output were released by Opticon Monitor software version 2.03 (Bio-Rad). Each sample was run in triplicate and the threshold cycle (C_T) of these technical replicates were averaged for each individual sampled. Each unknown sample was run in triplicate, and the threshold cycle (Ct) of these technical replicates were averaged for each individual sampled. The relative abundance of *Ca.* E. dacicola (R) was calculated according to Estes *et al.* (2012). The number of copies of *Ca.* E. dacicola 16S rRNA gene in oesophageal bulb samples of flies fed with

different types of honey (AC, CH and OR) was normalized relative to the number of copies of *Ca.* E. dacicola 16S rRNA gene in oesophageal bulb samples from flies fed with sugar (S) according to the formula:

$$R = E_S^{(Ct\ S)} / E_{sample}^{(Ct\ sample)}$$

Data were analysed by one-way analysis of variance (ANOVA) followed by Fisher least-significance difference (LSD) post-hoc test to assess the significance of differences between mean values ($p < 0.05$) by using Statistica software (Palo Alto, CA, USA). Adult mortality was evaluated with Kaplan-Meier survival analysis, using log rank (Mantel-Cox) test (95% confidence interval). Survival analyses were performed with IBM SPSS Statistics software, rel. 25.0.0 (Armonk, NY, USA).

Results

Molecular analyses

DNA from oesophageal bulbs was firstly tested by PCR-DGGE to confirm the presence of *Ca*. E. dacicola. Visual inspection of DGGE revealed the presence of a single dominant band in all samples with a similar migration pattern of wild flies used as marker (Figure 1). On the other hand, real-time PCR performed on positive samples showed a reduction of the relative abundance of *Ca*. E. dacicola from the first extraction (1 month) to the second one (2 months) within all honey treatments, comparing to the control (S), with the highest significant difference in the case of Acacia honey treatment (AC). No difference was highlighted among treatments, nor at 1 month neither at 2 months (Figure 2).

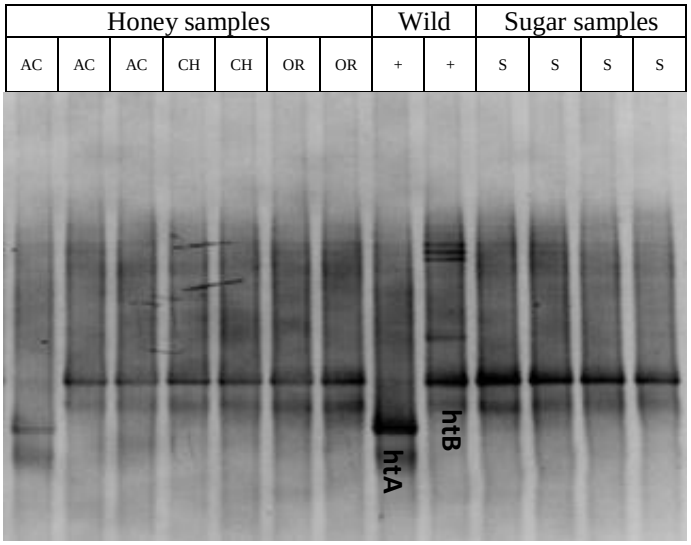

Figure 1 - DGGE performed with primers 63F-GC and 518R (El Fantroussi *et al.*, 1999). Positive controls are indicated with + and correspond to both *Ca.* E. dacicola haplotypes, htA and htB.

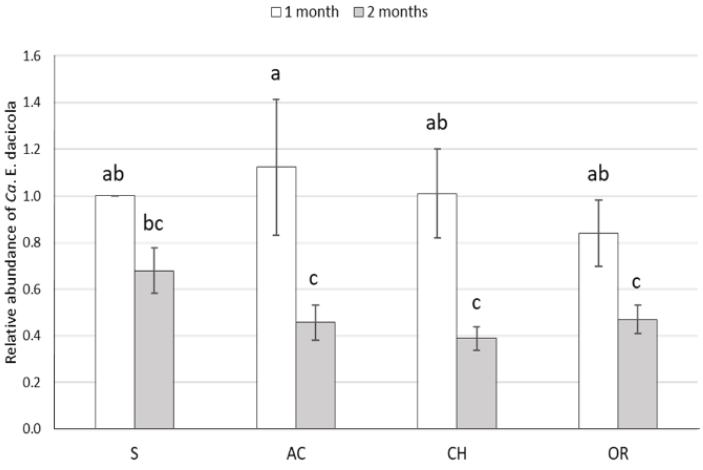

Figure 2 - Relative abundance of *Ca*. E. dacicola in samples treated with different types of honey (AC = acacia; CH = chestnut; OR = orange) vs sugar (S = sugar). Different letters indicate significant differences, test LSD ($P<0.05$).

Adult mortality

Log-Rank Mortality rates evidenced a significantly different survival probability between acacia and chestnut treatments comparing to the other two treatments (Log-rank $\chi 2 = 20.557$; d.f. = 3; $P < 0.000$) (Figure 3).

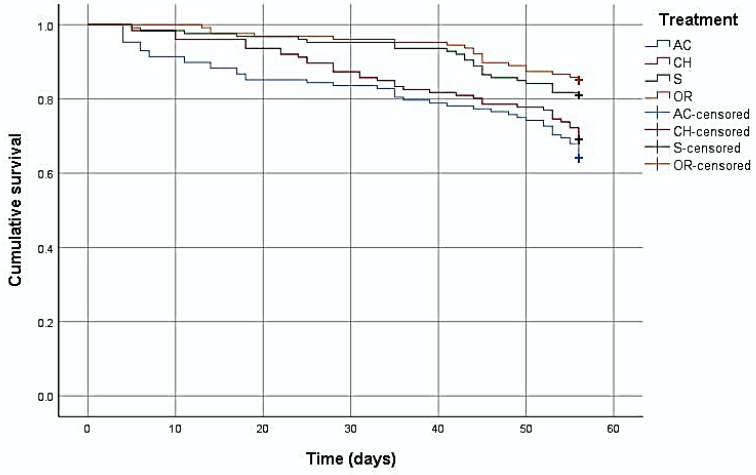

Figure 3 - Mortality rates analysis (Kaplan-Meier, log Rank test, $p < 0.05$) of wild *B. oleae* populations exposed to acacia honey (AC) chestnut honey (CH) sugar (S) and orange honey (OR).

Discussion

According to our preliminary results, a reduction of the relative abundance of *Ca*. E. dacicola was highlighted from the first extraction (1 month) to the second one (2 months) within all honey treatments, comparing to the control (S). So that, we can assume that time has been relevant in enhancing the effect within the treatment, in our case.

Moreover, the fact that acacia honey showed, in our case, the highest significant difference from the first extraction (1 month) to the second one (2 months) comparing to all treatments evidenced that this honey type is the more effective on the endosymbiont. Its antibacterial activity was already demonstrated with Gram-negative bacteria in previous studies (Zahoor *et al.*, 2014; Kim *et al.*, 2017).

Whereas, the bacterial content was reduced also in the control treatment from dissection no. 1 (1 month) and dissection no. 2 (2 months). Probably, we would have had to dissect flies also at the beginning of the experiment in order to have a better background of the bacterial content and to have a real evaluation of the effect of honey treatments. This lead us to plan further investigations with a different and more precise set up.

Another issue regarding this experiment was honey dilution. Several studies report that honey antibacterial properties depend on water dilution; if the solution is too diluted (more than 50%) honey could lose its antimicrobial activity (Olawuyi *et al.,* 2010) or that acacia honey requires concentrations of at least 25% to be efficient (Sayadi *et al*., 2015). Unfortunately, this research was planned to be compared with a previous experiment carried out at California State University East Bay (data not shown); in this previous set up, flies were fed with a honey solution 20% diluted with water, and that dilution was replicated to have comparable data.

As stated before, honey represents a natural mixture of carbohydrates, water, amino acids and other mineral and organic components (Garcia *et al.,* 1986; Cortes *et al.,* 2011; Khan *et al.,* 2017); it represents also a good food substrate for insect rearing and it is currently used in lab for several parasitoids species (Jervis and Kidd, 1996). That is why Berkeley researchers use to feed their wild *B. oleae* strains with diluted honey in lab rearing, but this could be involved with the modification of their associated microbial population, and this study represent the proof that this is not only a supposition.

However, this research contributed to open new perspectives for bacterial growth control in lab rearing procedures, highlighting that a sugar diet is preferred in order to maintain endosymbionts colonies as *Ca*. E. dacicola in wild *B. olea*e artificially reared strains.
Further investigation should be done in order to clarify antibacterial action of honey, comparing monofloral to multifloral honeys and using different diluting concentrations.

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Chapter Five

Other data: evaluation of two different irradiation doses on mating success and endosymbiont presence in wild *B. oleae* adults

Introduction

As above highlighted, the importance of improving SIT purposes to control the olive fly in field need to be explored since up to now the control methods for this pest belong to conventional methods, as chemical sprays. SIT would represent a species-specific method, increasing ecological sustainability.
Thus, another objective of this doctoral thesis was to evaluate the effect of irradiation on wild *B. oleae* adult males, both in terms of endosymbiont presence and mating success. Two different irradiation doses were tested on wild *B. oleae* pupae, 100 Gy and 150 Gy.

Methods

Insects

Wild pupae were obtained from infested olives collected in Vitolini (Vinci, Firenze) in March 2016. Olives were kept in open boxes to maintain their freshness; boxes had a fine layer of sterilized sand at the bottom to let exiting larvae to pupate in it. Pupae were collected and maintained at 15°C for few days until irradiation.

Irradiation

Irradiation was performed at the Calliope plant, Irradiation Facility of the ENEA-Casaccia Centre (Rome). Each samples or thesis (0, 100, 150 Gy) was represented by a Petri dish containing 10 g of wild pupae (1 g ≈ 170 pupae). Petri dishes were positioned at 35 cm from the irradiation source center Co_{60} (Baccaro *et al.*, 2005), according to the dosimetry no. 2/2012 with a dose rate of 384.93 Gy/h (H_2O), with absorption doses of 100 Gy e 150 Gy. Newly emerged irradiated adults were transferred in the designed experimental cages (BugDorm®). Each cage contained 75 wild males and 75 females. Cages were kept in an unconditioned rearing room with temperature 28±2 °C, RH 50±10%; photoperiod (L: D 11:13). Adults were provided water and sugar.

Molecular analyses

To detect the presence of *Ca*. E. dacicola after irradiation, oesophageal bulbs extractions were performed on irradiated flies. Dissections were

executed at four different ages (1, 5, 10 and 20 days-old) and oesophageal bulbs were extracted according to Bigiotti *et al*. (in press). Since we found *Morganella morganii* in previous bacterial detections in *B. oleae* adults of our lab strain (data not shown) bulbs were analysed by PCR using EdF1-EdEnRev (90bp) to detect the presence/absence of *Ca*. E. dacicola in oesophageal bulbs and EdF1-1507R (1400bp) primers (Estes *et al.*, 2009) to generate a nearly complete 16S rDNA fragment for confirming the presence of *Ca*. E. dacicola by ribosomal DNA restriction analysis (ARDRA), using the restriction enzyme *Cfo*I (Bigiotti *et al.*, in press).

Behavioural bioassays

Behavioural bioassays were performed in order to evaluate the effect of sterilization on mating success.
1 – Mating success in cage: three cages were set up containing 25 males and 25 females as shown (Figure 1) The bioassay lasted 2 hours. Formed couples were taken out of the cage as they formed. No. of couples, couple forming latency time and mating duration of each couple were counted.

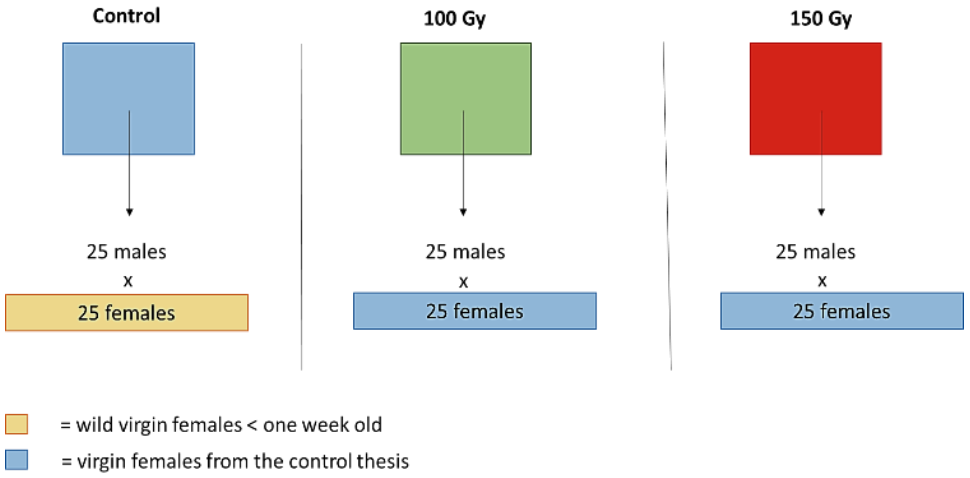

Figure 1 - Experimental design for mating success evaluation in cage.

2 – Mating success in arena: 30 minutes' bioassays were set up in arena (200 ml glass vial) to evaluate male competitiveness in arena with double choice (0 Gy irradiated male vs 100 or 150 Gy irradiated male). Bioassays were performed as shown (Figure 2). To distinguish irradiated males from

those 0 Gy irradiated, the formers were marked with acrylic colors (Polycolor®). Couple forming latency time, fights between males, wing vibrations in front of the female and other characteristic movements were noticed.

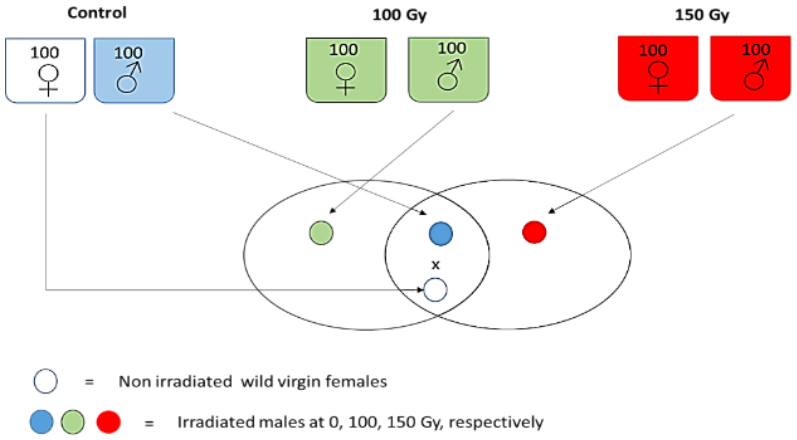

Figure 2 - Experimental design for mating success evaluation in arena.

Preliminary results

Molecular analyses

PCR at time 1 (1-day old) using EdF1-EdEnRev primers highlighted that the presence of *Ca* E. dacicola decreased as the irradiation dose increased. However, in the next three analyses (5, 10 and 20 days old) *Ca*. E dacicola has been found again in a higher concentration inside the oesophageal bulb (Figure 3). ARDRA analysis confirmed the presence of *Ca*. E. dacicola and the absence of *M. morganii* in our specimens (Figure 4).

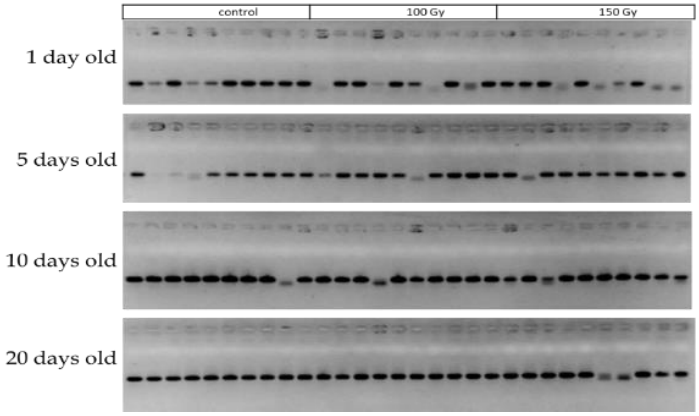

Figure 3 - PCR results on *Ca*. E. dacicola detection in oesophageal bulbs in wild irradiated adults newly emerged ad 5, 10 and 20 days' old.

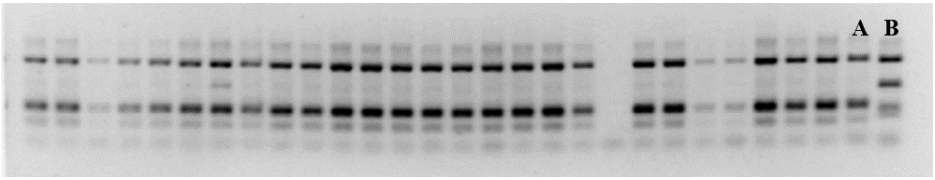

Figure 4 - *Ca*. E. dacicola presence confirmation by ribosomal DNA restriction analysis (ARDRA), using the restriction enzyme *Cfo*I (Bigiotti *et al.*, in press). A refers to the marker for *Ca*. E. dacicola (positive control) while B refers to *M. morganii*.

Behavioural bioassays

Afterwards, both bioassays in cage and in arena showed that control (0 Gy) and 100 Gy irradiated males mated more than 150 Gy irradiated ones, even if very few and non-relevant replicates. More specifically, in the case of the two-hour test in cage, 15 couples were formed in the 0 Gy and 100 Gy treatment while only 6 couples were formed in 150 Gy treatment. On the contrary, the bioassays in arena did not show satisfying results since only two couples were formed: in one case, the male was 0 Gy irradiated and in the other case it came from the 100 Gy treatment. No matings were evidenced in the case of 150 Gy irradiated males.

Discussion

Regarding *Ca*. E. dacicola presence in irradiated males, it seems that 100 Gy could be the right dose to irradiate flies without losing *Ca*. E. dacicola. Moreover, these preliminary experiments showed that *Ca*. E. dacicola content in irradiated flies seems to increase with fly age, without differences between the higher or the lower irradiation dose. Thus, since the endosymbiont did not disappear with irradiation, we might suppose that the irradiated fly would have the same "endosymbiosis benefits" as in not-irradiated wild flies in field, as longevity, male competitiveness and high fitness (Ben-Yosef *et al.,* 2010; Estes *et al.,* 2012). This let us believe in carrying out further research for SIT improvements: it will be suitable to repeat the experiment performing qPCR to quantify better *Ca*. E. dacicola loss.

Afterwards, during the bioassays in arena, we did not highlight consistent results since only two matings were observed. Probably 30 minutes was not enough for couples to form or maybe the weather conditions were not suitable (we performed bioassays in summer in an unconditioned room). However, even if they did not mate, observations carried out on males' behaviour led us to define a specific profile of irradiated males: the more they were irradiated the more they did not move, they did not show any interest toward the virgin females and they did not seem to compete with the 0 Gy irradiated male that, on the contrary, appeared to be more vital and active with the female.

Further research should be done in order to clarify the viability of *B. oleae* male sterilization trough irradiation to start SIT methods improvements.

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Chapter Six

Other data: *Candidatus* Erwinia dacicola in vitro cultivation attempts

Introduction

The relevance of *Ca*. E. dacicola in the entire *B. oleae*'s life was highlighted in several studies. It has been largely evidenced how this symbiont positively affects *B. oleae* population fitness, enhancing female fertility, pheromones production, oviposition success and larval survival in unripe olives (Estes *et al.*, 2009; Ben-Yosef *et al.*, 2015). The same studies showed some differences in the endosymbiont presence comparing olive-reared flies with artificial diet-reared ones; as a matter of fact, probably owing to the use of preservatives and antibiotics in the artificial diet, this endosymbiont disappears (Estes *et al.*, 2009), causing physiological and behavioural problems (Zervas and Economopoulos, 1982). To overcome this lack, probiotic diets would help in the endosymbiont acquisition in lab reared flies. Previous studies showed how probiotics seem to be very noteworthy for the olive fruit fly rearing (Sacchetti *et al.*, 2013 and 2014) and it would be useful to guarantee the endosymbiont presence, also in laboratory-reared adults, to increase perspectives for an efficient mass rearing. Unfortunately, *Ca*. E. dacicola has never been cultivated under artificial growth conditions, even if few scientists recently tried to achieve this goal (Piscedda, 2006; Estes, 2009). Thus, establishing the metabolic properties of *Ca*. E. dacicola in pure culture will enhance our knowledge about its physiology and how it adapts to symbiosis conditions.

Conventional cultivation of microorganisms is laborious, time consuming and, most important, very selective (Zengler *et al.*, 2002). Most part of microorganisms that could be found in the natural environment are refractory to cultivation. This phenomenon may be due to several issues such as the extremely high substrate concentrations, the lack of specific nutrients required for their growth (Zengler *et al.*, 2002), the difficulty to reproduce artificially essential aspects of their environment (pH, osmotic conditions, temperature, oxygen tension) (Stewart, 2012) and the adaptation of microorganisms to live together with other bacteria in *consortia*, interacting with each other and with other parts of the environment (Torsvik and Øvreås, 2002).

Hence, another objective in this study was to evaluate the possibility to cultivate *B. oleae* endosymbiont *Ca.* E. dacicola.

Methods

Two cultivation attempts were arranged, one in 2016 and one in 2017. Oesophageal bulb of wild *B. oleae* adults were used, in both cases, as *Ca*. E.

dacicola source. Place of the setup, flies' provenance and tested culture media changed. Details here below.

First bacterial cultivation attempt (2016) - the experiment was carried out at the Entomology Section of the Department of Agrifood Production and Environmental Sciences (DISPAA) of the University of Florence. Wild flies came out of infested olives collected in Vitolini (Vinci, Firenze) on March 2016. Olives were kept in open boxes to maintain their freshness; boxes had a fine layer of sterilized sand at the bottom to let exiting larvae to pupate in it. Pupae were collected and maintained at 15°C for few days. Newly emerged adults were then transferred in cages (BugDorm®) and kept in lab at room temperature with approximately 16±2 °C, RH 50±10%; photoperiod (L: D 10:14). Flies were dissected at 1 month old, keeping them 15 minutes at -20°C to ensure their death. The dissections procedure was performed under laminar flow hood in all its steps. At first, each specimen was washed with a 2% sodium hypochlorite solution and then rinsed twice in distilled sterile water. Secondly, each oesophageal bulb was extracted by cutting and opening heads under a stereoscopic microscope, using sterilized steel tools. Sex, sample's number and bulb aspect (transparent or milky) were noticed. Finally, each bulb was directly transferred in flasks containing 5 mL of different designed liquid media, one bulb per medium. The following substrates were tested: MacConkey, Bacto M Broth (MB; Difco), M9 (Sambrook *et al.*, 1989), Luria Bertani Broth (LB; Sambrook *et al.*, 1989), Tryptone Soy Broth (TSB; Oxoid) and Nutrient Broth (NB; Difco). To evaluate the role of the olive compounds on *Ca*. E. dacicola growth, M9 medium was tested also with or without carbon (C) source and addition of mashed olives or olive oil. Mashed olives were obtained by mashing 33 ripe olives in 10 mL of distilled water and then sterilizing by autoclaving (1mL solution per each flask) while, in the second case, 2 mL of non-sterilized olive oil were added to the culture flask.
Flasks were incubated at 25°C in both aerobiosis and anaerobiosis (liquid medium covered by 5 mL of sterile vaselline) conditions. See the table here below (Table 1) to have further details.
After 7 days, 4 mL of the bacterial suspension grown in each flask were centrifuged, and pellet was stored for further molecular analysis. DNA was extracted by using 50 µl of InstaGene Matrix (BioRad) The extracted DNA was then analysed by Polymerase Chain Reaction – Denaturing Gradient Gel Electrophoresis (PCR-DGGE) using the primers 986FGC-1401R (Felske *et al.*, 1996) and 63FGC-518R (El Fantroussi *et al.*, 1999).

Table 1 - Tested media characteristics and conditions.

Medium	Status	Additions	Air conditions	Temperature
MacConkey	liquid	none	Aerobic and anaerobic (with 5mL vaselline)	25 °C
Bacto M Broth (MB)	liquid	none	Aerobic and anaerobic (with 5mL vaselline)	25 °C
M9	liquid	none	Aerobic and anaerobic (with 5mL vaselline)	25 °C
M9 + C	liquid	Extra C source	Aerobic and anaerobic (with 5mL vaselline)	25 °C
M9 – C + oil	liquid	2 mL unsterile olive oil	Aerobic and anaerobic (with 5mL vaselline)	25 °C
M9 – C + olive	liquid	1 mL of mashed olives suspension	Aerobic and anaerobic (with 5mL vaselline)	25 °C
Luria Bertani Broth (LB)	liquid	none	Aerobic and anaerobic (with 5mL vaselline)	25 °C
Tryptone Soy Broth (TSB)	liquid	none	Aerobic and anaerobic (with 5mL vaselline)	25 °C
Nutrient Broth (NB)	liquid	none	Aerobic and anaerobic (with 5mL vaselline)	25 °C

The middle portions of several DGGE bands were aseptically excised from the gel and directly sequenced by Macrogen Service (Macrogen LTD, The Netherlands). The sequence chromatograms were edited using Chromas Lite software (v.2.1.1; Technelysium Pty Ltd.; http://www.technelysium.com.au/chromas-lite.htm) to verify the absence of ambiguous peaks and to convert them to FASTA format; DECIPHER's Find Chimeras web tool (http://decipher.cee.wisc.edu) was used to uncover chimeras in the 16S rRNA gene sequences. The sequences were analyzed via the web-based BLASTN tool (NCBI; http://www.ncbi.nlm.nih.gov/BLAST) of GenBank to identify bacterial species of highest similarity.

Second bacterial cultivation attempt (2017) - the experiment was set up at the Microbiology Department of California State University, East Bay (U.S.A) under the supervision of Prof. Carol R. Lauzon. Hundreds of dead wild flies preserved in mineral oil were obtained from California State University of Berkeley; 20 specimens of them were previously dissected and their bulb were analysed with 63f-518r and EdF1-1507r to confirm the presence of *Ca*. E. dacicola. After the endosymbiont presence confirmation, the remaining adults were dissected as described above. Finally, bulbs were directly transferred onto agar media or suspended in liquid media, one bulb per medium. The following substrate were tested: TSB, Blood agar (Tryptic Soy Agar with 5% Sheep Blood, Hardy Diagnostics), Hardy chrom + salmonella (Hardy Diagnostics), Hardy chrom + UTI (Hardy Diagnostics), UTI (Hardy Diagnostics), Urease Broth (Difco). To evaluate the role of the olive compounds on *Ca*. E. dacicola growth, TSB medium was also tested with the addition of Olive Fruit Extract (OFE) or Olive Leaf Extract (OLE), in different concentrations. OFE was obtained by mashing 30 ripe olives in 250 ml distilled water; the mixture was shaken and then stirred for 1 hour at 190 rpm. The solution was filtered, mashed olives were removed and the filtered liquid was added to agarose to create the culture medium. Besides, OLE was obtained by capsules of olive leaf extract (Nature’s way products, LLC Green Bay, WI 54311, USA) containing 12% oleuropein. Before use, OFE was tested with Mueller Hinton Agar test, confirming that it did not inhibit bacterial growth. Plates and tubes were incubated in order to test the following growth conditions: i) oxygen level: aerobic and anaerobic (in jar); ii) temperature: room temp. and incubator at 33°C. See the Table here below (Table 2) to have further details.

Bacterial growth in all liquid media was check out day by day, via spectrophotometer set up at 550 nm. Both colonies and cell suspensions were sampled and set for the DNA extraction, together with several microbiological evaluations, as McConkey test, Catalase-Oxidase test and API test.
At first, DNA extraction was performed using the DNEasy blood and tissue kit (®QIAGEN) but NanoDrop analysis (NanoDrop 2000, ThermoFisher Scientific, Waltham, Ma) highlighted that low bacterial DNA was extracted from bulbs. Hence, a new DNA Extraction Kit was used, the Extract-N-Amp™ Tissue PCR Kit; the NanoDrop analysis showed a good extracted DNA quantity that was analysed by PCR with 63f-518r and EdF1-1507r to evaluate the presence/absence of the symbiont.

Table 2 - Tested media characteristics and conditions.

Medium	Status	Additions	Air conditions	Temperature
TSB	liquid	none	Aerobic	Room and 33 °C
TSB + OFE	liquid and solid	Olive fruit extract (OFE)	Aerobic	Room and 33 °C
TSB + OLE	solid	Olive Leaf Extract (OLF)	Aerobic	Room and 33 °C
Blood agar	solid	none	Aerobic and anaerobic (in jar)	Room and 33 °C
Hardy chrom + salmonella	solid	none	Aerobic and anaerobic (in jar)	Room and 33 °C
Hardy chrom + UTI	solid	none	Aerobic and anaerobic (in jar)	Room and 33 °C
UTI	solid	none	Aerobic and anaerobic (in jar)	Room and 33 °C
Urease Broth	liquid	none	Aerobic	Room and 33 °C

Preliminary results

First cultivation attempt results

All media showed bacterial growth (Figure 1). However, even if DGGE and successively bands sequencing did not show a clear match between the positive control (*Ca*. E. dacicola) and our isolated strains (Figure 2), several minor bacteria were identified. The table here below (Table 1) shows their taxonomic classification.

Second cultivation attempt results

Spectrophotometer analysis highlighted cells growth in several culture media. In the Table 2 are reported two examples of bacterial cells readings. More specifically, among the tested media, OFE (liquid, 33 °C), OLE (solid, 33 °C), Urease Broth (liquid, 33 °C) and Hardy Chromogenic UTI (solid, 33 °C) showed a visible growth (Figure 3). McConkey test, Catalase-Oxidase test and API test highlighted that the above cited bacterial growths were Gram-negative bacteria that belonged to the Enterobacteriaceae family. Unfortunately, molecular analyses did not highlight positive bands for *Ca* E. dacicola with the above mentioned primers.

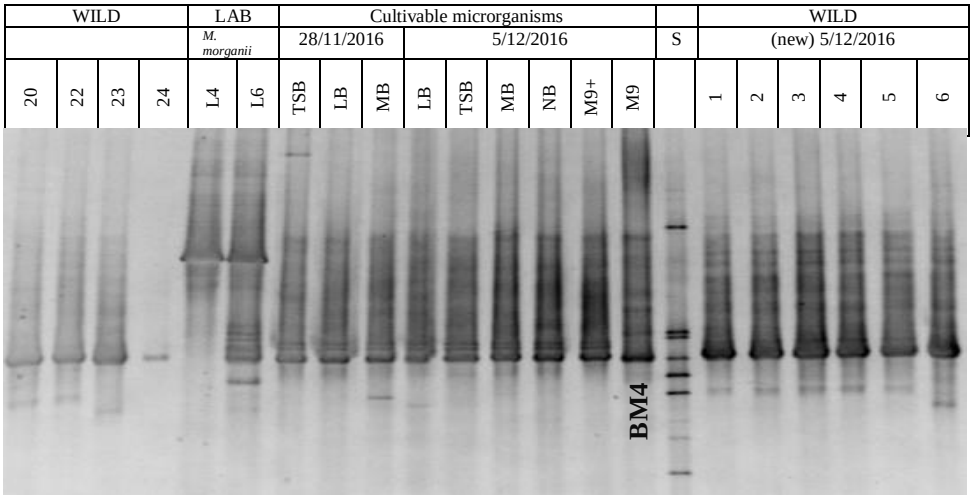

Figure 1 - DGGE with primers 986F – 1401R. The pointed band was sequenced (BM4). S = marker.

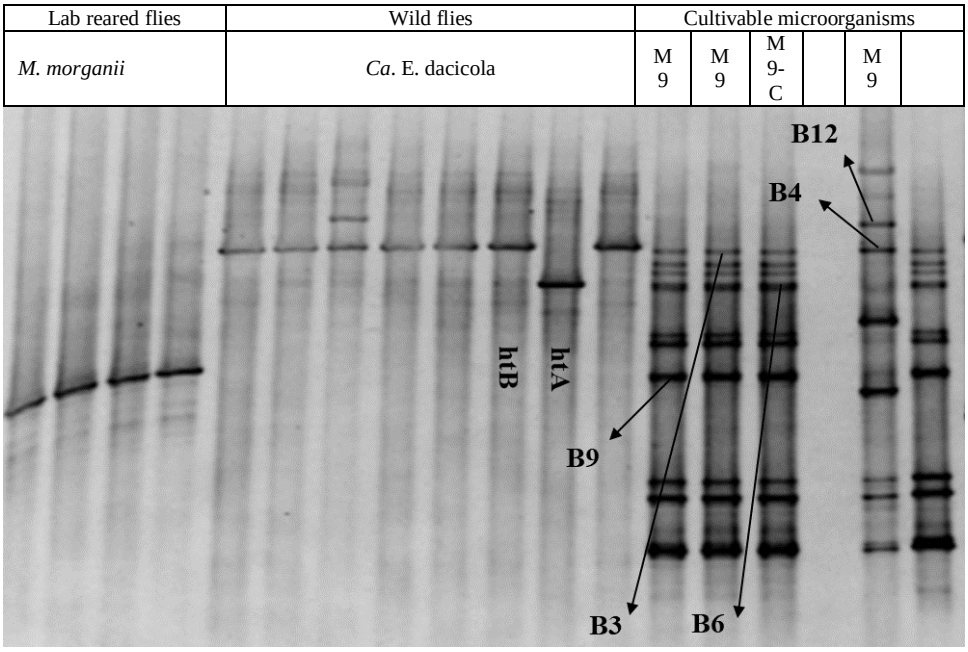

Figure 2 - DGGE with primers 63F – 518R. The pointed bands were sequenced. htB and htA refer to the two different *Ca*. E. dacicola haplotypes.

Table 1 - Identification of 16S rDNA fragments selected from PCR-DGGE from liquid cultures. Taxonomic identification was achieved by using different sequence similarity thresholds: a similarity ≥97% for a species level identification and 95%, 90%, 85%, 80% and 75% for assignment at the genus, family, order, class and phylum levels, respectively (Webster *et al.*, 2010).

Isolate PCR-DGGE gel band	Nearest match (GenBank accession no.; % sequence similarity)	Taxonomic classification
BM4	*Buttiauxella izardii* S3/2-191 (NR_025331; 100%)	*Buttiauxella izardii*
B7	*Cedecea lapagei* DSM4587 (NR_126318; 99%)	*Cedecea lapagei*
B3	*Erwinia persicina* NBRC102418 (NR_114078; 98%)	*Erwinia persicina*
B4	*Buttiauxella noackiae* NSW11 (NR_036919; 100%)	*Buttiauxella noackiae*
B6	*Erwinia persicina* NBRC102418	*Erwinia*

	(NR_114078; 98%)	*persicina*
B12	*Buttiauxella brennerae* S1/6-571 (NR_025328; 99%)	*Buttiauxella brennerae*
B9	*Kluyvera intermedia* NBRC102594 (NR_114153; 99%)	*Kluyvera intermedia*

Discussion

In any case, results on the culture of *Ca*. E. dacicola were not satisfying. However, several considerations could be done above this issue.
Regarding the first experiment, since we decided to extract DNA after 7 days for all of them, bacterial growth rate was different for each flask and in most cases many different kinds of bacteria grew in them. Moreover, also fungal growth was highlighted in most of the tested media.

Table 2 - Examples of two bacterial cells reading with the spectrophotometer. See how the number of bacterial cells increase during the days.

MEDIUM	OFE			
sample	1			
λ (nm)	550			
DAY	3-nov	4-nov	5-nov	6-nov
ABS/OD	0,214	0,223	0,242	0.291

MEDIUM	TSB			
sample	29			
λ (nm)	550			
DAY	3-nov	4-nov	5-nov	6-nov
ABS/OD	0,084		0,085	0.272

This might be due to the non-specificity of the culture media or because 7 days was a long time. We might suppose that, in seven days, other minor bacteria but with higher attitude to be cultured in vitro took over on *Ca*. E. dacicola. However, this situation does not happen inside the oesophageal bulb of the olive fly, where the endosymbiont seems to be able to overcome other bacteria's development and to make the most of the environmental conditions in which it is located. In addition, DGGE analysis represents a consistent screening method but it has the inconvenient of evidencing only dominant species, present in higher quantity (Muyzer *et al.*, 1993).

Hence, other bacteria were evidenced through DGGE band sequencing, for example: *Buttiauxella brennerae*, *Buttiauxella noackiae*, *Kluyvera intermedia* and *Erwinia persicina*. All of them grew up in M9 medium, in aerobic, anaerobic and with or without C source. While no literature shows existing relationships between *B. brennerae* or *B. noackiae* and the olive fly, *E. persicina* has been already found in association with *B. oleae* (Capuzzo *et al.,* 2005; Estes *et al.,* 2009). *Kluyvera intermedia* has been found in other insects' gut, ad in the case of the beetle *Diabrotica speciosa* (Perlatti *et al.,* 2017) or in *Dendroctonus ponderosae* (Winder *et al.,* 2010). These kind of genera have been already found in symbiosis with other fruit flies of the *Bactrocera* genus, as *Erwinia sp* and *Kluyvera sp* for *Bactrocera dorsalis* (Liu *et al.,* 2016). On the contrary, no matches with bacteria recently found in association with *Bactrocera cucurbitae* (Hadapad *et al.,* 2016) or *Bactrocera zonata* (Naaz *et al.,* 2016).

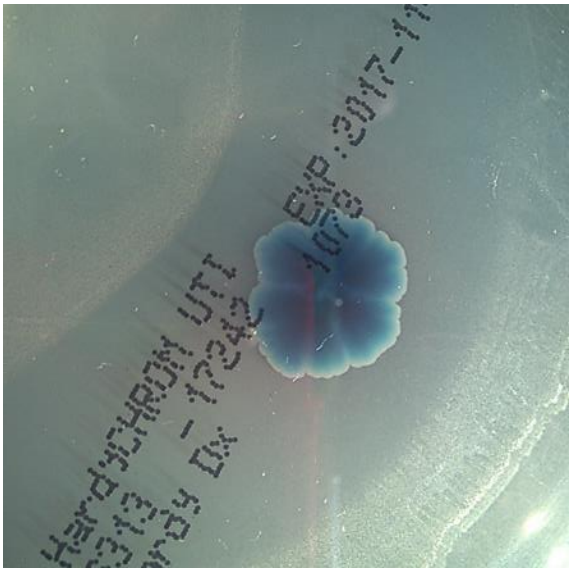

Figure 3 - "Blue" bacterial colony grown on a Hardy Chromogenic UTI plate (solid, 33°C).

It would be noteworthy to repeat the experiment with the same substrates maybe including different set up time and culture conditions sequencing all the growths.
Regarding the second experiment, aerobic conditions and a 33 °C temperature seemed to be the most favourable set for bacterial growth in lab

for the tested media, in this case. Moreover, the addition of OFE and OLE to medium could be considered good to promote Enterobacteriaceae growth. Microbiological tests in lab (API, UTI, Mc Conkey test) represent a good discrimination process and a consistent check to be added to molecular analyses. Unfortunately, we did not have the time to perform PCR on the last 4 plates of HardyChrom UTI, that showed the growth of an interesting "blue colony" (Figure 3). Thus, further trials should be set out in order to better clarify the usefulness of this medium for *Ca*. E. dacicola cultivation.
Last but not least, this experiment was hard to set up since wild flies' availability was not continuous during the year and we could not carry on analyses without fresh bulbs.

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CONCLUSIONS

The main aim of this dissertation has been to research the interaction between the olive fly *B. oleae* and associated bacteria, focusing on the endosymbiosis with *Ca.* E. dacicola, in order to improve the basic knowledge about the olive fly biology, its behaviour and to develop new biological control methods through OLF-bacteria study.

This work achieved the following positive results:

1 Horizontal transfer of *Ca.* E. dacicola, from adults of a wild *B. oleae* population to those of a lab reared strain; with this thesis we demonstrated that cohabitation of symbiotic wild flies and non-symbiotic lab flies allows the transfer of *Ca*. E. dacicola through adults. Actually, to our knowledge, the transmission of *Ca*. E. dacicola could have occurred through different methods, including mating, coprophagy or trophallaxis. Copulation between males and females was not directly verified; there is a high probability that the flies did mate, but we cannot be sure that this was the way through which the transfer occurred. The only thing we know is that the wild and lab flies stayed together for 15 days and they had time to perform several behaviours and to be in contact frequently in different ways. As a matter of fact, they had ample opportunities to regurgitate and defecate in the same cage. This observation allowed us to make a second hypothesis: perhaps not only the mating, but also the coprophagy and/or the trophallaxis behaviour between wild and lab flies during their cohabitation accounted for the horizontal transfer. Further trials assessing cohabitation between wildM x labM or wildF x labF could be set out in order to better clarify this finding. Moreover, PCR-DGGE analysis performed with the primer set 63F-GC/518R was shown to be a consistent method for screening the endosymbiont *Ca*. E. dacicola, also showing the potential to distinguish between the two haplotypes (htA and htB). This was the first successful attempt of a horizontal transfer of *Ca.* E. dacicola and the first step in acquiring a better knowledge of the endosymbiont physiology and its relationship with the olive fly. Our results could represent a starting point for the development of a laboratory symbiotic olive fly colony, improving perspectives for future applications of the Sterile Insect Technique.

2 To evaluate the antibacterial effects of copper, at two different concentrations (5% and 20%), and propolis on the presence of the symbiont *Ca.* E. dacicola in a wild population; our results suggest that

propolis and copper have several rebounds both on *B. ole*ae fitness and on the presence of its endosymbiont *Ca*. E. dacicola in oesophageal bulbs. Since both products displayed an effective antibacterial action on the symbiont, further investigations should be set out in order to evaluate if the lab tested products in this experiment may have the same effect in a natural ecosystem on wild *B. oleae* populations. Along with this, the effect of the tested products in this research should be evaluated also on the population density of beneficial organisms and useful insects, so as to verify their selectivity in field and their compatibility with biological control programs. Therefore, this study contributed to the investigation of the possible usage of two natural products against *B. oleae* populations spread in field, increasing perspectives for organic agriculture and low environmental impact control strategies, opening new possibilities for Integrated Pest Management programs.

3 To evaluate the effect of small-scale rearing procedures, such as the use of antimicrobials as propionic acid solution and a sodium hypochlorite + Triton X mixture (1:1) on the presence of bacteria on eggs laid by wild flies; focusing in the development of sustainable control methods for the olive fly such as SIT, it is important to notice that these applications rely on the availability of high-quality-mass-reared insects. Olive fly wild populations (larvae and adults) benefit from the endosymbiosis with *Ca*. E. dacicola, while those artificially lab-reared have a lower fitness, lacking the endosymbiont. So that, to establish a symbiotic laboratory strain of the olive fruit fly, *Ca*. E. dacicola must be maintained in all of the fly's developmental stages, in order to produce high performing males and females. This aspect has been investigated in this Ph.D. program, evidencing that common lab rearing procedures may reduce or remove the bacterial load under a minimum threshold symbiont egg load necessary to maintain the symbiotic relationship. We demonstrated that common disinfectants and antimicrobials used in egg collection, such as propionic acid, strongly affect the transmission of the endosymbiont, likely impacting on the vigour and the performances of obtained adults. However, no behavioural bioassays were set up in order to evaluate males' fitness or competitiveness, except the preliminary trials on irradiation (pp. 112-114, this document). Hence, since olive flies can be reared on artificial diet without antibiotics for eight generations and since *Ca*. E. dacicola can be transferred horizontally among adults through cohabitation, we could assert that it would be possible to create a stable symbiotic strain of the olive fruit fly, maintained under lab conditions, but it would be noteworthy to verify

males' performances. This would help in understand better the applicability of symbiotic-lab-reared males for SIT purposes, improving the development of new biological control methods.

4 To evaluate the natural antibacterial action of three different types of honey (chestnut, orange and acacia honey) on the presence of the symbiont *Ca*. E. dacicola in a wild population; the obtained results belong to a part of this research that does not have an applicable value in *B. oleae* biological control knowledge. In spite of this, our preliminary findings contributed to open new perspectives for bacterial growth control in lab rearing procedures, highlighting that a sugar diet is preferred in order to maintain endosymbionts colonies as *Ca*. E. dacicola in wild *B. oleae* artificially reared strains. Further investigation should be done in order to clarify antibacterial action of honey, comparing monofloral to multifloral honeys and using different diluting concentrations.

5 To evaluate the effect of irradiation on wild adult males, both in terms of endosymbiont presence and mating success; *B. oleae* male sterilization trough irradiation was one of the first aspects investigated during this Ph.D. thesis to start SIT methods improvements. However, no considerable results have been obtained through this research. Nevertheless, these preliminary results might represent a starting point to evaluate the effectiveness of irradiation for improving SIT purposes to control the olive fly in field, since up to now the control methods for this pest belong to conventional methods, as chemicals. SIT would represent a species-specific method, increasing ecological sustainability. This let us believe in carrying out further research for SIT improvements: it will be suitable to clarify irradiated males' viability, to repeat the experiment performing qPCR and to quantify better *Ca*. E. dacicola loss.

6 To cultivate in vitro *Ca*. E. dacicola; no considerable results have been obtained through this research. However, negative outcomes can always represent a good starting point for new experimental set up. As a matter of fact, our findings lead us to exclude several culture media and conditions for *Ca*. E. dacicola; it would be noteworthy to repeat the experiment with different substrates or to repeat it using the same growth media with different set up times and culture conditions. All the colonies should be sequenced in order to understand the selectivity of the media and their application. Establishing the metabolic properties of *Ca*. E. dacicola in pure culture will enhance our knowledge about its physiology and how it

adapts to symbiosis conditions. This will help in the establishment of a symbiotic laboratory strain of the olive fruit fly, increasing the availability of high-quality-mass-reared insects to be destined to SIT purposes.

We can finally affirm that *B. oleae* is still considered one of the major insect pests for the olive tree and that the study of its behaviour, its biology and its physiological needs is important to figure out the best method to control its population spread in field. This dissertation wanted to investigate the development of new biological control methods through OLF-bacteria study, exploring the possibility of SIT applications. This work clarified several important aspects of this issue and our findings could represent a starting point for future practical applications.