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# Symbiosis disruption in the olive fruit fly, *Bactrocera oleae* (Rossi), as a potential tool for sustainable control

Martina Sinno,<sup>a\*</sup> Annie Bézier,<sup>b</sup> Francesco Vinale,<sup>c,d</sup> David Giron,<sup>b</sup> Stefania Laudonia,<sup>a</sup> Antonio P. Garonna<sup>a</sup> and Francesco Pennacchio<sup>a</sup>



## Abstract

**BACKGROUND:** The olive fruit fly *Bactrocera oleae* (Rossi) (OLF) is a major agricultural pest, whose control primarily relies on the use of chemical insecticides. Therefore, development of sustainable control strategies is highly desirable. The primary endosymbiotic bacterium of OLF, '*Candidatus Erwinia dacicola*', is essential for successful larval development in unripe olive fruits. Therefore, targeting this endosymbiont with antimicrobial compounds may result in OLF fitness reduction and may exert control on natural populations of OLF.

**RESULTS:** Here, we evaluate the impact of compounds with antimicrobial activity on the OLF endosymbiont. Copper oxychloride (CO) and the fungal metabolite viridiol (Vi), produced by *Trichoderma* spp., were used. Laboratory bioassays were carried out to assess the effect of oral administration of these compounds on OLF fitness and molecular analyses (quantitative polymerase chain reaction) were conducted to measure the load of OLF-associated microorganisms in treated flies.

CO and Vi were both able to disrupt the symbiotic association between OLF and its symbiotic bacteria, determining a significant reduction in the endosymbiont and gut microbiota load as well as a decrease in OLF fitness. CO had a direct negative effect on OLF adults. Conversely, exposure to Vi significantly undermined larval development of the treated female's progeny but did not show any toxicity in OLF adults.

**CONCLUSIONS:** These results provide new insights into the symbiotic control of OLF and pave the way for the development of more sustainable strategies of pest control based on the use of natural compounds with antimicrobial activity.

Supporting information may be found in the online version of this article.

## 1 INTRODUCTION

The olive fruit fly *Bactrocera oleae* Rossi (OLF) causes severe yield losses and reduced quality of olives and derived products,<sup>1–3</sup> with an estimated 15% reduction in world production per year.<sup>4</sup> During the past 40 years, control strategies for *B. oleae* have been based on the wide use of chemical insecticides, both as bait and as cover spray.<sup>5–8</sup> However, insecticide misuse has promoted the development of resistance,<sup>6,7,9–13</sup> with a negative impact on non-target arthropods<sup>14</sup> and olive oil quality.<sup>15</sup> Therefore, sustainable control strategies for OLF are highly desirable,<sup>16,17</sup> and in line with the inspiring principles of EU Directive 128/2009 on the sustainable use of pesticides.

Currently available alternatives to chemical pesticides are the attract-and-kill method, mass trapping devices, and use of biological control agents (BCA) or sterile insect techniques (SIT), with effects that are not always satisfactory in all instances.<sup>16,18–26</sup> Unfortunately, olive cultivars showing resistance/tolerance to OLF are not available,<sup>27</sup> even though this could be a promising area of research.<sup>28–30</sup>

An alternative new opportunity for pest control relies on disruption of insect symbiosis, also known as 'symbiotic control'.<sup>31–34</sup> Insect life and evolution are strongly influenced by microorganisms associated with the insect.<sup>35,36</sup> The insect is, therefore, a

metaorganism (or holobiont) controlled by a complex network of interactions with its associated microbiota, which can be manipulated and/or suppressed by targeting either the insect or the microbial component.<sup>31,32,35,37–39</sup> The symbiotic control of insect pests has recently been reviewed by Arora and Douglas.<sup>33</sup> Among the different options that can be pursued, disruption of vertical transmission (mother to offspring) of an obligate endosymbiont required for nutrition, insect growth, reproduction or survival, seems to be an amenable target.

Implementation of symbiotic control strategies can be fostered by the discovery of novel natural molecules with antimicrobial

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activity. This appears to be a promising research area, as indicated by the successful use of antimicrobial peptides against the primary endosymbiont of *Sitophilus* sp. (Schoenherr) or *Buchnera*, the bacterial symbiont of the pea aphid (*Acyrtosiphon pisum* Harris) and the green peach aphid (*Myzus persicae* Sulzer).<sup>40–42</sup>

Since the beginning of the last century, *B. oleae* has been known to harbor endosymbiotic bacteria.<sup>43</sup> A study by Capuzzo *et al.* confirmed that a single, non-culturable bacterial species, ‘*Candidatus Erwinia dacicola*’ (Enterobacteriaceae: Gammaproteobacteria), represents nearly the entire symbiotic population associated with OLF.<sup>44</sup> Constant association of this symbiont with all life stages of different widespread OLF populations,<sup>44–50</sup> its vertical transmission to offspring,<sup>46</sup> and its specific localization, within both larvae and adults,<sup>46,47</sup> collectively indicate a highly specific and long-term symbiotic relationship.<sup>47,51,52</sup>

During mass-rearing, the standard artificial diet routinely used contains antibiotics that block the natural transmission of endosymbionts, which are replaced by environmental bacteria. In these conditions, both the adult and larval stages show a remarkable decrease in fitness.<sup>48,53,54</sup> To alleviate this, the development of antibiotic-free and probiotic-supplemented diets, as well as the horizontal transfer of the endosymbiont from wild flies to laboratory-reared strains have been successfully pursued.<sup>55–58</sup>

Recent studies suggested that ‘*Ca. Erwinia dacicola*’ is able to use many different nitrogenous sources present in the diet of wild OLF populations to synthesize essential amino acids, which are then made available to the host to complement its poor nutritional substrate, to support both development and reproduction.<sup>59–61</sup> Furthermore, a capacity to influence the oviposition behavior of its insect host has recently been demonstrated.<sup>62</sup>

Unlike most fruit fly species, which lay eggs in ripe and nutritionally suitable fruits,<sup>63–67</sup> OLF larval stages can also develop in unripe olives, exploiting a broader temporal window for multiple generations on a single host.<sup>68,69</sup> Unripe fruits are generally resistant to herbivores and pathogen attack thanks to the high secondary metabolite content, which has antimicrobial, anti-nutritive and toxic effects.<sup>70,71</sup> The unripe fruit of *Olea europaea* (L.) is no exception; in fact, it contains several secondary metabolites, the most abundant of which is oleuropein, a bitter phenolic glycoside that can contribute up to 14% of fruit dry weight.<sup>29,71,72</sup> Ben-Yosef *et al.* have lately shed light on the role of bacterial symbionts in overcoming the nutritional constraints imposed by phenolic compounds present in the unripe olive fruits.<sup>53</sup> In fact, whereas symbiotic larvae were able to develop in unripe olive fruits, their aposymbiotic counterparts could not reach the pupal stage, demonstrating that ‘*Ca. Erwinia dacicola*’ is essential for the development of *B. oleae* larvae in unripe olive fruits.<sup>53,73</sup>

Thus, ‘*Ca. Erwinia dacicola*’ is thought to play a key role in OLF larval stage survival,<sup>53,59,60,73</sup> and disruption of this symbiotic association can offer new opportunities for pest control. A recent study by Bigiotti *et al.*, showed fitness reduction in adult flies with reduced symbiont loads in the esophageal bulb as a consequence of exposure to antimicrobial compounds, such as copper and propolis.<sup>74</sup> However, no data are available on the transgenerational effects of antimicrobial treatments, and, thus, on their potential to limit larval development in unripe olive fruits, which should be the major goal of a symbiotic control strategy.

Here, we contribute to this research by investigating insecticide activity on OLF adult and larval stages, and the concurrent impact on its microbiota, exerted by antimicrobial compounds of differing origin. We focused our attention on viridiol (Vi), a secondary metabolite biosynthesized by beneficial fungi belonging to the

genus *Trichoderma* (Hendrik).<sup>75–78</sup> Vi is a steroid-like molecule with broad antimicrobial activity both *in vivo* and *in vitro*; it is produced by *T. virens* (Mill), *T. viride* (Pers.), *T. hamatum* (Bonorden), *Hypocrea virens* (Chaverri) and certain *Gliocladium* (Corda) species.<sup>79–81</sup> *Trichoderma* is one of the most studied fungal bio-control agents and its activity is also related to the variety of metabolites they produce. These metabolites have been found to directly inhibit pathogens, increase disease resistance, and also enhance plant growth in a field experiment.<sup>82</sup>

We also focused on copper oxychloride (CO), which is widely used in olive groves for disease control. Field observations of reduced OLF infestations associated with the use of CO led to the hypothesis that this compound may act as a symbioticicide,<sup>83–91</sup> which was corroborated by a recent laboratory study.<sup>74</sup> A remarkable negative effect of copper treatments on larval growth has been reported,<sup>90</sup> along with a deterrent effect on OLF oviposition.<sup>91</sup> Furthermore, CO was found to be moderately toxic when orally administered both to adults and larval stages of several insect species, including Diptera.<sup>92–95</sup> Here, we try to assess if these negative effects of CO on OLF can be due in part to its impact on associated microbiota.

Laboratory bioassays were carried out on wild populations to assess the suitability of these compounds for OLF symbiotic control. We recorded the mortality and fecundity of adult flies and larval development of their progeny in unripe olive fruits. To measure any concurrent change in the associated microbiota, we also estimated the endosymbiont load in the esophageal bulb and the midgut, as well as the overall microbiota resident in the midgut using a quantitative polymerase chain reaction (qPCR) approach.

## 2 MATERIALS AND METHODS

### 2.1 Sampling, origin and maintenance of *B. oleae* wild population

Olives were collected from trees in a pesticide-free experimental field of *Olea europaea* at the Department of Agricultural Sciences, University of Naples Federico II (Portici, NA, Italy). Infested olives were collected weekly, from September to December, in 2015 and 2016, placed in plastic trays, bottom lined with paper, and incubated in a controlled rearing room (24 ± 2 °C, 60 ± 10% relative humidity, and 12:12 h light/dark photoperiod). Mature larvae and puparia were collected daily and caged; emerged adults were sexed and maintained separately in groups of 30 flies, using cylindrical cages (20 cm diameter, 15 cm height).

### 2.2 Viridiol production and purification

For Vi production, *T. virens* strain GV41, obtained from actively growing margins of potato dextrose agar (PDA; Hi Media, Mumbai, India) cultures, was used. The fungal strain was maintained on PDA slants at room temperature, and subcultured every 2 months. Two 7-mm diameter plugs of the abovementioned strain were inoculated into 5 L conical flasks containing 1 L of sterile potato dextrose broth (PDB; Hi Media). Stationary cultures were incubated for 31 days at 25 °C and then filtered under vacuum through filter paper (Whatman No. 4, Brentford, UK). Subsequently, the filtrates were stored at 2 °C for 24 h. Two liters of the filtered culture broth of strain GV41 were extracted with ethyl acetate (EtOAc). The combined organic fractions were dried (Na<sub>2</sub>SO<sub>4</sub>) and evaporated under reduced pressure at 35 °C. The recovered yellow residue was then subjected to column chromatography (Si gel; 50 g), eluted with a gradient of EtOAc/petroleum ether (from 8:2 to 10:0). Similar thin layer chromatography (TLC) profiles fractions were combined and

subsequently purified with preparative TLC separation (Si gel; EtOAc/petroleum ether, 6:4) or silica gel flash chromatography (EtOAc/petroleum ether, 8:2 to 10:0). Preparative silica gel TLC of fractions 2, 3 and 4 yielded 6 mg of Vi. Analysis of the mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectra finally revealed the expected signals previously described for such compound, confirming the purity of the obtained product.<sup>79</sup>

## 2.3 Bioassays

### 2.3.1 Adult survival

The basic diet for OLF adults was sucrose (0.5 g) solubilized in water (200  $\mu$ L), in which were dissolved the following experimental compounds: CO (0.5%, 0.1% and 0.02% w/w sucrose) and Vi (0.5% and 0.1% w/w sucrose). As negative control, the sucrose diet completed with the antibiotic piperacillin (100  $\mu$ g mL<sup>-1</sup>; Sigma-Aldrich, S. Louis, MO, USA), abbreviated to 'antibiotic' in this study, was used to clear the endosymbiotic bacteria from the fly's gut,<sup>59,60</sup> while plain diet (sucrose only) acted as a positive control. In all cases, the diet was dried in an oven at 50 °C for 3 h and offered in a solid form to the experimental insects.

Newly emerged adults were sexed then kept in groups of 30 individuals for each sex, with *ad libitum* access to one of the different experimental diets; for each diet/treatment three groups of females and three groups of males were used, giving a total of 1260 experimental flies. From the onset of the experiment to sexual maturity, which was attained after 14 days,<sup>96</sup> the percentage of surviving flies was recorded daily along with the diet consumption per fly/day. This was obtained by calculating the difference between the weight of the diet on day 1 and that recorded on day 14, which was divided by the average number of flies found alive every day.

### 2.3.2 Adult reproduction

On the 14th day, mature adults (at least 15 females and 15 males) were allowed to mate. Non-infested and unripe olive fruits were offered to mated females for the oviposition (one olive/female). The olives in the cage were replaced after 3 days to assess any time-related change in reproductive activity. One half of the olives from these two groups was sectioned to assess the number of laid eggs per female, while the second half was incubated for 3–4 weeks to allow larval development.

At the end of the oviposition tests, all treated adults were stored at -80 °C, until further processing.

### 2.3.3 Larval development

The number of eggs laid by reared females that completed development and gave rise to adults was scored daily. The number of offspring/female was used to assess the successful development in unripe olives, which requires the presence of primary endosymbionts.<sup>53</sup>

## 2.4 DNA extraction

Frozen flies were surface-sterilized by vortexing for 15 s in a 1% sodium hypochlorite 0.1% Triton X solution, rinsed twice with distilled water and then dissected under sterile conditions in a laminar flow hood to isolate the head and the abdomen, where the esophageal bulb and midgut reside, respectively.

After dissection, pooled samples of four heads and four abdomens were separately used for DNA extraction to assess the level of bacterial load within the esophageal bulb and the midgut. DNA was extracted using a PureLink® Genomic DNA Mini Kit (Sigma-Aldrich), following the manufacturer's instructions. The quality

and concentration of the extracted DNA were evaluated using Varioskan (Thermo Fisher Scientific, Finland) and samples with a 260/280 nm ratio < 1.6 and 230/260 nm ratio < 1.5 were discarded. All the remaining DNA samples were stored at -80 °C until further processing.

## 2.5 Quantification of the endosymbiotic bacterial titer by qPCR

The amplification of 'Ca. Erwinia dacicola' 16S rDNA region was obtained with primers EdEnRev and EdF1.<sup>47,48</sup> These primers were previously validated in a study on the relative abundance of 'Ca. Erwinia dacicola' across life stages of *B. oleae*.<sup>48</sup> Even though the specificity of these primers was recently questioned,<sup>58</sup> it did not significantly affect the current study because the esophageal bulb is colonized almost exclusively by the primary endosymbiont,<sup>44,61</sup> and the gut environment of the experimental population was also predominantly colonized by the primary endosymbiont (see Fig. S1).

Moreover, for midgut samples only, total bacterial load was assessed using a pair of primers, designed with Primer Express software (Thermo Fisher Scientific), within the 16S rDNA region shared by all bacteria.<sup>97</sup> Of the three tested couples of candidate primers, only the most efficient, 16SuniF and 16SuniR, was used for further analyses.

To normalize data, different *B. oleae* housekeeping genes were used for the two body parts.  $\beta$ -actin gene was used for data normalization in the esophageal bulb samples and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used for data normalization in midgut samples, according to the study of Sagri *et al.*<sup>91</sup> Amplification of a portion of the  $\beta$ -actin gene was obtained with a pair of primers designed with Primer Express. Three pairs of primers were designed, and their efficiency was tested. The most efficient pair, Act2F and Act2R, was chosen. Amplification of a portion of the *gapdh* gene was obtained with the pair of primers GapF and GapR.<sup>98</sup> All primers are reported in Table 1.

Quantitative PCR experiments were carried out with SYBR® Green PCR Master Mix (Thermo Fisher Scientific, Lithuania) in a 13  $\mu$ L total reaction volume, containing 3  $\mu$ L of diluted genomic DNA, 6.5  $\mu$ L of Master Mix and a solution of primers with a final concentration of 300 nM. The amplification procedure used by Estes *et al.* was tested and adapted.<sup>48</sup> The experiments were performed with a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min. Reactions were followed by a quality control melting curve and terminated by cooling.

For quantification, standard curves were generated using purified amplicons of each selected target, produced with the above-mentioned primers, as a template. First, classic PCR was performed with DreamTaq PCR Master Mix (Thermo Fisher Scientific) in a total volume of 50  $\mu$ L, using 3  $\mu$ L of genomic DNA extracted from the control samples and 2  $\mu$ L of primers (10 mM each). The following conditions were used for the PCR: initial denaturation at 95 °C, 2 min; 35 cycles of denaturation at 95 °C for 30 s; annealing at 60 °C for 30 s and extension at 72 °C for 60 s; final extension at 72 °C for 10 min. PCR products were separated by electrophoresis on a 1.2% agarose gel and the expected bands were successively excised from the gel and purified with QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The concentration of purified amplicons was determined using the Qubit 2.0 Fluorometer (Life Technologies, Singapore).

**Table 1.** List of the primers used in molecular analyses

Target genes	Primers	Amplicon length (bp)
' <i>Candidatus</i> <i>Erwinia dacicola</i> ' 16S rDNA	EdF1: 5'-CTAATACCGCATAACGCTCTCG-3' EdEnRev: 5'-CCACCTACTAGCTAATCC-3'	90
Bacteria universal segment 16S rDNA	16SuniF: 5'-GACGTTACCCGAGAAGAA-3' 16SuniR: 5'-CGCCAGTAATCCGATTA-3'	198
Housekeeping genes		
<i>β-actin</i>	Act2F: 5'-GCAGAGCAAACGTGGTAT-3' Act2R: 5'-TGTGATGCCACACTTCT-3'	91
<i>gapdh</i>	GapF: 5'-GGTGTCTTTACAACAATCG-3' GapR: 5'-TAGATACGACCTTCATGTCAG-3'	148

The following equation was used to calculate the numbers of amplicon copies into the purified template with a predetermined concentration: weight of PCR fragment ( $\text{g } \mu\text{L}^{-1}$ )/(660  $\text{g mol}^{-1} \times$  pair bases number of the PCR fragment)  $\times (6.023 \times 10^{23}) =$  number of genomic copies per microliter.<sup>99</sup>

Taking the number of amplicon copies of the purified templates per microliter, five 10-fold serial dilutions were made, ranging from  $100\text{E} + 07 = 10^7$  to  $100\text{E} + 03 = 10^3$  number of amplicon copies. These serial dilutions were used to generate the standard curves. All the standard curves had very similar efficiency and slope allowing the use of  $2^{-(\Delta\Delta\text{Ct})}$  method for the relative quantification.<sup>100</sup>

In the esophageal bulb samples, the abundance of '*Ca. Erwinia dacicola*' 16S rDNA was calculated relative to the *B. oleae β-actin* reference, whereas in midgut samples, the abundance of '*Ca. Erwinia dacicola*' 16S rDNA and the abundance of the universal bacterial segment of 16S rDNA were calculated relative to the *B. oleae gapdh* reference gene.

The  $\Delta\text{Ct}$  between the reference gene (*B. oleae β-actin* and *gapdh*) and the target gene ('*Ca. Erwinia dacicola*' 16S rDNA and 16S rDNA universal bacterial segment) was calculated for each sample as:  $\Delta\text{Ct} = \text{Ct}_{\text{target gene}} - \text{Ct}_{\text{reference gene}}$ . The  $\Delta\Delta\text{Ct}$  between the  $\Delta\text{Ct}$  of the treated samples and the average  $\Delta\text{Ct}$  of the control groups was then calculated using as:  $\Delta\Delta\text{Ct} = \Delta\text{Ct}_{\text{treatment}} - \text{mean } \Delta\text{Ct}_{\text{control}}$ . Finally, the fold change  $2^{-(\Delta\Delta\text{Ct})}$  was calculated and averaged. Obtained data were log-transformed and the logarithm of the fold change  $2^{-(\Delta\Delta\text{Ct})}$  was used as an index of the relative abundance of the bacterial loads in comparison with the control.

## 2.6 Statistical analysis

The analyzed parameters were: adult mortality rate, daily diet consumption, fecundity, number of offspring/female, relative bacterial load of '*Ca. Erwinia dacicola*' and of total bacteria.

For both fitness and molecular data, distributions were analyzed, and the summary statistic calculated. Boxplots and bar charts of each distribution were generated.

One-way analysis of variance (ANOVA) was carried out for each parameter. Levene's test for homogeneity of variance from means and medians was performed to assess whether the assumption of homoscedasticity, required for ANOVA, was verified and  $H_0$  was accepted with a  $P$ -value  $\geq 0.05\%$ . The normality distribution of the residual was verified with the Shapiro–Wilk test with  $W \geq 0.05$  and  $P$  normal  $\geq 0.05$ . Tukey's pairwise *post hoc* test was used to identify the treatments that were statistically different and Dunnett's simultaneous test for level mean *versus* control mean revealed the treatments that statistically differ from the control. When data were not normally distributed, they were analyzed using the non-parametric Kruskal–Wallis test. Where

significant differences were observed, *post hoc* comparisons were performed using Mann–Whitney *post hoc* tests.

Analysis of covariance (ANCOVA) was performed to investigate whether the variability in the analyzed parameters was linked with some variables. Sex and treatment concentration were used as covariates for bioassay data.

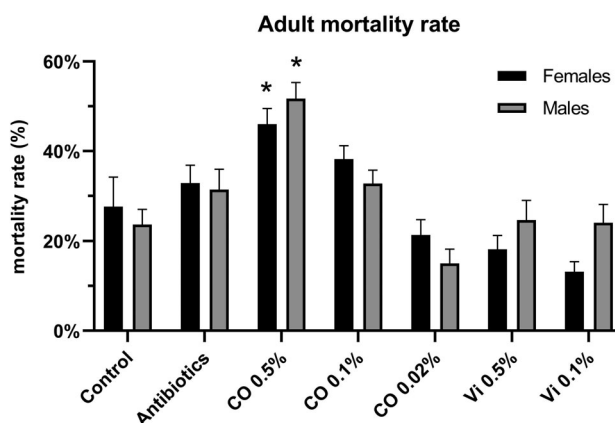
Statistical analyses of the results were carried out with Minitab 18 Statistical Software®, GraphPad Prism 8®, PAST 3® and Microsoft Excel®.

## 3 RESULTS

### 3.1 Impact of experimental treatments on OLF fitness

Taking into account all the recorded parameters, the overall outcome of the bioassays clearly indicates that both CO and Vi negatively affect OLF fitness.

The highest experimental concentration of CO (0.5%) led to a significant increase in adult mortality rate for both sexes (ANOVA,  $F_{6,28} = 7.621$ ; Dunnett's pairwise *post hoc*,  $P = 0.0224$ ,  $P = 0.0001$ ; for females and males respectively) (Fig. 1). Moreover, a dose-dependent mortality trend was observed for CO (ANCOVA,  $F_{2,16} = 16.00$ ,  $P = 0.00011$ ) (Fig. 1), which further corroborates the occurrence of direct toxicity of this compound on adult flies. None



**Figure 1.** Mean ( $\pm$ SE) adult mortality rate recorded on the 14th day of treatment. Bars marked with an asterisk were significantly different from the mean registered for controls of the same sex (Dunnett's pairwise *post-hoc* test,  $P < 0.02$ ). Control flies were fed on a compound-free diet. Antibiotics cohorts were fed on a diet containing piperacillin. CO-treated flies were fed on a diet containing copper oxochloride at different concentrations (0.5%, 0.1%, 0.02% w/w). Vi-treated flies were fed on a diet containing viridiol at different concentrations (0.5%, 0.1% w/w).

of the other experimental treatments had a significant impact on adult mortality (Fig. 1).

CO and Vi treatments had an overall negative impact on daily diet consumption, with an inverse correlation between treatment concentration and dietary intake (ANCOVA: treatment concentration:  $F_{1,10} > 6.64$ ,  $P < 0.030$ ). Reduced daily diet consumption, compared with controls, was observed for both sexes at higher CO doses (ANOVA,  $F_{6,14} = 12.67$ ; Dunnett's pairwise post-hoc  $P = 0.00015$  and  $P = 0.00028$  for females and males, respectively), and for females only for CO 0.1% and Vi 0.5% ( $P = 0.0029$  and  $P = 0.0040$ , respectively) (Fig. 2). Moreover, the dietary intake for each treatment was sex-related, with a higher level of consumption in females (ANCOVA sex,  $F_{1,10} > 7.10$ ,  $P < 0.026$ ).

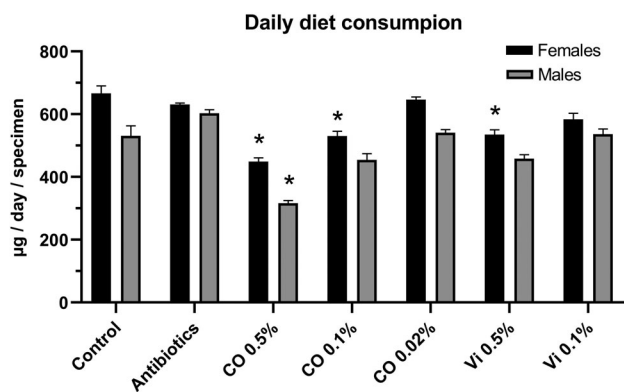
Overall fecundity (sum of the mean number of laid eggs/female recorded in the two oviposition tests) was significantly reduced only by the two higher CO concentrations (ANOVA,  $F_{6,21} = 10.44$ ; Dunnett's pairwise  $P = 0.00017$  and  $P = 0.00134$  for 0.5% and 0.1%, respectively) (Fig. 3A).

The number of offspring *per* female was statistically lower for all compounds tested (ANOVA,  $F_{6,21} = 45.21$ ; Dunnett's pairwise  $P < 0.05$ ) (Fig. 3B).

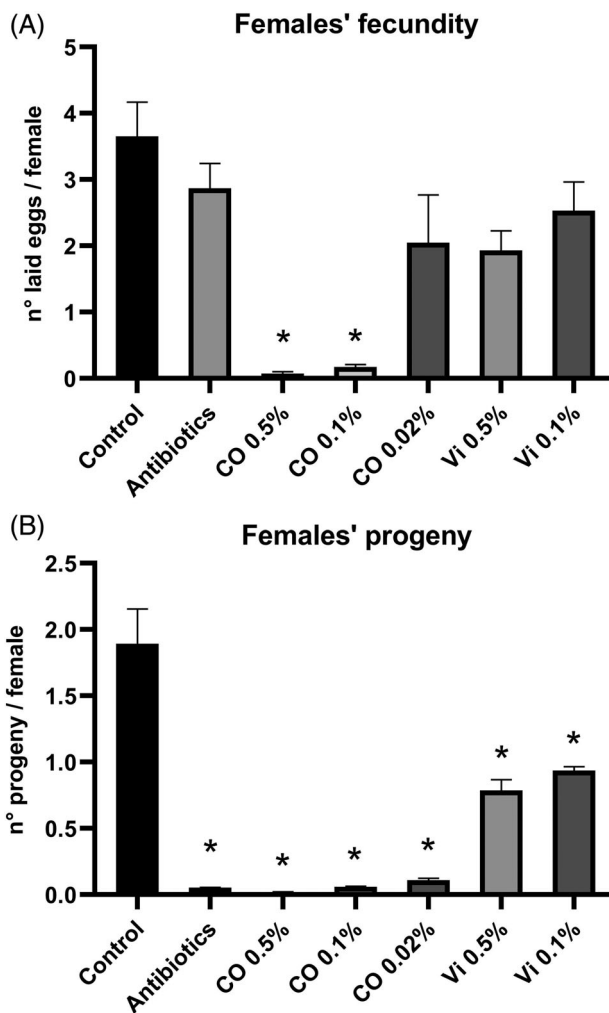
The results reported above indicate the occurrence of a direct negative impact of higher concentrations of the experimental compounds tested on OLF. However, their negative impact at lower concentrations on progeny development only, mirroring the antibiotic response, indicates that part of their overall effect on fitness is likely mediated by the negative impact they may have on OLF-associated microbiota, particularly the symbiont, which is essential for larval development.

### 3.2 Alteration of associated bacterial community

The qPCR results showed a widespread negative impact exerted by several experimental treatments on the load of the main bacterial endosymbiont of *B. oleae*, '*Ca. Erwinia dacicola*', in both the esophageal bulb (Fig. 4A) and the midgut (Fig. 4B). Endosymbiotic bacterial load in esophageal bulb samples was significantly lower (Kruskal–Wallis,  $P < 0.036$ ) in all experimental treatments, compared with controls, except for the lowest CO concentration (0.02%) (Fig. 4B). The endosymbiotic bacterial titer in the midgut



**Figure 2.** Mean ( $\pm$ SE) daily diet consumption. Bars marked with an asterisk were significantly different from the mean registered for controls of the same sex (Dunnett's pairwise post-hoc test,  $P < 0.004$ ). Control flies were fed on a compound-free diet. Antibiotics cohorts were fed on a diet containing piperacillin. CO-treated flies were fed on a diet containing copper oxychloride at different concentrations (0.5%, 0.1%, 0.02% w/w). Vi-treated flies were fed on a diet containing viridiol at different concentrations (0.5%, 0.1% w/w).



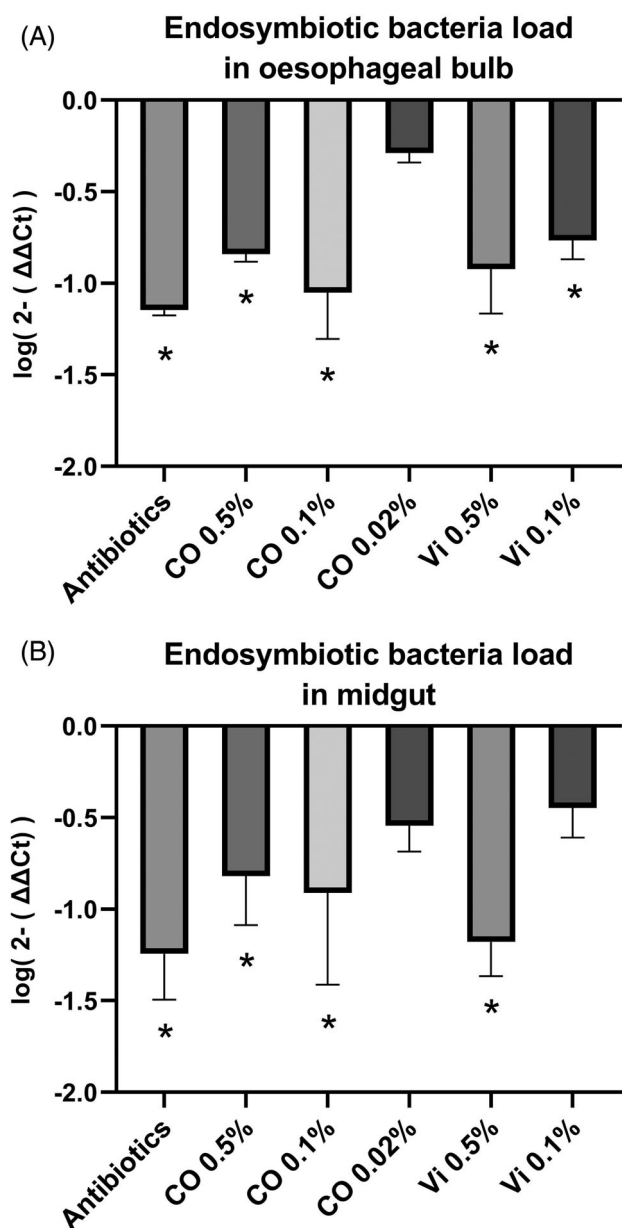
**Figure 3.** Mean ( $\pm$ SE) females fecundity (A) and mean number of offspring/female (B). Bars marked with an asterisk were significantly different from the control mean (Dunnett's pairwise post-hoc test,  $P < 0.03$ ). Control flies were fed on a compound-free diet. Antibiotics cohorts were fed on a diet containing piperacillin. CO-treated flies were fed on a diet containing copper oxychloride at different concentrations (0.5%, 0.1%, 0.02% w/w). Vi-treated flies were fed on a diet containing viridiol at different concentrations (0.5%, 0.1% w/w).

was significantly lower in all treated insects, except for the lowest concentration of CO and Vi (0.02% and 0.1%, respectively) (Kruskal–Wallis,  $P < 0.036$ ) (Fig. 4B). These results are in tune with the reduced fitness recorded in the bioassay, which is associated with a widespread decrease in the endosymbiont titer.

The total bacterial community resident in the midgut was significantly lower in all experimental treatments (Kruskal–Wallis,  $P < 0.0121$ ), except for the antibiotic and the lowest concentration of CO (0.02%) (Fig. 5). These results suggest that CO and Vi have a broad and more intense effect on the gut microbial community, whereas the high specificity of piperacillin on Enterobacteriaceae may likely allow development of resistant bacteria harbored in the gut.

## 4 DISCUSSION AND CONCLUSION

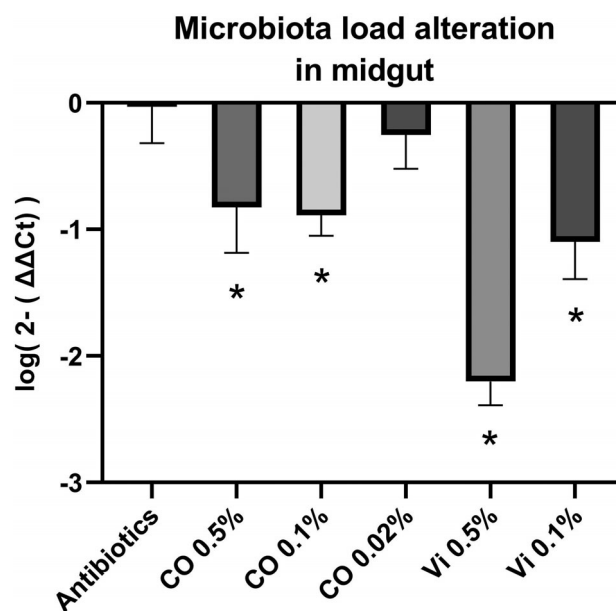
To impair the fitness of insect pests, several authors suggested the use of symbiocide compounds to interrupt vertical transmission



**Figure 4.** Mean ( $\pm$ SE) endosymbiotic bacterial load resulted from qPCR and expressed as  $\log(2^{-(\Delta\Delta Ct)})$  in oesophageal bulb (A) and midgut (B) samples of *Bactrocera oleae* females. The zero-level corresponds to the mean control load. Bars marked with an asterisk were significantly different from the control mean in the Mann–Whitney's pairwise post-hoc test. Control flies were fed on a compound-free diet. Antibiotics cohorts were fed on a diet containing piperacillin. CO-treated flies were fed on a diet containing copper oxychloride at different concentrations (0.5%, 0.1%, 0.02% w/w). Vi-treated flies were fed on a diet containing viridiol at different concentrations (0.5%, 0.1% w/w).

of primary insect endosymbionts.<sup>31–33</sup> Here, we test the potential of symbiosis disruption for control of *Bactrocera oleae*, one of the major agricultural pests of olive orchards.

Bioassays performed in this study, across two subsequent generations, allowed us to tease apart: (i) the direct negative impact (i.e. mortality, reduced food intake, reduced fecundity) of the experimental compounds; and (ii) indirect effects mediated by alteration in the symbiotic load and gut-associated microbial community (i.e. larval progeny development, number of



**Figure 5.** Mean ( $\pm$ SE) total bacterial load resulted by qPCR and expressed as  $\log(2^{-(\Delta\Delta Ct)})$  in midgut samples of *Bactrocera oleae* females. The zero-level corresponds to the mean control load. Bars marked with an asterisk were significantly different from the control mean in the Mann–Whitney's pairwise post-hoc test. Control flies were fed on a compound-free diet. Antibiotics cohorts were fed on a diet containing piperacillin. CO-treated flies were fed on a diet containing copper oxychloride at different concentrations (0.5%, 0.1%, 0.02% w/w). Vi-treated flies were fed on a diet containing viridiol at different concentrations (0.5%, 0.1% w/w).

offspring). Reduced survival and fecundity of treated flies are due to a direct negative impact of the administered compound. In fact, it is known that the presence of '*Ca. Erwinia dadicola*' is irrelevant for *B. oleae* females' fecundity when flies are reared on a nitrogen-free diet, as in our experimental bioassays.<sup>59,60</sup> This was also confirmed by our results because the survival and fecundity of aposymbiotic females, treated with antibiotics, were not statistically different from controls. On the contrary, the survival and fecundity of CO-treated flies were highly reduced in a dose-dependent manner, as expected based on previous studies demonstrating copper toxicity on insects.<sup>92–95</sup> By contrast, Vi treatment did not affect female survival and fecundity, displaying no significant toxic effect.

The presence of '*Ca. Erwinia dadicola*' is essential for *B. oleae* larval survival in unripe olive fruit.<sup>53</sup> Our results demonstrate that the reduction in fitness induced by some of the experimental treatments may be exclusively due to their negative impact on insect-associated microorganisms. Indeed, the number of offspring per female clearly decreases in treated insects, suggesting an anti-symbiotic effect of the administered compounds. Antibiotic treatment led to a drastic reduction in the number of offspring of up to 97%, compared with the control cohort, in agreement with previous results by Ben-Yosef *et al.*<sup>53</sup> In addition, all the administered compounds led to a significant reduction in offspring, with a decrease ranging from 99% to ~50%, for CO 0.5% and Vi 0.1%, respectively. It is worth noting in the case of Vi, these results are associated with a total absence of direct negative effects on OLF. This is an interesting issue when considering the direct toxic effect this compound may have on non-target arthropods.

In the case of CO, the higher experimental concentrations used (0.5% and 0.1%) had a direct toxic effect, which reduced the



number of eggs laid by females, further reinforcing the negative impact on insect fitness mediated by a reduction in symbionts. Our study provides additional evidence for an anti-symbiotic effect of CO on OLF and a possible explanation for the high larval mortality reported in CO-sprayed olive fruits.<sup>83–90</sup> In the current study, there is strong evidence that the oral administration of CO can affect both OLF adult and larval fitness, as a result of both a toxic and a symbiocide effect. The symbiocide effect persists at the lowest concentration used, whereas a strong toxic effect occurs when higher doses are used supporting the toxicity of copper for insects in a dose-dependent manner.<sup>84–87</sup> The double effect of CO could make this compound particularly useful for OLF control, as suggested by previous field observations.<sup>92–95</sup> A recent study by Bigiotti *et al.* demonstrated the symbiocide effect on OLF of two different copper products after oral administration.<sup>74</sup>

The quantification of microbial loads in OLF exposed to the experimental treatments clearly indicates that the capacity of larvae to develop in unripe olives is highly dependent on the presence of primary symbionts, which are affected to a differing extent by the compounds tested. Changes in progeny development can be linked to substantial changes in 'Ca. *Erwinia dacicola*' load but treatments also strongly affected the entire microbiome composition. If and how alterations of the whole bacterial community compared with 'Ca. *Erwinia dacicola*' are partially responsible for the observed fitness reduction remains to be studied.

Our results shed light on the possible use of natural products for symbiotic control of OLF. Indeed, Vi has a strong negative impact on endosymbiont load and on the overall load of gut microbiota, which is associated with a reduced OLF larval survival. Vi has shown remarkable antibiotic activities against some bacteria and phytopathogenic fungi,<sup>76–82</sup> but, to our knowledge, this is the first time that a fungal metabolite has been used for symbiotic control of OLF, via the disruption of its endosymbiotic bacterial load. Interestingly, the fungal metabolite 3-O-methylfunicone from a beneficial endophytic strain of *Talaromyces pinophilus* displayed toxic effects against the pea aphid *Acyrtosiphon pisum* (Homoptera, Aphididae).<sup>76</sup>

In conclusion, our results pave the way for use of this natural compound to disrupt *B. oleae* endosymbiosis, fostering the development of more sustainable strategies of OLF control. However, even though the absence of direct negative effects on OLF adults exerted by the lower concentration of CO and Vi appears promising in terms of possible toxic effects on non-target arthropods, further studies are needed to assess the risk associated with use of these compounds.

## AUTHOR CONTRIBUTIONS

FP, APG, SL, DG, FV, and MS conceived and designed the study. MS conducted experiments. AB assisted in the qPCR set-up. FV provided the fungal metabolites. FP and MS wrote the manuscript, and APG, SL, AB, FV, and DG revised and improved the manuscript. All authors reviewed and approved the final version of the manuscript.

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## SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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