
University of Thessaly

Master's Degree Program Host-Microbe Interactions

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# Development of RNAi-Based Insecticides Against the Mediterranean Fruit Fly *Ceratitis capitata*: Gene Target Screening and Microbiome Effects

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Larissa, 2025

This Master's thesis was conducted at the Laboratory of Plant and Environmental Biotechnology, Department of Biochemistry and Biotechnology, University of Thessaly, in Larissa, under the supervision of Professor Kalliopi Papadopoulou.

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# Abstract

The Mediterranean fruit fly, *Ceratitis capitata* (Diptera: Tephritidae), is a major agricultural pest, causing significant crop losses worldwide. RNA interference (RNAi)-based insecticides represent a promising, species-specific pest control strategy due to their sequence-based mode of action, which minimizes impacts on non-target organisms. To identify potential RNAi target genes, we utilized a large-scale RNAi screen of over 1.500 genes in *Tribolium castaneum* and selected 13 candidate genes for testing via oral delivery in *Ceratitis capitata*. Of these, three genes-*ETI3A*, *CSB*, and *SF3B1*-showed statistically significant downregulation. Further validation by dsRNA injection confirmed significant silencing for *ETI3A*, while *SF3B1* and, to a lesser extent, *CSB*, also showed reduced expression levels. Phenotypic analysis of dsETI3A- and dsSF3B1-treated individuals revealed a reduction in the number of larvae developing into pupae and in adult emergence, suggesting a functional impact of gene silencing. We then explored whether RNAi efficiency was associated with alterations in the gut microbiome. While bacterial community composition shifted between the 2$^{nd}$ and 3$^{rd}$ instar larval stages, no clear differentiation was observed between treatment groups. However, some bacterial taxa varied between treatments, suggesting a potential interaction between host RNAi response and gut microbial dynamics.

# Introduction

## RNAi Insecticides

### Mechanism of Action

RNAi mechanism was first described in petunia in 1990 (Napoli et al., 1990) and characterized as gene silencing in the nematode *Caenorhabditis elegans* only a few years later (Fire et al., 1998). Since its discovery, RNAi has become a powerful tool across diverse fields, including agriculture. RNAi pesticides, which are part of the biological pest control methods, have been studied as enhancers of plant resistance against viruses, viroids, nematodes, insect pests, and fungi (Dalakouras et al., 2019).

RNAi is a mechanism that is conserved across eukaryotic cells and serves as natural gene regulator and antiviral defense mechanism (Hernández-Soto & Chacón-Cerdas, 2021). In insects and other animals, RNA interference is comprised of three main pathways, each mediated by a distinct class of small RNAs: small interfering RNAs (siRNAs), microRNAs (miRNAs), and Piwi-interacting RNAs (piRNAs) (Zhu & Palli, 2020). Double-stranded RNA (dsRNA) of either endogenous or exogenous origin initiates the siRNA pathway, which is essential for transposable element suppression and antiviral defense. The miRNA pathway is activated by endogenously transcribed single-stranded RNAs (21–24 nucleotides long) that regulate gene expression levels. These two pathways are active throughout the whole insect body and their mechanism of action has several similarities (Figure 1a and b): both dsRNA and pre-miRNA are cleaved by the endoribonuclease Dicer and the RNA fragments are loaded onto the Argonaute (Ago) protein, forming the RNAi induced silencing complex (RISC). This complex then guides the RNA to find its supplementary mRNA target, leading to its cleavage. Regarding the piRNA pathway, it is mediated by slightly longer single-stranded RNAs (23–36 nucleotides) and functions primarily to silence transposons, and is mainly active in germline cells (Figure 1c) (Zhu & Palli, 2020).

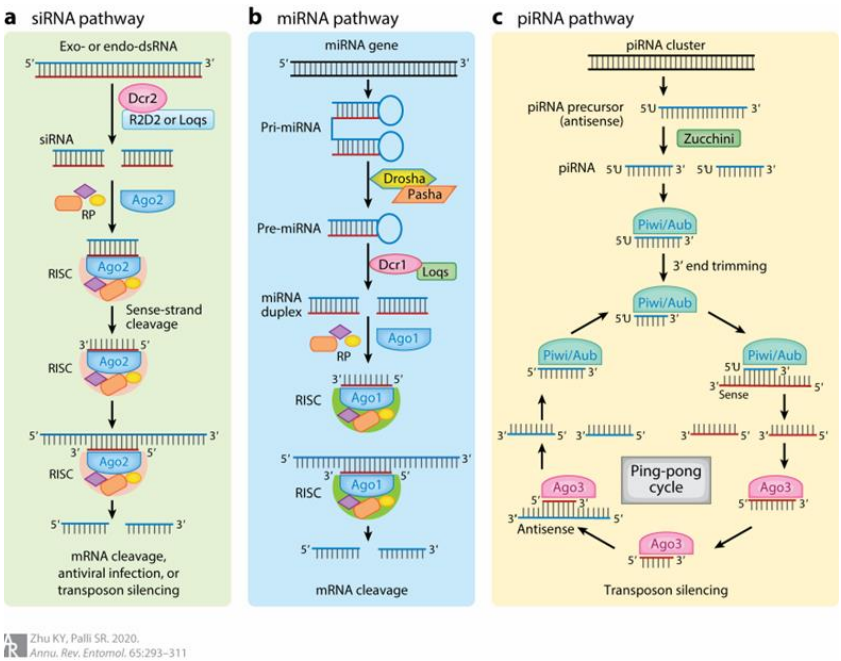



*Figure 1: RNAi pathways in insects (Zhu & Palli, 2020)*

## Application methods

RNAi-based biopesticides are divided into two categories: plant-incorporated protectants (PIPs) and non-plant-incorporated protectants (non-PIPs). In the case of PIPs, plants are genetically modified to produce dsRNA, which target specific pest genes. On the other hand, non-PIPs refer to the external application of dsRNA without genetic modification of the plant. This exogenous application can be performed through various methods such as foliar spraying, trunk injection or root irrigation (Luo et al., 2024).

In the case of plant-incorporated protectants, numerous studies have demonstrated the effectiveness of genetically modified plants in producing dsRNAs for pest control (Germing et al., 2025). In these systems, the plant genome is engineered to express dsRNAs either from the nuclear or chloroplast genome. This approach provides long-term protection, as the dsRNA expression is stably inherited across generations. Maize (*Zea mays*) and its insect pests represent the most extensively studied model, while other crops such as wheat, barley, rice, tobacco, soybean, and tomato have also been genetically modified to express dsRNAs targeting specific pests (Germing et al., 2025).

The most well-known example of RNAi-based protection against an insect pest, through plant genetic modification, is that of western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte. In that case, transgenic maize was engineered to express dsRNA targeting the *DvSnf7* gene, which is essential for the survival of WCR. This system was first described in 2007 by Baum et al., who demonstrated that maize expressing *DvSnf7* dsRNA significantly reduced plant damage caused by the pest (Baum et al., 2007) . The success of this strategy led to its regulatory approval in the United States in 2017 (Darlington et al., 2022).

In the case of non-PIPs, dsRNA can be applied more easily since these methods do not require genetic modification of plants and therefore bypass GMO-related legislations (Figure 2). The most common application method is foliar spraying, which is particularly effective for chewing insects that feed directly on treated surfaces, or for insects that come into contact with the dsRNA, allowing absorption through the cuticle (Hoang et al., 2022). The first commercialized sprayable dsRNA product for insect control, was registered in the USA in 2023. The product's name is Calantha and is produced in commercial scale by GreenLight Biosciences (Narva et al., 2025). This is about a dsRNA product for the control of Colorado potato beetle, *Leptinotarsa decemlineata,* which devastates potato plants when larvae feed on their leaves, and is designed to target the *Proteasome Beta Subunit 5* of the beetle.

Another exogenous application method is trunk injection. With this technique, dsRNA is delivered directly into the plant's vascular system, allowing it to move throughout the plant's vasculature. Trunk injection has traditionally been used to apply pesticides, insecticides, and nutrients to woody plants (Archer et al., 2022). The delivery of dsRNA molecules in the upper parts of woody plants has been proven in apple and vine trees (Dalakouras et al., 2018; Wise et al., 2022). It has also been shown that, in these trees, the hpRNAs are transferred through the apoplasts upwards, without being processed by plant's DCL so they could trigger RNAi in pests (Dalakouras et al., 2019).

Finally, root irrigation offers an alternative method for applying dsRNA for pest control. Through this approach, plants absorb dsRNA via their roots and transmit it to herbivorous insects feeding on them. For instance, dsRNA targeting arginine kinase persisted in citrus trees for up to 57 days and was later detected in psyllids and leafhoppers feeding on treated plants. In maize and rice, root irrigation with dsRNAs reduced target gene expression and increased

mortality in pests, like the Asian corn borer and brown planthopper. In tomato, soaking roots in dsRNAs targeting neural genes, caused up to 80% mortality in *Tuta absoluta* (He et al., 2022).

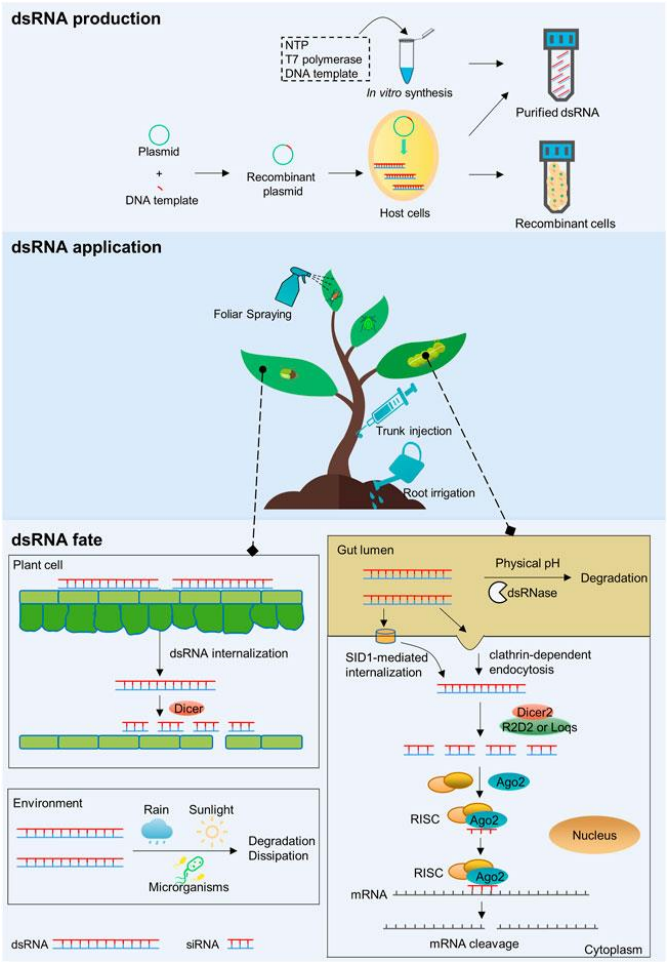



*Figure 2: Production, application and fate of dsRNA molecules (He et al., 2022).*

## Barriers

Silencing through the siRNA pathway includes three main steps: uptake of dsRNA by the insect, silencing of the target gene, and systemic spread of the silencing signal throughout the organism (Darlington et al., 2022). Especially when dsRNA is delivered through feeding, it must stay intact in the insect gut to reach the cytoplasm of target cells. Several factors can influence RNAi effectiveness across different insect species, including: (1) dsRNA degradation before or after uptake; (2) poor uptake into cells; (3) weak or incomplete RNAi machinery; (4) limited spread of the silencing signal; and (5) target genes that are difficult to silence (Figure 3) (Cooper et al., 2019). However, not all insects respond equally well to ingested dsRNA. Coleopteran insects exhibit the highest susceptibility to RNAi via oral delivery, while species from other orders—such as Lepidoptera, Diptera, and Hemiptera—tend to show more variable and often reduced responses (Christiaens et al., 2020).

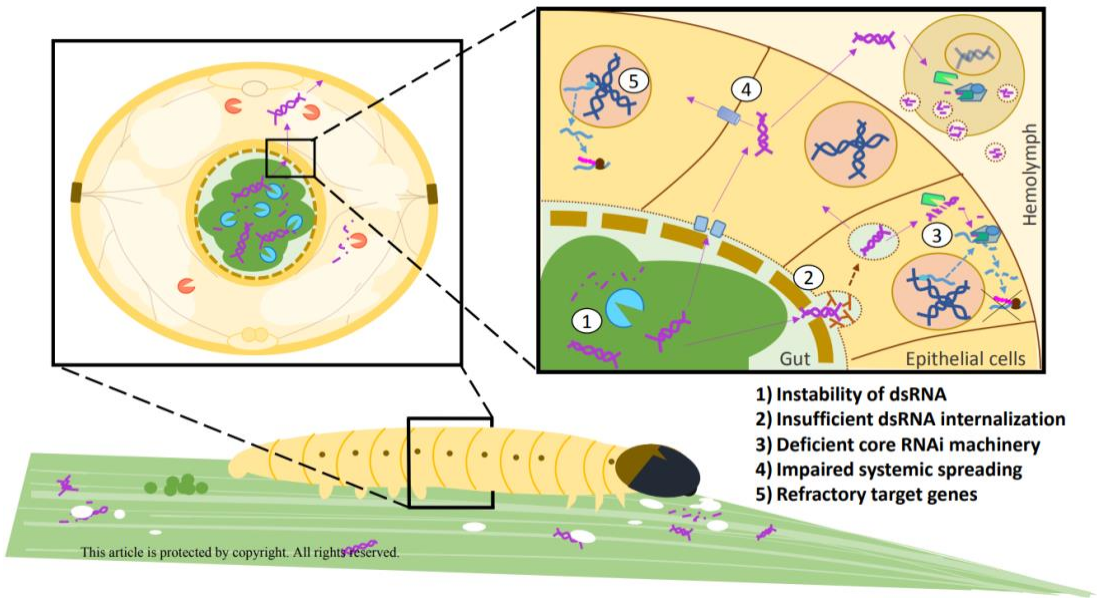



*Figure 3: Proposed mechanisms of low RNA interference (RNAi) efficiency in insects (Cooper et al., 2019)*

Regarding dsRNA uptake, it is known that in the nematode *Ceanorhabditis elegans,* a transmembrane protein named Sid-1 is responsible for cellular uptake and systemic spread of RNAi (Winston et al., 2007). Although Sid-related genes have been found in insect species, nothing relevant is present in the Diptera genome (C. Maktura et al., 2021).

Studies in *Droshophila melanogaster* S2 cells have shown that the uptake of dsRNA molecules occurs through clathrin mediated endocytosis (Saleh et al., 2006). Additionally, this process is mediated by two scavenger receptors: SR-Cl and Eater (Rocha et al., 2011). In Tephritidae species, transcriptome analysis of *Anastrepha fraterculus* revealed the presence of all known components involved in dsRNA uptake, except for Sid-1. This suggests that tephritid flies likely rely on clathrin-mediated endocytosis to initiate the RNAi response. They also possess orthologs of scavenger receptors, which are believed to assist in dsRNA uptake, similar to mechanisms observed in Drosophilidae (C. Maktura et al., 2021).

### Improvement methods

A strategy that improves silencing and phenotype is the use of formulation, in order to protect dsRNAs from environmental factors, as well as to improve the cellular uptake from gut cells. This can be achieved with several methods. Liposomes can enhance uptake but are often costly and potentially toxic. Chitosan, a natural polysaccharide, is widely used due to its low cost and biocompatibility. Inorganic nanoparticles such as hydroxyapatite, silica, and layered double hydroxide (LDH) seem to cause low toxicity and high RNA loading efficiency. Protein-based carriers like branched amphipathic peptide capsules (BAPCs) and cell-penetrating peptides (CPPs) provide promising alternatives, improving dsRNA delivery, stability, and systemic response in various insect species (Ortolá & Daròs, 2024).

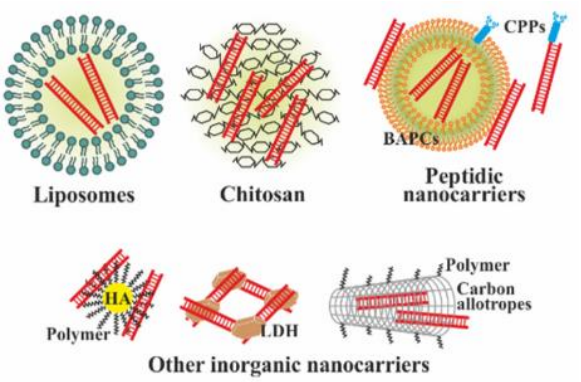



*Figure 4: methods for dsRNA formulation (Ortolá & Daròs, 2024)*

A successful example of nanocarrier application in *Bactrocera dorsalis* (Diptera: Tephritidae) involved the use of a star polycation (SP) to deliver dsRNA targeting the Wingless gene, essential for normal wing development (Guo et al., 2021). In a simulated experiment, artificial baits containing an artificial diet, dsWingless, and the SP nanocarrier were provided to the flies. The number of offspring emerging from pupae developed in these baits was significantly reduced compared to the control group.

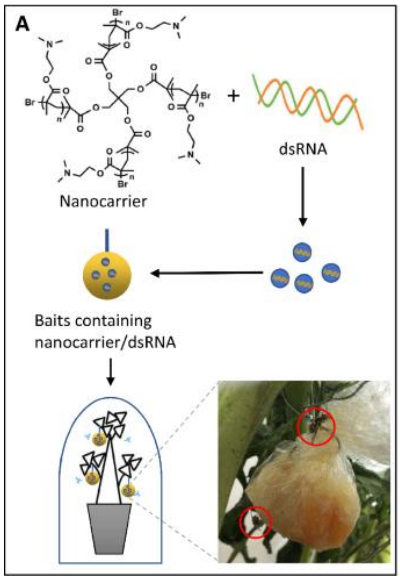



*Figure 5: Nanocarriers and artificial bait application in Bactrocera dorsalis (Guo et al., 2021)*

Another strategy to enhance RNAi efficiency is the simultaneous silencing of both the target gene and intestinal nucleases, which are major contributors to the variability of RNAi responses across insect species (Tayler et al., 2019). In *Ceratitis capitata*, co-administering dsRNA targeting an ATPase gene along with dsRNA against RNases in adult diets, led to increased mortality (Volpe et al., 2024). Similarly, in the tephritid fruit fly *Bactrocera tryoni*, co-feeding both larvae and adults with dsRNA against gut RNases resulted in a marked reduction in target gene expression (Tayler et al., 2019). In both studies *ex vivo* dsRNA degradation assays using gut extracts were also performed, demonstrating that co-silencing intestinal nucleases significantly reduces dsRNA degradation.

Another example is the melon fly, *Zeugodacus cucurbitae*, where a co-silencing RNAi approach yielded promising results. DsRNA targeting the ZcCOPI-alpha gene was rapidly degraded by gut RNases, particularly ZcRNase1. Silencing ZcRNase1 reduced dsRNA degradation, enhanced RNAi efficiency, and increased larval mortality (Ahmad et al., 2024).

#### *Target gene selection*

One important factor also affecting the effectiveness of the RNAi response is the selection of the appropriate target genes. In the context of designing RNAi-based insecticides, it is critical to choose genes whose silencing leads to a lethal phenotype. Even though many insect genes are essential for their survival, only a few cause significant mortality when knocked down (Schmitt-Engel et al., 2015). In this context, a large scale RNAi screening was performed in the beetle *Tribolium castaneum* (Buer et al., 2025a; Ulrich et al., 2015). In total, 15.530 genes were targeted (using injection method), and scored for lethality. Using screening and clustering techniques, this study concluded in 34 superior target genes, that cause lethality upon oral application.

## Insect Gut Microbiome

### Composition and Function

Insects form symbiotic relationships with gut microbes that offer important physiological and ecological benefits. Gut microbiota are found in insects across all taxonomic orders and play key roles in providing nutrients, aiding digestion, and facilitate regulate growth, metabolism, and immunity, by influencing various signaling pathways. Recent advances in metagenomics and bioinformatics have led to the identification of many microbial species living in insect guts. The most common bacterial groups found are Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes. These microbes can affect insect feeding behavior and support survival, by participating in the break down of plant toxins, chemical pesticides, and even microplastics. (Yasika & Shivakumar, 2025). In fruit flies, a high prevalence of Enterobacteriaceae has been observed, particularly species from the genera Klebsiella and Enterobacter (Raza et al., 2020).

A study by M. Darrington et al. (2022) used 16S rRNA sequencing to analyze the gut microbiota of *Ceratitis capitata* from both laboratory-reared and wild populations, raised on various diets and collected from different natural plant hosts. The study found that the medfly gut contains a relatively simple microbial community, and that its composition is mainly influenced by the larval diet. Despite the variation in host plants or artificial diets*, Klebsiella* species consistently emerged as the dominant genus in the gut microbiome, suggesting a strong and stable association between *Klebsiella* and the medfly across different environments (Darrington et al., 2022).

### Safety Considerations and Risk Assessment

RNAi is designed to be highly sequence-specific, but studies have shown that siRNAs and dsRNAs can sometimes silence non-target genes if there are short regions of sequence similarity. Even partial matches, like 7–8 identical bases or over 80% similarity, can trigger unintended effects. This happens because Dicer can cut dsRNA at multiple points, creating a range of siRNAs that may match off-target genes. Such off-target effects have been observed

in both vertebrates and insects, and in some cases, many unrelated genes were unintentionally downregulated (Luo et al., 2024).

Insects can also show sequence-independent effects in response to exogenous dsRNA. These effects are often linked to immune system activation, as seen in several species where non-specific dsRNA triggers the upregulation of immune-related genes. For example, immune responses have been observed in honeybees, *Tribolium castaneum*, *Bombus terrestris*, and *Bombyx mori*, suggesting that insects may recognize dsRNA as viral material and respond by boosting immune gene expression (Luo et al., 2024; Niu et al., 2024).

Recent studies have shown that conventional pesticides may have unintended effects on the gut microbiota of humans and other animals. The gut microbiota plays an important role in digestion, immune system function, and overall health. Pesticides can enter the body through food, water, or contact with the environment. Some studies have found that exposure to these chemicals can disrupt the balance of gut bacteria, which may lead to health problems such as inflammation, metabolic disorders, and increased risk of chronic diseases (Sharma et al., 2023).

RNAi-based plant protection methods, such as spray-induced gene silencing (SIGS), are also being studied for their possible effects on microbiota. One study tested how spraying plants with dsRNA to control *Fusarium graminearum* affected the microbes on the surface of wheat and barley. The results showed that bacterial communities changed depending on the type of dsRNA applied and the plant species, while the fungal communities remained mostly stable. This suggests that while less common microbes might be affected by dsRNA sprays, the core plant microbiota remains mostly unchanged (Sundararajan et al., 2025).

### Effect of Gut Microbiota on RNAi Efficacy

When insects ingest dsRNA through feeding, the molecules must pass through the gut, before entering epithelial cells and reaching the systemic circulation—provided they are not degraded along the way. Several factors can influence this initial interaction, including the gut microbiota. In a study on the leaf beetle *Plagiodera versicolora*, researchers explored whether the gut microbiome affects RNAi efficiency. By comparing axenic (germ-free) and non-axenic larvae, they found that while both groups showed similar gene silencing levels, non-axenic larvae experienced significantly faster mortality. This suggests that the gut microbiota does not influence RNAi at the gene expression level but may enhance RNAi-induced mortality, possibly by interacting with dsRNA degradation products or by shifting from a commensal to a pathogenic role, as observed with *Pseudomonas putida* (Xu et al., 2021).

### Mediterranean Fruit Fly, *Ceratitis capitata*

The Tephritidae family of fruit flies includes around 4,300 recognized species across 500 genera worldwide. These insects are major agricultural pests, known for infesting a wide variety of fruits and vegetables and causing large economic damage. Among them, the Mediterranean fruit fly (*Ceratitis capitata*, Wiedemann) stands out as the most widespread and damaging species (Rao et al., 2024). Originating in tropical Africa, *Ceratitis capitata* has now spread across the rest of Africa, Mediterranean countries, Central and South America, and parts of Western Australia (Szyniszewska & Tatem, 2014).

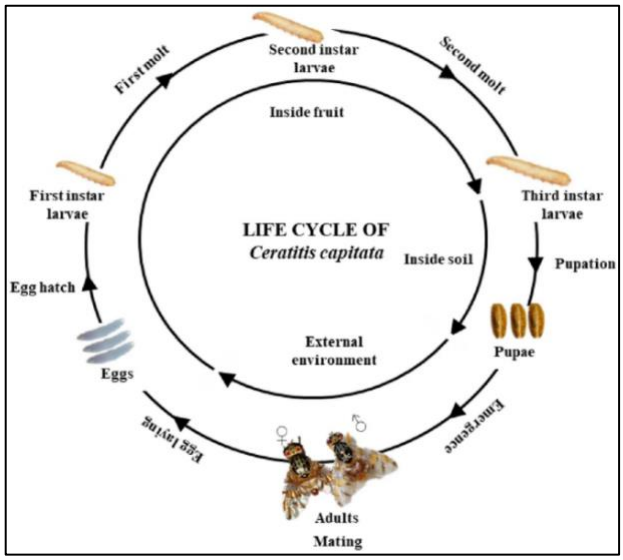



*Figure 6: Ceratitis capitata life cycle (Elqdhy et al., 2024)*

Regarding its life cycle, female *Ceratitis capitata* lay eggs beneath the skin of more than 300 types of fruits and vegetables (Thomas et al., 2004). After hatching, the larvae feed on the fruit flesh, until they reach the third instar stage. At this point, they drop to the ground, burrow into the soil, and pupate. The pupae remain in the soil for several days before emerging as adult flies, ready to mate and continue their life cycle (Figure 6) (Elqdhy et al., 2024). The crop damage is caused primarily by larval feeding, which creates large holes and tunnels inside the fruit. When the larvae exit, these openings allow secondary infections by bacteria and fungi, leading to rapid fruit decay.

Various methods have been used to suppress Mediterranean fruit fly populations, with chemical insecticides being the most commonly applied. Among them, organophosphates such as malathion and carbamates are widely used, particularly in the Mediterranean region, to control medfly outbreaks. Other approaches include bait sprays combining attractants with toxins, which the flies ingest and die. Biological control alternatives involve the use of parasitoid wasps that oviposit in *Ceratitis capitata* eggs, as well as entomopathogenic fungi, bacteria, and nematodes. Additionally, plant extracts and essential oils have been explored as environmentally friendly pest management strategies (Hallouti et al., 2024). Finally, the release of sterile Mediterranean fruit fly males, as part of the Sterile Insect Technique (SIT), has been successfully applied in many countries (Elqdhy et al., 2024).

# Aim-Objectives

This MSc thesis is structured around two main objectives. The first objective is to investigate the RNA interference (RNAi) efficiency of various candidate target genes with the aim of developing RNAi-based insecticides for *Ceratitis capitata*. The second objective is to explore the interaction between the gut microbiome and double-stranded RNA (dsRNA) molecules, and to assess whether this interaction influences the gene-silencing phenotype. Ultimately, this research aims to contribute to the development of effective RNAi insecticides for *Ceratitis capitata* and to enhance our understanding of their mode of action.

# Materials-Methods

## Insect rearing and maintenance

Mediterranean fruit fly (*Ceratitis capitata*) individuals used in this study belonged to the EGII laboratory strain, which was reared in the insectary facility of the Department of Biochemistry and Biotechnology at the University of Thessaly. Adults were kept in cages 40x40 cm inside a chamber set at 23 ± 2 °C, 65 ± 5% relative humidity, and a photoperiod of 14:10 h (light:dark).

The adult diet consisted of a mixture of 20% Yeast Hydrolyzed Enzymatic (MP Biomedicals) and 80% granulated sugar. 500ml larva diet include 450ml tap water, 30g granulated sugar, 30g Brewer's Yeast (MP Biomedicals), 30g cotton wool, 10ml Cholesterol solution (5% w/v in 23,8% v/v EtOH) (AppliChem) , 10ml Hydrochloric Acid solution (5,4% w/v in dH2O) (Chemlab), 10ml Sodium Benzoate solution (10% w/v Sodium Benzoate in 57% v/v EtOH) (AppliChem).

## Selection of Target Genes, Primer Design

The 13 candidate target genes were selected based on the genome-wide RNAi screening study conducted by Bucher et al. in the beetle *Tribolium castaneum (Buer et al., 2025a; Ulrich et al., 2015)*. In these studies, a total of 15,530 genes were screened via RNAi injections, resulting in the identification of 34 high-confidence target genes, which were also screened in another beetle (Buer et al., 2025b). From this list, orthologous genes were identified in *Ceratitis capitata* genome. DsRNAs were then designed to target these orthologs, based on two key criteria: (i) the absence of paralogous genes in the *Ceratitis capitata* genome to minimize off-target effects, and (ii) the basic biochemical and structural characteristics for dsRNA design and construction. Selected genes and their Gene IDs in *Ceratitis capitata* and *Tribolium castaneum* are listed in Table 1.

| | Gene name | *Ceratitis capitata* Gene ID (NCBI) | *Tribolium castaneum* Gene ID (iBeetle-Base) |
|---|---|---|---|
| 1 | *PSBT4* | 101455602 | TC008617 |
| 2 | *PRS4* | 101457730 | TC009675 |
| 3 | *PRS10B* | 101456446 | TC010321 |
| 4 | *PSBT1* | 101453980 | TC000069 |
| 5 | *SRP54* | 101461502 | TC002574 |
| 6 | *PREB* | 101455681 | TC014725 |
| 7 | *PNARS6* | 101452840 | TC006375 |
| 8 | *PNARS1* | 101450012 | TC033036 |
| 9 | *ETI3A* | 101450563 | TC012303 |
| 10 | *SF3B1* | 101458592 | TC034312 |
| 11 | *CSB* | 101451892 | TC000641 |
| 12 | *PRS7* | 101453108 | TC006492 |
| 13 | *PRS6B* | 101455254 | TC007999 |
| 14 | *RNase1* | 101448568 | - |
| 15 | *RNase2* | 101463362 | - |
| 16 | *RPL19* | 101460909 | - |

*Table 1: list of 13 candidate genes that were selected for targeting when providing orally dsRNA molecules in 2nd instar Ceratitis capitata larvae, their NCBI gene ID for Ceratitis capitata and the IDs of their orthologs in Tribolium castaneum from the iBeetle database. Also, the NCBI gene IDs for the 2 Ceratitis capitata gut RNase genes, and the housekeeping RPL19 are listed.*

## dsRNA synthesis

Double-stranded RNAs (dsRNAs) were synthesized using PCR products that included T7 RNA polymerase promoter sequences at both ends. To generate these templates, gene-specific primers containing T7 promoter overhangs (5'-TAATACGACTCACTATAGGGAGA-3') were used to amplify target gene fragments from *Ceratitis capitata* cDNA. Amplification was performed using Q5® High-Fidelity 2X Master Mix (New England Biolabs, M0492S). Primer sequences and PCR conditions are provided in Table 2. PCR conditions were adjusted based on product length (extension time) and the annealing temperature was calculated from the official NEB website.

| | Target gene | Primer type | PCR primer sequence (including T7 overhang) 5' → 3' |
|---|---|---|---|
| 1 | *PSBT4* | Forward | TAATACGACTCACTATAGGGAGAGGTCCAAACGTTCATCATCAC |
| | | Reverse | TAATACGACTCACTATAGGGAGACCATTTGAAATTTGTTCACGTACC |
| 2 | *PRS4* | Forward | TAATACGACTCACTATAGGGAGAAGCATGCTCCTTCTATTGTTT |
| | | Reverse | TAATACGACTCACTATAGGGAGAATAAATACAATCCCTCGACTGTG |
| 3 | *PRS10B* | Forward | TAATACGACTCACTATAGGGAGACGACCATCAACCCTGCATAA |
| | | Reverse | TAATACGACTCACTATAGGGAGATCTAATTTCTTATTGTCGGCTACCT |
| 4 | *PSBT1* | Forward | TAATACGACTCACTATAGGGAGAACTGTGCTCGGTTCAACT |
| | | Reverse | TAATACGACTCACTATAGGGAGACACCTCTTCCTCCGTGATG |
| 5 | *SRP54* | Forward | TAATACGACTCACTATAGGGAGACTTAGTAAGGCGACGGTCATT |
| | | Reverse | TAATACGACTCACTATAGGGAGACCACAATAATAATTTCGAATCCTTCAC |
| 6 | *PREB* | Forward | TAATACGACTCACTATAGGGAGACTTGTGGCAGGTGGTG |
| | | Reverse | TAATACGACTCACTATAGGGAGAAAATCTGTTTGCACTGAATCTC |
| 7 | *PNARS6* | Forward | TAATACGACTCACTATAGGGAGAGAGGCCATCGAAGATGCG |
| | | Reverse | TAATACGACTCACTATAGGGAGAGAATTTCTGCGCCTTTCACTAC |
| 8 | *PNARS1* | Forward | TAATACGACTCACTATAGGGAGAGCGCACTCGCTTTCCTTA |
| | | Reverse | TAATACGACTCACTATAGGGAGAATCTCAAAGTTAGGCTCAGGTTC |
| 9 | *ETI3A* | Forward | TAATACGACTCACTATAGGGAGAGCTTACTCCGAAACT |
| | | Reverse | TAATACGACTCACTATAGGGAGATACTCTTACATATATCTTCC |
| 10 | *SF3B1* | Forward | TAATACGACTCACTATAGGGAGAGGTACTGAAACCTCTCTGGAAG |
| | | Reverse | TAATACGACTCACTATAGGGAGAATGTCTGCAGCTCCCAAAT |
| 11 | *CSB* | Forward | TAATACGACTCACTATAGGGAGACTAGCTGCTATATTGAGTTGATCGT |
| | | Reverse | TAATACGACTCACTATAGGGAGAGTTACTTCCAATATCTGCTCCT |
| 12 | *PRS7* | Forward | TAATACGACTCACTATAGGGAGACTGTAGCACCAACAGATATTGAGG |
| | | Reverse | TAATACGACTCACTATAGGGAGAAAACGAGCACCACCAATCG |
| 13 | *PRS6B* | Forward | TAATACGACTCACTATAGGGAGACATTGGATGAGTTGGATATTG |
| | | Reverse | TAATACGACTCACTATAGGGAGACAATTCGAAATGTGTCAGA |
| 14 | *RNase1* | Forward | TAATACGACTCACTATAGGGAGAGAGCGCGTCCTGTGTAAG |
| | | Reverse | TAATACGACTCACTATAGGGAGACTGCTCGGTGCCATAATCAA |
| 15 | *RNase2* | Forward | TAATACGACTCACTATAGGGAGACCTACGCCACCATCACTAAT |
| | | Reverse | TAATACGACTCACTATAGGGAGAAGGAAATACTGCTTCTGCAAAC |
| 16 | *GFP* | Forward | TAATACGACTCACTATAGGGAGAGTCAGTGGAGAGGGTGAAGGTG |
| | | Reverse | TAATACGACTCACTATAGGGAGACTTTTGCTTGTCGGCCATG |

*Table 2: Primer sequences (including T7 promoter sequence with red colors) using for synthesizing the dsRNAs.*

PCR products were run on a 1% agarose gel, and the desired bands were excised. DNA was extracted from the gel slices and purified using the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel), following the manufacturer's protocol. The concentration of the purified DNA was determined using the Qubit 2.0 Fluorometer (Invitrogen) with the Qubit dsDNA BR Assay Kit (Invitrogen, Q32850).

The dsRNAs used for the experiments were synthesized using the MEGAscript® RNAi Kit (Thermo Scientific, AM1626), following the manufacturer's instructions. After synthesis and purification, dsRNAs were quantified using a Qubit 2.0 Fluorometer (Invitrogen) with the Qubit RNA BR Assay Kit (Invitrogen), and their integrity was assessed by electrophoresis on a 1.7% agarose gel.

## Droplet Feeding Assay

This droplet feeding method has been successfully applied in Coleopteran species capable of ingesting the droplet content, as demonstrated in previous studies (Rodrigues et al., 2017; Dhandapani et al., 2019). In our experiments, we also assessed the effectiveness of this approach, by exposing larvae to droplets containing apple juice supplemented with blue food dye. Successful ingestion was confirmed by visualization of the blue dye within the larvae, as shown in Figure 7. In the same figure is also shown an example of droplet feeding of 2$^{nd}$ instar medfly larvae.

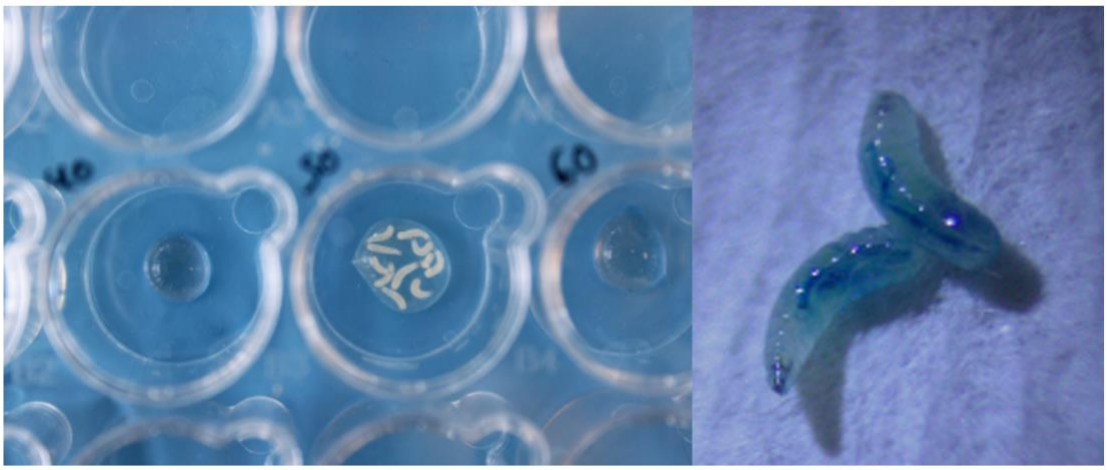


*Figure 7: droplet feeding assay of 2$^{nd}$ instar Medfly larvae and effective ingestion of blue food coloring dye mixed with apple juice, after method application.*

In the 13-gene screening experiment, each droplet contained 1 µg of dsRNA diluted in freshly prepared apple juice to a final volume of 50 µL. For the chitosan-dsRNase experiments, the dsRNA and chitosan quantities used per droplet are detailed in the table 3. Each droplet consisted of 15 larvae.

To obtain larvae for the feeding experiments, eggs were collected on day 0 and transferred to the standard larval diet. After four days of development, second-instar larvae were subjected to a 1-hour starvation period prior to the droplet feeding assay. After 5 hours of exposure to

the RNAi droplets, larvae were transferred back to their standard diet, where they remained until sampling. Each droplet (50µl or 100µl) contains 15 2nd instar larvae. When more larvae needed, more droplets with equal conditions were used.

## Injections

Injections were performed to 8 days adult flies using a mixed population of male and female flies, with a Nanoject II Injector (Drummond Scientific Company) and with flies immobilized using $CO_2$ anesthesia. Each fly was injected with 64,4 nL of dsRNA at a concentration of 5 µg/µL, corresponding to a total of 322 ng of dsRNA per individual. A total of 20 flies were injected per condition. Each fly represented a single biological replicate. Following injection, flies were transferred to cages 19x19 cm for recovery and maintained until sampling.

## RNA extraction and analysis of gene silencing by RT-qPCR

Total RNA was extracted from whole-body samples using TRItidy G™ reagent (AppliChem, A4051), following the manufacturer's instructions. Sampling was conducted at 24, 48, and 72 hours post-dsRNA administration, except for the chitosan/dsRNase feeding experiment, where samples were collected only at 24 and 48 hours post-dsRNA administration. Injected adult flies were transferred into 200 µL while second-instar larvae into 50 µL of TRItidy G™, and stored at –20 °C, until RNA extraction. Insects from both life stages were homogenized using sterilized pestles, and additional TRItidy G™ was added, in order to reach final volumes of 500 µL for adults and 200 µL for larvae.

Following homogenization, samples were centrifuged at 12,000 × g for 5 minutes at 4 °C, to remove residual tissue debris. The resulting supernatant was transferred to a new tube, and 1/5 volume of chloroform was added. After centrifugation at 12,000 × g for 15 minutes at 4 °C, the upper aqueous phase was transferred to a new tube. An equal volume of isopropanol was then added, and the samples were incubated at –20 °C for 1 hour. RNA was precipitated by centrifugation at maximum speed for 30 minutes at 4 °C, and the resulting pellet was washed twice with 75% ethanol. RNA pellets were dissolved in sterilized dd$H_2O$ and quantified using a Quawell Spectrophotometer (Q3000).

For DNA removal, 500 ng of total RNA were treated with DNase I as previously described. Subsequently, 10 ng of DNase-treated RNA were used as input for reverse transcription and quantitative PCR using the Luna® Universal One-Step RT-qPCR Kit (New England Biolabs, E3005S).

RT-qPCR reactions targeting 13 genes of interest and the housekeeping gene *rpl19* (Sagri et al., 2017) were performed using a Bio-Rad CFX system. Gene expression was analyzed using the Livak (2^–ΔΔCt) method. Briefly, Ct values were normalized to the expression of the housekeeping gene *rpl19*, to calculate ΔCt values. These were then compared to the ΔCt values of control samples to obtain ΔΔCt and fold change expression values. Primer sequences and detailed qPCR conditions are provided in Table 3.

| | Target gene | Primer type | qPCR primer sequence 5’ → 3’ |
|---|---|---|---|
| 1 | *PSBT4* | Forward | CCAAATATTCCGTGGCAGTTTC |
| | | Reverse | GCCCTTAATCATAGTAGCGAGTG |
| 2 | *PRS4* | Forward | AGCGCCTCAGGAAACTTATG |
| | | Reverse | GCGGTAATTCGACGGATTCT |
| 3 | *PRS10B* | Forward | GACGTTATTAGCCAGAGCAGTAG |
| | | Reverse | CGTGCACTCTCACCAATGTA |
| 4 | *PSBT1* | Forward | TCAAACGGAGGATCTATTGTGG |
| | | Reverse | TGTGGATTGTGTAACCACTACTC |
| 5 | *SRP54* | Forward | TTATGACTAAGGGTGGTGAACAG |
| | | Reverse | TCTAATTCGCCATCGGACATAC |
| 6 | *PREB* | Forward | GTGCAGCGTAGTGATGTAGAAG |
| | | Reverse | GGCCATCTTTCGAGATTGTGA |
| 7 | *PNARS6* | Forward | GGCTGATGTAGAGCTGGTATTG |
| | | Reverse | GCAGTTGAGTGTAAGCAAGAATG |
| 8 | *PNARS1* | Forward | GTGCCCTTCAGTTGTTAGTTTG |
| | | Reverse | CAAGCAATACCCAAAGCCATAG |
| 9 | *ETI3A* | Forward | AAGGAGCGAGCTTCGTTATAC |
| | | Reverse | AGTGCTAATAGGCGCTTACG |
| 10 | *SF3B1* | Forward | CTCCTTCAATGACACCCTCTTC |
| | | Reverse | GTAATGTGACCTGGAGTGGTATG |
| 11 | *CSB* | Forward | TGCACGGACACTGAGTTTAG |
| | | Reverse | GCAAATCGGTGAAGGTTGTG |
| 12 | *PRS7* | Forward | CCCGATTTAGAAGGACGTACAC |
| | | Reverse | CACAGTCGTGCAAGCAATTC |
| 13 | *PRS6B* | Forward | TTCCCACTGCCTGATAGAAG |
| | | Reverse | GTGCTACGAAATCCTCCAAATC |
| 14 | *RNase1* | Forward | GTGCAGACGCCCTTATATTTG |
| | | Reverse | CAATCACGACGCGGAAATAC |
| 15 | *RNase2* | Forward | AACCTCGCGAATGGTTACTC |
| | | Reverse | GACCGGTTGTGTGCAAATTC |
| 16 | *RPL19* | Forward | AACAAACGTGTACTGATGG |
| | | Reverse | CACGTACTTTATGTCGTCTG |

*Table 3: Primers used for qPCR in order to quantify target gene expression levels. None of them overlaps with the corresponding dsRNA region of each gene. Primers for housekeeping RPL19 were taken from: (Sagri et al., 2017).*

## Statistical analysis of RNAi experiment data

To determine whether differences in expression levels were statistically significant, each treatment group was compared to its corresponding control. The Shapiro–Wilk test was first used to assess the normality of the data. For datasets that did not follow a normal distribution, comparisons were made using the Wilcoxon rank-sum test. If the data of both groups were normally distributed, an F-test was conducted to evaluate the equality of variances between them. When variances were equal, a Student’s t-test was performed; otherwise, Welch’s t-test was used to account for unequal variances.

## 16S rRNA amplification and amplicon sequencing

A droplet feeding assay was repeated for four target genes, under the same conditions as the previous 13-gene screening experiment (droplet feeding assay section). Gut samples were collected and prepared for 16S rRNA amplicon sequencing. Specifically, three genes (SF3B1, ETI3A, and CSB) were targeted, along with two control conditions: dsGFP and a NULL control (feeding exclusively with apple juice). All dsRNAs were diluted in apple juice.

Larval guts were dissected at three time points post-feeding: 24, 48, and 72 hours. Dissections were carried out under sterile conditions. Larvae were immobilized by incubation at −20°C for 10 minutes. Afterwards, they were surface-sterilized with sterilized 1× PBS, followed by two washes with 75% ethanol, and a final rinse with sterilized 1× PBS to remove residual ethanol.

Dissections were performed under a stereoscope. The dissection forceps and cutting surfaces were sterilized with 75% ethanol before and between each sample. Dissected guts were immediately transferred to 1.5 ml tubes and snap-frozen in liquid nitrogen. Each biological replicate consisted of one gut.

DNA extraction was carried out using the NucleoSpin Tissue DNA extraction kit (Macherey-Nagel, 740952.50), following the manufacturer's protocol. Guts were lysed in the provided buffer until complete tissue degradation. DNA was then purified and eluted in 40 µl of elution buffer. DNA concentration was quantified using a Qubit 2.0 Fluorometer (Invitrogen) with the Qubit dsDNA BR Assay Kit (Invitrogen).

In order to characterize the bacterial communities in larval guts, amplicon sequencing targeting the V4 region of the 16S rRNA gene was undertaken. Sequencing was performed using a NextSeq 2x300 bp paired-end reads Illumina Sequencing System, and was carried out in the Agricultural Research Institute (ARI) Nicosia, Cyprus. Bacterial 16S rRNA genes were amplified with the primer set 515f-806r targeting the V4 region of the 16S rRNA gene (Walters et al., 2016). PCR amplifications were carried out using Q5® High-Fidelity DNA Polymerase (NEB, Massachusetts, USA). All samples were initially amplified using the primers mentioned above, followed by a PCR using the same primers but with extensions at the 5' end of the forward primer 515f used for sample barcoding for multiplex sequencing, according to our in-house protocol (Vasileiadis et al., 2022). PCR conditions are listed in Table 5. In total, 66 indexes were used for 60 biological samples and 6 PCR negative controls. All PCR reactions were prepared in a laminar flow hood to minimize contamination. PCR cycling conditions for both steps are detailed in Table 5.

1st PCR Conditions

| Reagents | V for 1 Sample |
|---|---|
| $ddH_2O$ | 2,8 µl |
| Q5 mix 2x | 5 µl |
| 515f (F) 10µM | 0,5 µl (0,5µM) |
| 806r (R) 10µM | 0,5 µl (0,5µM) |
| BSA (20 mg/ml) | 0,2 µl (0,4 µg/µl) |
| **Template (0,5 ng/µl DNA)** | **1 µl (1ng)** |

| Step | Temperature | Duration | Cycles |
|---|---|---|---|
| **Initial Denaturation** | 98°C | 30 sec | 1 |
| **Denaturation** | 98°C | 10 sec | 28 |
| **Primer Annealing** | 50°C | 30 sec | |
| **Extension** | 72°C | 15 sec | |
| **Final Extension** | 72°C | 1 min | 1 |
| **Final Hold** | 4°C | ∞ | 1 |

2nd PCR Conditions

| Reagents | V for 1 Sample |
|---|---|
| $ddH_2O$ | 6 µl |
| Q5 mix 2x | 10 µl |
| 515f (F) 10µM indexed | 1 µl (0,5µM) |
| 806r (R) 10µM | 1 µl (0,5µM) |
| **Template (from 1st PCR)** | **2 µl** |

| Step | Temperature | Duration | Cycles |
|---|---|---|---|
| Initial Denaturation | 98°C | 30 sec | 1 |
| Denaturation | 98°C | 10 sec | 7 |
| Primer Annealing | 50°C | 30 sec | |
| Extension | 72°C | 30 sec | |
| Final Extension | 72°C | 1 min | 1 |
| Final Hold | 4°C | ∞ | 1 |

*Table 4: PCR condition for 16S amplification and barcoding*

## Bioinformatic Analysis of Amplicon Sequencing Data

The 16S rRNA gene sequencing data were initially demultiplexed following the established pipeline (Vasileiadis et al., 2022). Subsequent processing was carried out in R using the dada2 v1.30.0 package (Callahan et al., 2016). Quality filtering involved trimming and removing reads that contained ambiguous nucleotides, exceeded a maximum error rate of 0.7 per 100 base pairs, or were shorter than 150 bp after trimming. High-quality reads were dereplicated and denoised through dada2's error correction algorithm, and paired-end reads were merged into amplicon sequence variants (ASVs). Chimeric sequences were identified and removed using the default de novo chimera detection method. Taxonomic assignment of ASVs was performed using the naïve Bayesian classifier (Wang et al., 2007) with the SILVA reference database v138 applying an 80% bootstrap confidence cutoff. The resulting annotated ASVs were imported into a phyloseq v1.46.0 object (McMurdie and Holmes, 2013) for downstream community analysis, after filtering out non-target taxa.

## Statistical Analysis on alpha and beta diversity

The finalized phyloseq objects were subjected to a range of statistical analyses at both α-diversity and β-diversity levels. Alpha diversity metrics - including Shannon diversity, inverse Simpson's index, Fisher's alpha, observed species richness, and the abundance-based coverage estimator (ACE) - were calculated using the vegan v2.6.10 (Dixon, 2003) and entropart v1.6.13 (Marcon and Hérault, 2015) packages in R. Differences in diversity across experimental groups were evaluated using hypothesis testing via one-way ANOVA followed by Tukey's post hoc test, or alternatively, the Kruskal-Wallis and Wilcoxon rank-sum tests, implemented in the agricolae v1.3.7 package (de Mendiburu, 2021). For β-diversity analysis, Bray-Curtis dissimilarities were calculated, and their effects were assessed using permutational multivariate analysis of variance (PERMANOVA) to evaluate the influence of treatment and timepoint on community composition (vegan package). Sample clustering patterns were further examined through non-metric multidimensional scaling (NMDS) ordination, based on Bray-Curtis distances, to visualize similarities or dissimilarities in microbial communities across groups (Bray and Curtis, 1957).

# Results

## Screening of 13 candidate target genes

The graphs below show the results of the screening experiment for 13 candidate target genes at three timepoints: 24, 48, and 72 hours after dsRNA feeding. Fold change values are presented relative to the target gene expression in dsGFP control group, at each corresponding timepoint. Each group (treatment per timepoint) consists of 3 biological replicates, and the graph bars show the mean values with their standard error. Among the tested genes, only three showed a statistically significant reduction in expression: *SF3B1* at 24 hours with 89% downregulation, *ETI3A* at 48 hours with 71% downregulation and *CSB* at 48 hours with 41% downregulation, all relatively to expression in dsGFP control treatment, which is presented as 100%. All Ct values of the target genes were normalized to the expression of *RPL19* housekeeping gene.

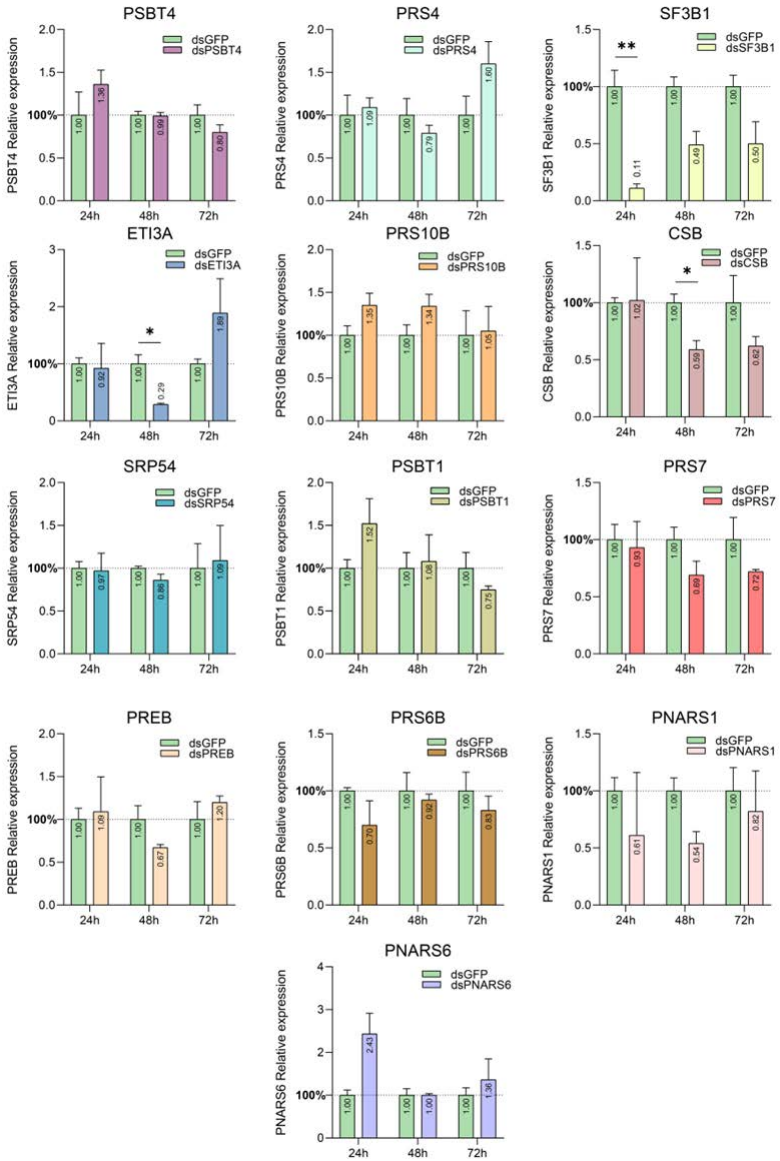


*Figure 8: RNAi results of the screening of 13 target genes, after feeding 2nd instar larvae for 5 hours with 1μg of dsRNAs using the droplet feeding assay. Each bar represents the mean fold change values of 3 biological replaces with error bars (standard error). Statistical analysis of each treatment compared to each respective control, with stars indicate the statistical significance (* p-value < 0.05, ** p-value <0.01). Ct values of target genes were normalized to RPL19 housekeeping gene.*

## Injections

The three promising target genes were then targeted by injecting the dsRNAs. Below can be observed the expression levels in the same 3 timepoints as before. Graphs show the mean values of 4 biological replicates and their standard error. All Ct values of target genes were normalized to the housekeeping gene RPL19. Statistically significant drop in expression levels is observed for ETI3A target gene in 24h and 48h post injection, with 44% and 58% drop respectively, relatively to expression in dsGFP control groups. However, the drop in expression levels recovers 72hours post injecting. A tend for downregulation is also observed for the other two genes in the first timepoints, however without being statistically significant.

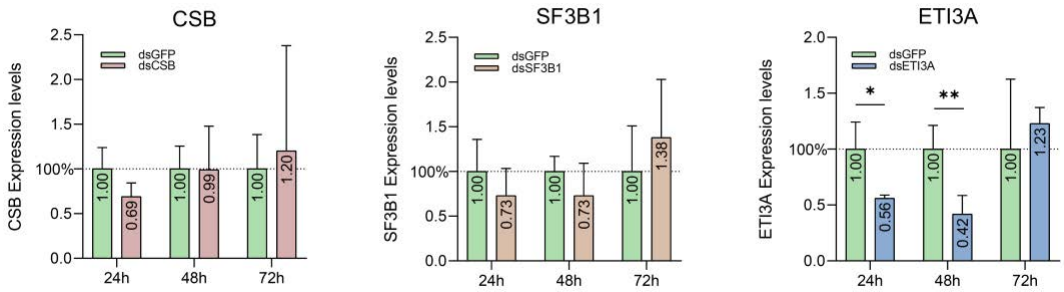



*Figure 9: RNAi results of the injections of the 3 target genes selected from the screening, using 322ng of dsRNA, in adult flies. Each bar represents the mean fold change values of 3 biological replaces with error bars (standard error). Statistical analysis of each treatment compared to each respective control, with stars indicate the statistical significance (* p-value < 0.05, ** p-value <0.01). Ct values of target genes were normalized to RPL19 housekeeping.*

## Phenotypic analysis

We then selected the 2 best target genes regarding their silencing effectiveness and proceeded to phenotypic analysis. Droplet feeding assay was repeated same as in the screening experiment. Each condition (dsSF3B1, dsETI3A, dsGFP and NULL) included 15 larvae, and two phenotypic features were measured: the number of larvae that successfully pupated and the number adult flies that emerged from these pupae. No delay was observed in the transition between developmental stages between treated and control groups.

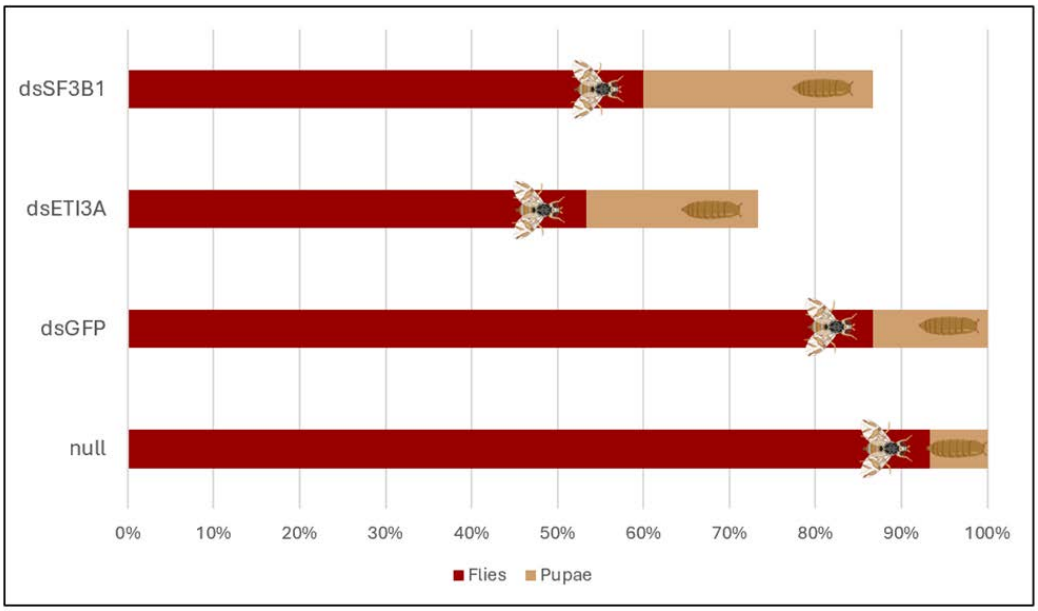



*Figure 10: phenotypic analysis in different developmental stages of Ceratitis capitata after larvae ingest dsRNA molecules*

We observed a reduction in pupal transition and adult emergence in both treatments compared to controls. Specifically, for the dsSF3B1 treatment larvae transformed to pupae 87% (13/15) and 60% to flies (9/15) and for dsETI3A treatment we had 73% pupae (11/15) and 53% flies (8/15). For the control groups we observed 100% pupal transition (15/15) and 93% (14/15) and 87% (13/15) fly emergence for NULL and dsGFP respectively.

## dsRNA effect in Bacterial communities

After getting these results from the screening experiment, we were interested in investigating whether the ingestion of the dsRNAs that show a statistically significant reduction in expression levels after feeding (dsCSB, dsSF3B1, dsETI3A), affects the composition of the larvae gut bacterial community. Firstly the expression levels of the 3 target genes in gut and carcass were measured with qPCR in order to see how these genes are expressed in the gut. As can be observed in figure, *SF3B1* has the lowest expression level in gut, then follows *CSB* and *ETI3A* which has the highest expression. 3 biological replicates were used, and expression is normalized to *RPL19* housekeeping.

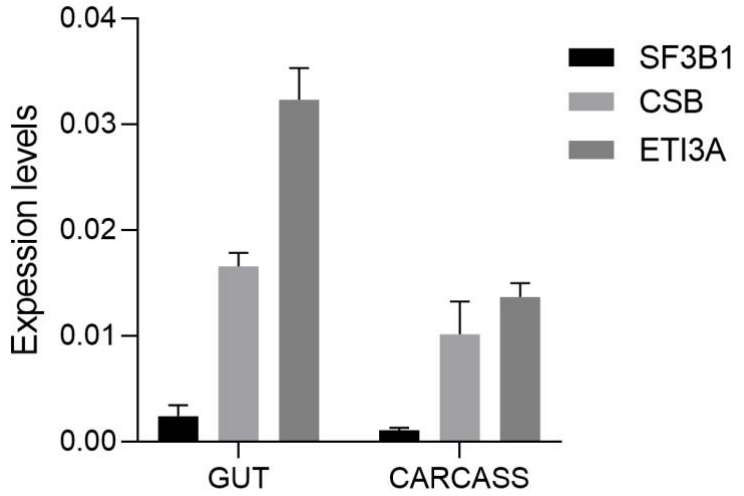



*Figure 11: Expression levels of the 3 Ceratitis capitata target genes, in gut and carcass samples. 3 biological replicates were used, and Ct values were normalized to the expression of RPL19 housekeeping gene. Each biological replicate contains guts and carcass of 2 flies*

Regarding the 16S amplicon sequencing, gut samples were collected at the same timepoints that the expression levels in the screening experiment (24, 48 & 72 hours post dsRNA feeding) were measured. Each sample consisted of 1 gut, and 4 biological replicates were used for the analysis. After 16S amplicon sequencing 1827 bacterial quality ASVs were obtained . As shown in Figure 10, refraction curves reach a plateau, which means that sequencing depth represents the gut bacterial community with high coverage.

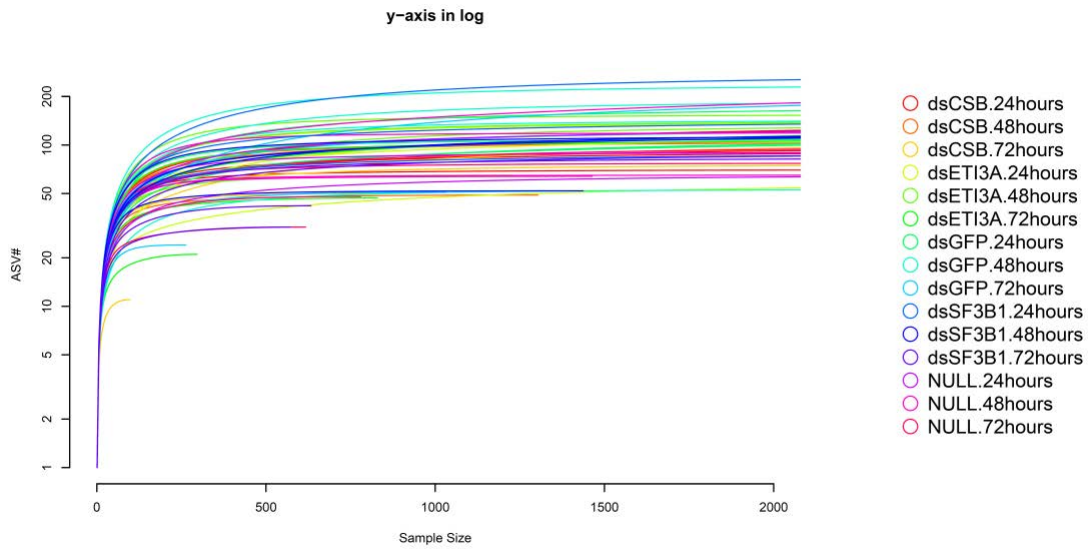



*Figure 12: Rarefaction curves for all samples that were analyzed*

## Alpha diversity

Within sample diversity or alpha diversity indices were calculated for the group of samples seen below: in the first row in all timepoints between the different treatment groups (dsCSB, dsETI3A, dsGFP, dsSF3B1, NULL) and in the second row in all groups between the different timepoints (24, 48 and 72 hours post dsRNA oral administration). No differences are observed between the treatments, whereas a reduction in all alpha diversity metrics is statistically significant in timepoint 72hours post dsRNA oral administration. Statistical analysis was performed using a parametric ANOVA followed by Tukey's HSD or a non-parametric Kruskal-Wallis test, based on the results of the Shapiro-Wilk Normality test.

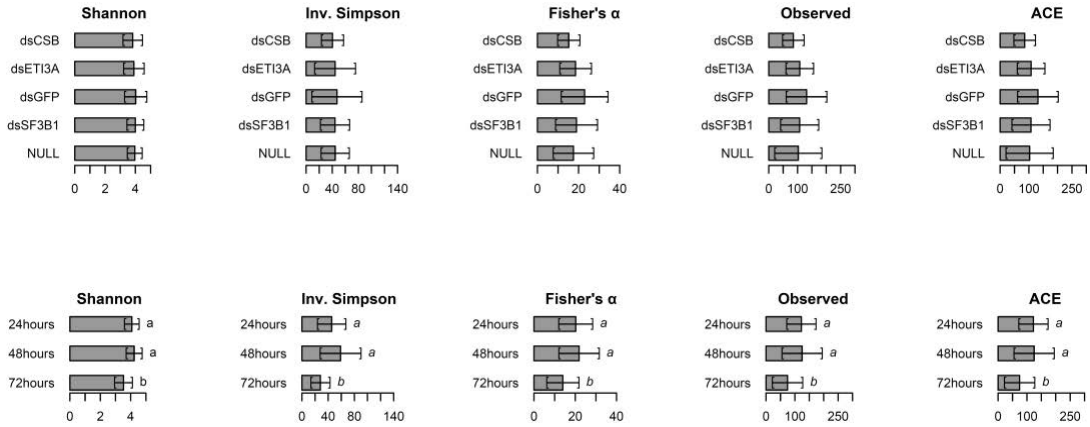



*Figure 13: The 5 alpha diversity metrics calculated for each condition: in the first row comparison between the different treatments, in the second row comparison between the timepoints.*

## Beta-diversity

To compare differences between sample groups, beta diversity was ordinated using non-metric multidimensional scaling (NMDS) based on Bray–Curtis dissimilarity. In Figure all treatments are plotted together, and the time is the separation factor (colour). A statistically significant separation between the three timepoints can be observed (p-value=0.001) with the time affecting 9% of the community differences (R2=0.09), using PERMANOVA. Pairwise PERMANOVA comparisons between all timepoints were also statistically significant (p.adjusted=0.01). On the other hand in figure that samples are separated by colour based on treatment, no grouping is observed with treatment p-value > 0.05.

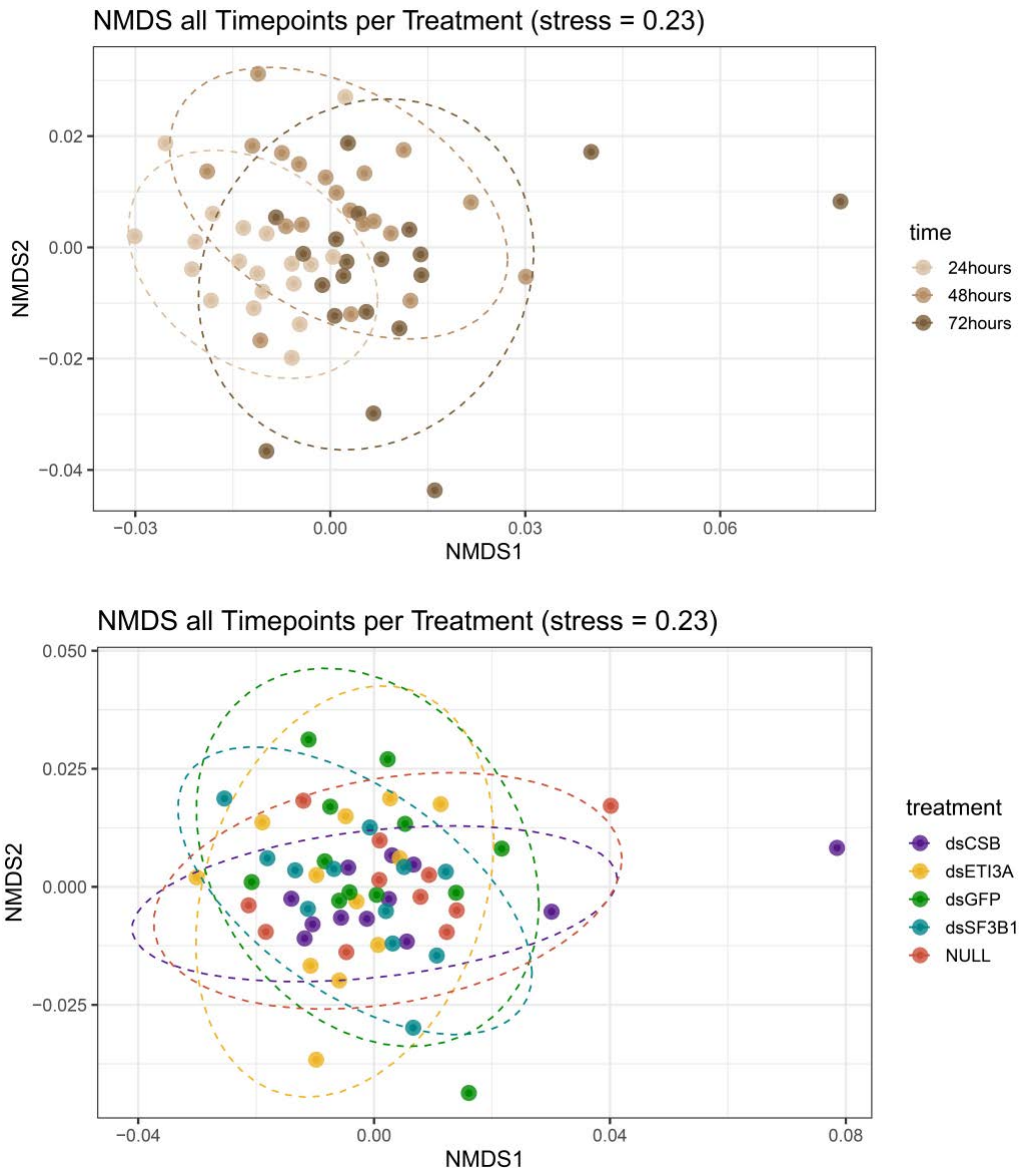



*Figure 14: NMDS plot-comparisons between timepoints and treatments*

| Term | Df | $R^2$ | F | Pr(>F) |
|---|---|---|---|---|
| Treatment | 4 | 0.059021 | 0.901663 | 0.849 |
| Time | 2 | 0.090031 | 2.750822 | **0.001** |
| Residual | 52 | 0.850948 | *NA* | *NA* |
| Total | 58 | 1.000000 | *NA* | *NA* |

| Comparison | Df | F.Model | $R^2$ | p-value | p.adjusted | Significance |
|---|---|---|---|---|---|---|
| 24h vs 48h | 1 | 3.839583 | 0.094016 | 0.001 | **0.001** | ** |
| 24h vs 72h | 1 | 2.789016 | 0.070095 | 0.001 | **0.001** | ** |
| 48h vs 72h | 1 | 1.798461 | 0.045189 | 0.001 | **0.001** | ** |

*Table 5: Permanova results for 2 factors Treatment and Time, and Pairwise Permanova for all Time groups*

## Barplots

In the following Barplot, are presented the relative abundances of the 20 most abundance bacterial Genus of each group (treatment per timepoint).

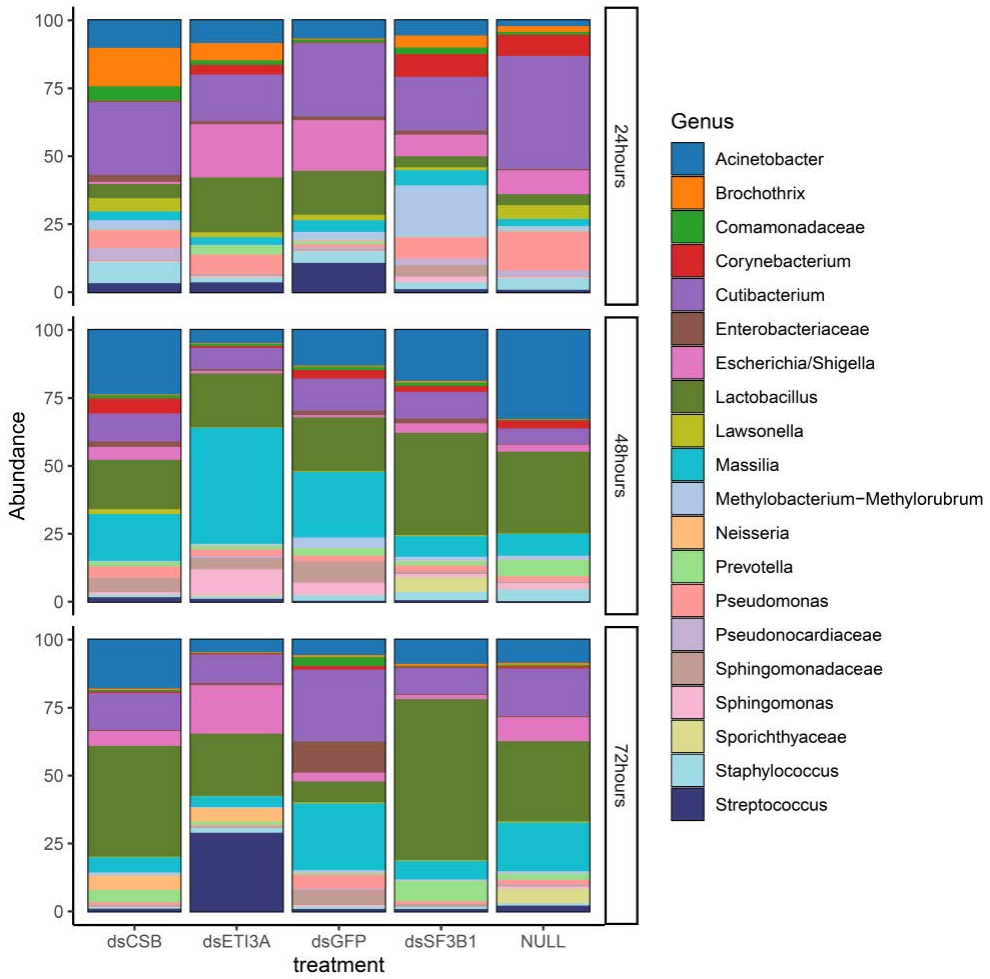
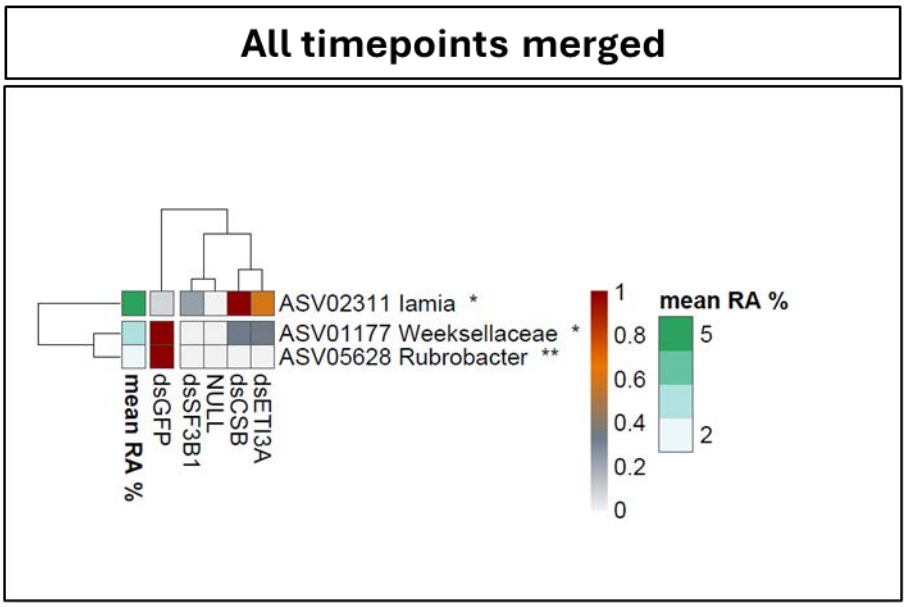


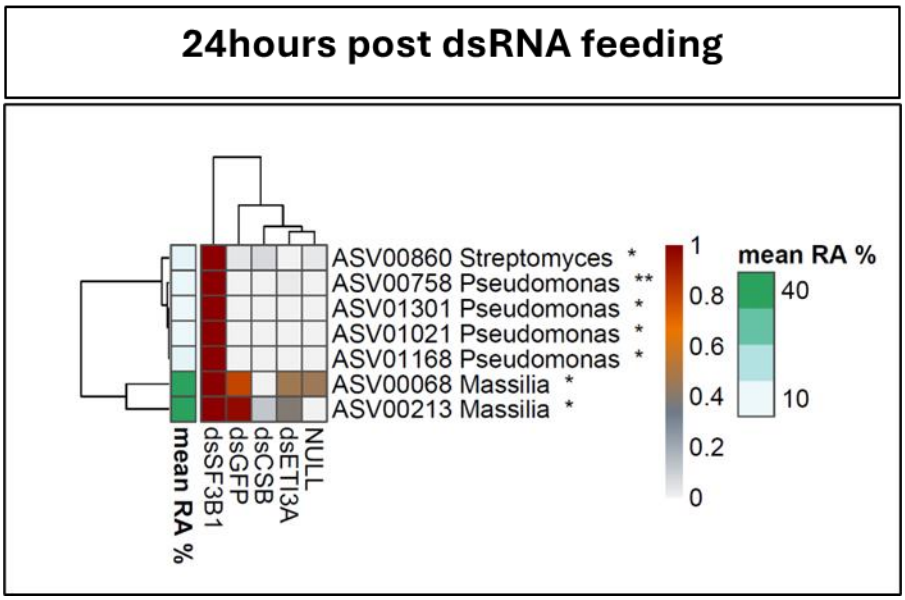

*Figure 15: Barplots showing the relative abundance of top 20 Genera*

We observe a high relative abundance of *Cutibacterium spp.* 24 hours post treatment (2nd instar larvae), a high relative abundance of *Lactobacilus spp.* and *Massilia spp.* 48h post treatment (early 3rd instar larvae) and a high relative abundance of *Lactobacilus spp* 72 hours post treatment (late 3rd instar larvae).

## Heatmaps

We then checked if there are ASVs or Genera that differ significantly between the treatment groups. To visualize these differences we constructed heatmaps that show both the relative abundances of a specific ASV in each treatment group and the mean relative abundance of this ASV within the groups, indicating whether it is high or low abundant. Kruskal-Wallis test was used to find statistically significant differences between groups.



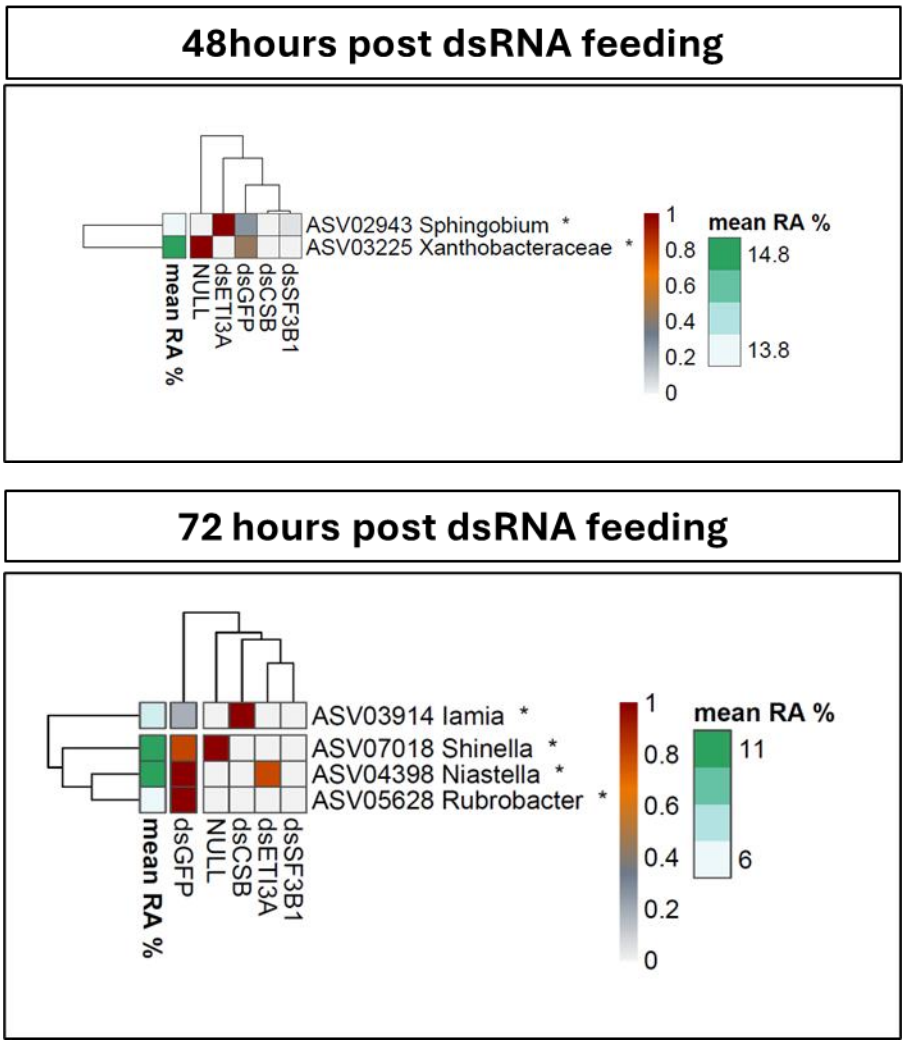



*Figure 16: Heatmaps showing the ASVs that statistically significantly differ between the treatment groups. Stars indicate p-values (without p adjustment).*

When we compare the samples of all timepoints of each treatment group, we find that the *Iamia spp* are highly abundant in dsTARGET groups – especially dsCSB and dsETI3A - compared to the dsGFP an NULL controls. When we subset the samples per timepoint, we observe a higher abundance of 4 *Pseudomonas spp*., 2 *Massilia spp*. and 1 *Streptomyces spp* in dsSF3B1 treated samples compared to the rest groups, 24hours post administration. 48hours post administration, we observe a higher relative abundance of *Sphingobium spp*. compared to the rest groups and a high reduction of *Xanthobactteraceae spp*. in all dsRNA treated samples - which is higher for the dsTARGET treated ones - compared to the null samples. 72 hours post administration, we observe a higher relative abundance of *Iamia spp* in dsCSB treated samples, of *Shinella spp*. in control groups (dsGFP and null) of *Niastella spp*. in dsETI3A and dsGFP-treated samples and Rubrobacter in dsGFP treated samples.

# Discussion

*Ceratitis capitata* (Diptera: Tephritidae), commonly known as the Mediterranean fruit fly or medfly, is a pest of major economic significance due to its broad host range and destructive impact on fruit production. In the context of Integrated Pest Management (IPM), there is a growing effort to develop and implement innovative biological control strategies. The integration of such methods aims to achieve effective pest suppression while simultaneously reducing reliance on chemical insecticides.

RNA interference (RNAi)-based insecticides are emerging as a promising tool for plant protection, with the first sprayable dsRNA product recently entering the U.S. market. This product, named Calantha, is developed by GreenLight Biosciences and targets the *Proteasome Beta Subunit 5* gene of the Colorado potato beetle, *Leptinotarsa decemlineata*, demonstrating the potential of RNAi technology for pest management at a commercial scale (Narva et al., 2025).

However, a significant challenge in applying RNAi insecticides to *Ceratitis capitata* is its relatively low susceptibility to RNAi, especially when compared to coleopteran species. Among insect orders, Diptera and Lepidoptera are known to exhibit the least sensitivity to RNAi, primarily due to the presence of highly active intestinal RNases that degrade dsRNA molecules before they can trigger gene silencing (Christiaens et al., 2020).

In our study, we used the droplet feeding assay as the method of dsRNA delivery, aiming to identify the most effective candidate target genes for RNAi-based control of *Ceratitis capitata* and also to investigate whether the dsRNA uptake and the RNAi efficiency associated with changes in the gut microbiome.

In order to select candidate target genes, we based it on a large-scale RNAi screening study done for the beetle *Tribolium castaneum* (Buer et al., 2025b). This team proceeded to an initial large-scaale screening of 15.530 genes by injections and scoreded the lethality rates. Subsequently they validate a part of these genes using difference dsRNA concentrations and finally they ended up with 34 high-confidence lethal targets. These targets were shown to induce mortality not only in *Tribolium castaneum* castaneum but also in a second coleopteran species upon oral delivery. From this list of 34 genes, we selected for targeting 13 *Ceratitis capitata* orthologs. These target genes code for 8 Proteasome Subunits (*PSBT4, PRS4, PRS10B, PSBT1, PNARS6, PNARS1, PRS7, PRS6B*) 1 Signal Recognition Protein (*SRP54*), 1 Prolactin Regulatory Protein (*PREB*), 1 Translation Initiation Subunit (*ETI3A*), 1 Splicing Factor Subunit (*SF3B1*) and 1 Coatomer Subunit (*CSB*).

After screening these 13 candidate target genes using the droplet feeding assay, we observed statistically significant downregulation in three of them: *ETI3A, CSB,* and *SF3B1*. Specifically, dsSF3B1 feeding caused a 89% downregulation of this gene 24hours post administration, dsETI3A feeding a 71% downregulation 48hours post feeding and dsCSB feeding a 41% downregulation also in 48hours. The observed gene silencing suggests their potential as effective RNAi targets in *Ceratitis capitata*.

We proceeded by injecting dsRNA corresponding to the three selected target genes (*ETI3A, CSB,* and *SF3B1*) into adult *Ceratitis capitata* flies to further validate their silencing efficiency. Among the three, only *ETI3A* showed a statistically significant downregulation at the first two timepoints (24 and 48 hours post-injection). For *CSB* and *SF3B1*, a non-significant trend toward

reduced expression was observed, particularly at 24 hours, suggesting a possible but less consistent RNAi effect.

After these results we performed a phenotypic analysis in two out of three RNAi targets (SF3B1 and ETI3A) that had the best RNAi performance both in feeding and in injection experiments. We observed a reduction in pupal transition and adult emergence in both treatments compared to controls. Specifically, for the dsSF3B1 treatment larvae transformed to pupae 87% (13/15) and 60% to flies (9/15) and for dsETI3A treatment we had 73% pupae (11/15) and 53% flies (8/15). For the control groups we observed 100% pupal transition (15/15) and 93% (14/15) and 87% (13/15) fly emergence for NULL and dsGFP respectively. This indicates that the downregulation of these 2 genes affects the flies normal development.

Even though silencing appears within 48 hours post administration, the phenotype seems to appear in a range of developmental stages. An interesting question could be whether the phenotypic changes are promoted by alteration in gut microbiota. This question was firstly raised for the leaf beetle where they found that: i) the presence of gut bacteria accelerates mortality induced by RNAi targeting host essential genes and ii) the dsRNA degradation products are able to cause bacterial overgrowth, since bacteria use them as nutrient sources (Xu et al., 2021).

In the case of the leaf beetle, a species belonging to the order Coleoptera, the high susceptibility to RNA interference (RNAi) allows for 100% lethality to be achieved just a few days after dsRNA ingestion. In contrast, in Diptera such as *Ceratitis capitata*, where RNAi efficiency is generally lower, the application of RNAi insecticides—based on our results—leads only to increased mortality during later developmental stages compared to control groups. Complete lethality has not been observed in studies involving oral delivery of dsRNA to adult flies indicating that achieving a fully lethal phenotype in this species remains challenging (Ortolá & Daròs, 2024b; Volpe et al., 2024).

In this context we were interested to see whether the silencing that we observe in the 3 target genes causes alterations in gut microbiome, when we know also that the 3 target genes are expressed in insect gut cells (Figure 11). It is important to note down that in the leaf beetle study that they observed bacterial overgrowth, leaf beetles were constantly eating dsRNA for 3 days until sampling. In our case we were able to feed larvae with dsRNA for 5 hours.

Our results revealed that bacterial diversity within the larval gut was significantly influenced by developmental time, rather than by dsRNA treatment. This has been mentioned before for *Ceratitis capitata*, with developmental stage to be a factor shaping the gut microbiome (Bel Mokhtar et al., 2022; Malacrinò et al., 2018). This was indicated by both the alpha diversity and the beta diversity analysis of the groups. Regarding beta-diversity, the results indicate that time was a significant factor affecting community structure (PERMANOVA $p = 0.001$), with all timepoints differing significantly in pairwise comparisons (adjusted $p = 0.001$). In contrast, treatment did not significantly impact the microbiome (PERMANOVA $p = 0.849$). This is consistent with the fact that bacteria, lacking RNA interference machinery, are unlikely to be directly affected by dsRNA exposure.

Interesting are also the relative abundances of the 20 most abundant genera observed with the bar plot (Figure 15), where it is clearer how the composition of the bacterial groups changes across time. 24 hours post treatment we have late 2$^{nd}$ instar larvae and *Cutibacterium spp*. have a high relative abundance, which is then decreased in 3$^{rd}$ instar larvae (48 & 72h

post treatment) and *Lactobacillus spp.* and *Massilia spp*. become highly relative abundant. There is no any other report of the bacterial composition of the Egypt II (EGII) strain of *Ceratitis capitata* in the literature. Most published data refer to wild *Ceratitis capitata* populations and the guts of these flies are dominated by a Genus that is totally absent in our Egypt II (EGII) laboratory strain: *Klebsiella spp.* Interestingly these bacteria species as well as *Citrobacter spp.* and *Pantoea spp*. which are considered core microbiome taxa for wild populations (Bel Mokhtar et al., 2022), are mostly related to nitrogen fixation and provide to their host nitrogen resources which otherwise they are unable to receive from a nitrogen-poor diet. On the other hand the diet of the larvae of our EGII laboratory stain contains Brewer's Yeast which is rich in amino acids, providing plenty of nitrogen. This could be the reason why these bacteria are not necessary for the host any more. There is also one study of wild type populations that also do not find at all *Klebsiella spp.* (Malacrinò et al., 2018).

It is also known that bacterial composition and diversity are significantly influenced by several factors apart from developmental stage, such as laboratory adaptation, diet, and rearing environment (Bel Mokhtar et al., 2022). Especially for a polyphagous animal like *Ceratitis capitata*, that can be fed with several types of fruits and vegetables and adapt to wide range of climates, microbiome adaptations are considered normal. Our results validate that bacterial community composition changes in 24h when *Ceratitis capitata* larvae pass from the 2$^{nd}$ instar stage to the 3$^{rd}$ under controlled laboratory conditions.

Then, in order to investigate whether the different treatments affect the gut bacterial communities, we focused on the significantly differentially abundant ASVs between the treatments. When we merge all timepoints together, we observe a higher abundance of *Iamia spp.* in dsTARGET-treated samples compared to dsGFP-treated and NULL samples. These Bacteria have been only reported where they discovered, in a see cucumber in Japan (Kurahashi et al., 2009). Also when doing the same procedure for each timepoint separately, we find 24 hours post feeding, an increase in abundance of some *Pseudomonas spp*., a *Massilia spp*. and a *Streptomyces spp*. in dsSF3B1-treated samples. We also observed a high downregulation in this timepoint, which could probably associate with the presence of these bacteria. From the literature we know that some *Pseudomonas spp*. could be entomopathogenic (Behar et al., 2008) , *Streptomyces spp*. play a role in both digestion and defense (Chevrette et al., 2019) and *Massilia spp.* are mostly found in plants and rhizosphere (Han et al., 2024). Some *Massilia sp.* Though are able to degrade chitin, a polymer that exists in the insect gut, but none have been reported to exist in insect microbiome (Adrangi et al., 2010). 48hours post administration, where dsETI3A and dsCSB are downregulated we observe a high relative abundance of *Sphingobium spp..* These are mainly found in soil, but have been reported also in the Silkworm, *Bombyx mori* without having a clear role (Li et al., 2020). 72hours post administration, *Iamia spp* are present in dsCSB-treated samples , and *Shinella spp.* have high relative abundance only in control conditions. These bacteria are usually found in environmental samples (Lin et al., 2008). Finally *Niastella spp.* are highly abundant in dsGFP treated and dsETI3A treated samples. These bacteria are found in soil but are also able to degrade chitin *Rubrobacter spp* are present in dsGFP treated samples, and have been found in mosquitoes .

A potential next step would be to investigate alterations in the gut microbiome at later time points, in order to better assess whether the lethal phenotype is associated with microbiome disruption. For example, in the case of the leaf beetle, larvae were continuously exposed to dsRNA for three days. On the third day, microbial analysis was performed, and the dsActin

treatment resulted in 100% lethality by the fourth day. It would also be valuable to examine whether the composition of fungi and protists is affected, as these eukaryotic organisms could be directly influenced by RNAi, given that the silencing mechanism may be activated within their own cells.

# Acknowledgments

First and foremost, I would like to express my deepest gratitude to my supervisor, Prof. Kalliopi Papadopoulou, and to the other members of my three-member committee, Dr. Athanasios Dalakouras and Asst. Prof. Sotirios Vasileiadis, as well as Prof. Konstantinos Mathiopoulos, for their continuous support, guidance, and encouragement throughout the course of this thesis.

I would also like to thank the members of the Plant and Environmental Biotechnology Laboratory at the University of Thessaly for providing a stimulating and collaborative research environment. Special thanks go to Venetia Koidou for her technical assistance, helpful discussions, and support during both the experimental and writing phases.

Last but not least, I would like to thank my family and friends for their constant support, understanding, and encouragement throughout this journey.

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