AMERICAN UNIVERSITY OF BEIRUT

ANALYSIS OF SERINE PROTEASES INVOLVED IN *DROSOPHILA MELANOGASTER* IMMUNITY AND IDENTIFICATION OF POTENTIAL BIOCONTROL AGENT

by
SANA ALLAM JABER

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by

SANA ALLAM JABER

Approved by:

Dr. Zakaria Kambris, Associate Professor — Advisor
Department of Biology
American University of Beirut

Dr. Khouzama Knio, Professor — Member of Committee
Department of Biology
American University of Beirut

Dr. Mike Osta, Associate Professor — Member of Committee
Department of Biology
American University of Beirut

Dr. Georges Nemer, Professor — Member of Committee
College of Health and Life Sciences
Hamad Bin Khalifa University

Dr. Dani Osman, Associate Professor — Member of Committee
Faculty of Sciences
Lebanese University

Dr. Assia Hijazi, Associate Professor — Member of Committee
Faculty of Sciences
Lebanese University

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Title: Analysis of Serine Proteases involved in *Drosophila melanogaster* immunity and identification of potential biocontrol agents

Although the fruit fly *Drosophila melanogaster* is not an agricultural pest and does not transmit pathogens to humans, it has been used as a model to decipher innate immune responses and to study host-pathogens interactions. Antimicrobial peptides are a major component of insects' immunity and their production is mainly controlled by two pathways: Toll and IMD. In response to fungal or Gram-positive bacterial infections, the Toll signaling pathway is activated by a cascade of Serine Proteases (SPs) leading to the cleavage of the cytokine Spz, the Toll ligand. Several SPs have been shown to act upstream of Toll, linking microbial recognition to the cleavage of Spz. Other SPs have also been shown to mediate the flies' melanization reaction. Serine Protease inhibitors or serpins (SPN) are negative regulators of melanization and AMPs production. Large numbers of SPs and several serpins remain unexplored and the complexity of proteolytic cascades operating upstream of Toll during the flies' immune response is not completely understood. Using an *in vivo* RNAi approach, we screened the unexplored SPs for their potential role in Toll activation. We also collected different naturally occurring fungi from dead arthropods and tested their pathogenicity on lab insect models in the aim of a potential utilization as new biological pest control agents.

CONTENTS

FIGURES

TABLES

ABBREVIATIONS

AMPs	Antimicrobial peptides
B. bassiana	*Beauveria bassiana*
CRO	Croquemort
Cys	Cysteine
DAP-type	Diaminopimelic acid-type peptidoglycans
DDT	Dichlorodiphenyltrichloroethane
dsRNA	Double-stranded RNA
E. carotovora 15	*Erwinia carotovora carotovora 15*
E. coli	*Escherichia coli*
E. faecalis	*Enterococcus faecalis*
F1	First filial generation
g	Gramme
h	Hours
GNBP	Gram negative bacteria binding-protein
IR	Inverted repeat
ITS	Internal Transcribed Spacer
KD	Knockdown
LPS	Lipopolysaccharides
Lys-type	Lysine-type peptidoglycans
M. luteus	*Micrococcus luteus*

mL	Milliliter
Nec	Necrotic
ng	Nanogramme
nL	Nanoliter
PAMPs	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
PDA	Potato Dextrose Agar
PGRP	Peptidoglycan recognition protein
PO	Phenoloxidase
PPO	Prophenoloxidase
PRRs	Pattern recognition receptors
Psh	Persephone
RCL	Reactive center loop
RNAi	RNA interference
ROS	Reactive-oxygen-species
Rp49	Ribosomal protein 49
RT	Reverse transcriptase
S. aureus	*Staphylococcus aureus*
Ser	Serine
SP	Serine protease
SPE	Spätzle-processing enzyme
SPH	Serine protease homolog
SPN	Serine protease inhibitor/serpin

Spz	Spaetzle
TG	Tranglutaminase
Thr	Threonine
TLRs	Toll-like receptors
UAS	Upstream Activation Sequence
W1118	Wild-type flies
µL	Microliter
%	Percent
*	Crossed to
^{o}C	Degrees Celsius

CHAPTER I

INTRODUCTION

A large variety of adequate biological defenses has evolved to defend vertebrates against infection, parasitism, and neoplasia. These defense responses rely on both innate and adaptive components. But understanding innate immune responses has gained a lot of interest in the last two decades, since it is the first line of host defense mechanisms to confine pathogen infections in a wide range of organisms, and it plays an important role in stimulating the adaptive response [1, 2]. Toll-like receptors (TLRs) count among the key molecules that alert the immune system, and are named for their similarity to Toll, a receptor first identified in the fruit fly *Drosophila melanogaster* [3]. In addition, *Drosophila* was the first multicellular organism to have its genome sequenced and well known, and studies revealed that around 75% of the genes that cause diseases in humans have functional orthologs in flies [4-6]. This highlights the value of *Drosophila* as a model organism to decipher the evolutionarily conserved immune pathways and to study biological processes in relation to humans. Hence, studying the immune defenses in insects open new ideas in understanding the host-pathogen interactions, which help us to develop tools in order to reduce disease transmission by these vectors, and to identify new insect biological control agents that preserve the environment.

A. *Drosophila* innate immunity

Over a decade, and because of the absence of adaptive immune response, the fruit fly *Drosophila melanogaster* has been used as a powerful model for studying innate immunity. Our general knowledge about innate immunity comes mostly from studies that were initially carried out in *Drosophila* and other insects. *Drosophila* invokes efficient innate immune responses to fight microbial pathogens, many of which are shared with higher organisms. Hence, this model is used in order to understand how these immune responses are regulated at the level of whole organism. These immune responses involve: i) epithelial immunity that includes the lining of the trachea, the gut and the reproductive system, and coagulation; ii) cellular reactions mediated by blood cells present in the hemolymph that promote phagocytosis and encapsulation of pathogens, and; iii) humoral reactions of antimicrobial peptides (AMPs) produced by the fat body. In addition, melanization reaction is a prominent immune response to combat infection in *Drosophila*.

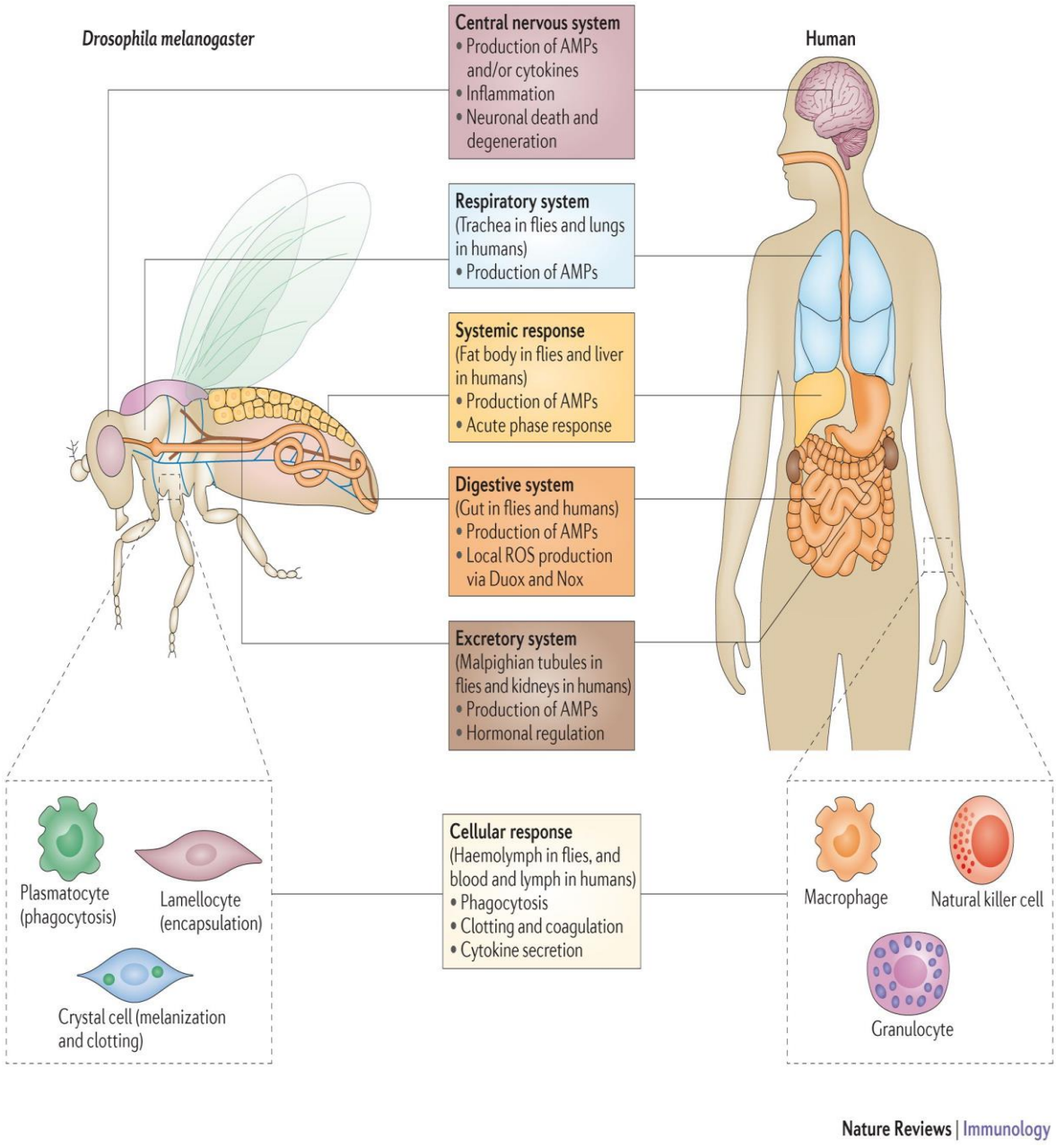


Figure 1: Evolutionary conserved innate immune pathway from *Drosophila*, an invertebrate model, to humans.

Drosophila melanogaster presents important innate immune responses, of which many are shared with vertebrates, especially humans. The gut and the fat body of the fruit fly function similarly to the liver and adipose tissue in mammals, and the malpighian tubules are analogous to mammalian kidney. Many organ systems in the fruit fly are contributing to the innate response. Concerning cellular response, haemocytes in flies play the role of blood cell types in mammals. Plasmatocytes, which are macrophage-like cells, phagocyte

the microbial invaders upon infection, crystal cells have a role in melanization, generating bactericidal reactive oxygen species (ROS) at infection sites and promoting coagulation, and lamellocytes encapsulate large pathogens as the role of natural killer human cells. The humoral response of *Drosophila melanogaster* corresponds to the inducible synthesis and secretion of antimicrobial peptides (AMPs) into the hemolymph representing a systemic reaction. In response to local microbial infection, the barrier epithelial cells, trachea, malpighian tubules, and gut can also produce tissue-specific AMPs. The generation of ROS by NADPH oxidases in the gut, such as dual oxidase (Duox) and NADPH oxidase (Nox), play a role in fighting infection and regulating the gut microbiota. The central nervous system of the fruit fly is similar to both organism physiology and immunity by the secretion of different hormones. Adapted from [7].

1. Epithelial barrier and coagulation

1.1. Physical barrier

Like all vertebrates, insects including *Drosophila*, live in an environment, where unquantifiable number of microorganisms exist. They are exposed to a huge amount of pathogens as they feed, lay their eggs and develop on fermenting medium. Upon infection with invaders, insects rely on their physical barriers that act as the first line of defense, by preventing the access of microbes to the hemolymph [8]. The cuticle is considered the most protective layer from invading pathogens of the external environment [9, 10]. It is a chitinous hydrophobic material forming the exoskeleton on the insect, and lining the foregut and the hindgut. An injury to that cuticle leads to the entry of pathogens to the body cavity [11, 12]. The oral path is another possible route of entry for pathogens. Hence, epithelial cells that line the digestive (gut), and the respiratory (trachea) systems, in addition to those of the epidermis, and the reproductive systems, play an important role in

preventing the access of microbes to the internal tissues and limiting the spread of pathogens [13-15]. They are also involved in antimicrobial recognition and in triggering the activation of local immune responses that act as an early control system [16-18].

1.2. Clotting reaction

After the breaching of the primary epithelial barrier, the clotting reaction forms a secondary barrier to infection. It limits the loss of hemolymph, and it initiates wound healing as soon as the tissue is injured [19]. In the clot, various proteins participate in the process of increasing viscosity and forming characteristic filaments, which immobilize the bacteria and promote their killing [20]. The major identified proteins are Hemolectin, a large protein produced by plasmocytes that constitute the major component of the clot [21]; Fondue, an abundant hemolymph protein regulated by Toll pathway and involved in cross-linking of clot fibers [22], the pro-coagulants lipophorin, hexamerin, and its receptor called fat body protein 1 [23], and the Tranglutaminase (TG), which provides the connection between the clot matrix and the bacteria [24].

Once these physical barriers are breached, a series of host immune responses are activated to eliminate the invading pathogens.

2. Cellular immune responses

2.1. Cellular effectors

The cellular responses are mediated by immune cells called hemocytes. The body cavity of *Drosophila* is filled with a circulating hemolymph containing three cell types of hemocytes: plasmocytes, lamellocytes and crystal cells [25]. Plasmocytes are the predominant type by representing 90%-95% of all mature hemocytes. These phagocytic cells carry receptors for recognizing bacteria, viruses, yeast and apoptotic cells. Pathogen recognition is followed by internalization and destruction of the engulfed target within phagosomes. Rather than their phagocytic role, they are considered an important player during the fruit fly innate immunity, as they can produce cytokines, clotting factors, antimicrobial peptides, and extracellular matrix components [26-29]. The second type of hemocytes is lamellocytes, which are non-phagocytic, large, flat, and adherent cells. They function in encapsulation of foreign bodies that are too large to be phagocytosed [30]. These cells differentiate only in response to specific immune conditions such as wasp parasitism or stress mediated by an increase of reactive-oxygen-species (ROS) [31, 32]. Finally, crystal cells represent 5% of the larval hemocytes. They are involved in melanization process as they constitute storage cells for prophenoloxidase (PPO) [33].

2.2. Phagocytosis

Phagocytosis is an evolutionary conserved process that plays an important role in host defense and tissue homeostasis [34]. It is a rapid response used to remove pathogens and particles bigger than 0.5μm through the formation of a membrane-derived vesicle, known as the phagosome. After the internalization of the pathogen into the phagosome, this latter fuses with lysosome, and then hydrolytic enzymes digest the particle [35]. In *Drosophila*, plasmocytes (macrophages) function as professional phagocytes, as they engulf pathogens, apoptotic cells, and dendrite debris. Other cells, usually known as tissue resident cells, have also been shown to engulf foreign particles in areas where circulating macrophages are less accessible [36]. They are characterized as nonprofessional phagocytes since they display a reduction in the phagocytic ability, and they mediate, mainly, the clearance of apoptotic cells (effrocytosis). Ovarian follicle epithelial cells are an example of these nonprofessional phagocytes [37].

This response is usually initiated when microbial associated molecular pattern (MAMPs), expressed on pathogens or apoptotic cells, are recognized by specialized phagocytic receptors. These MAMPs include peptidoglycans (PGN) and lipopolysaccharides (LPS) found in bacteria, and the fungal β-1,3 glycan. In addition, phosphatidylserine (PS), a phospholipid membrane that can be found in the inner leaflet of the plasma membrane, is exposed on the surface of apoptotic cells [38].

Several receptors have been identified for their role in phagocytosis [39]. These phagocytic receptors include evolutionary-conserved receptors such as Croquemort (CRO)

[40-43] and Draper [44-48], members of the scavenger receptor family dSR-CI [49-52] and Peste [53, 54], peptidoglycan PGRP-LC [55-57], members of the Nimrod family the EGF-domain protein Eater [58-61], Nimrod C1 [62-65],and NimC4/SIMU [9, 66], and the IgSF-domain protein Dscam [67-70], and finally β-integrins [71]. In addition, *Drosophila* S2 cells are highly phagocytic [72-74].

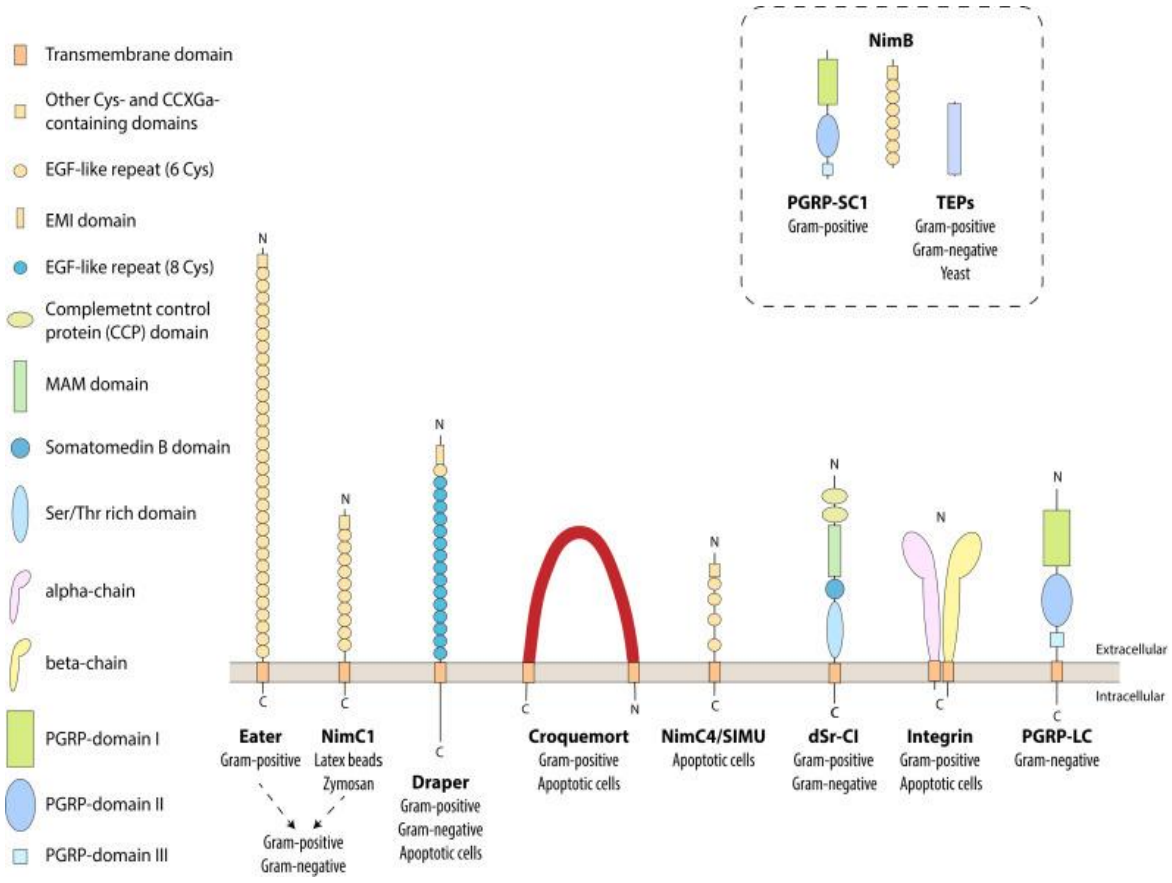


Figure 2: Plasma membrane phagocytic receptors found in *Drosophila*.

Bacterial infection, apoptotic bodies, or fungal spores are eliminated from the hemolymph of the *Drosophila melanogaster* through receptor-mediated recognition and phagocytosis.

This process represents a model for mammalian phagocytosis [75]. In fact, the evolutionary-conserved receptors such as Croquemort (CRO, CD36 paralogue), and Draper (LPS recognition protein (RP) paralogue) recognize dead cells during development. In addition, S2 cells of the flies, which share some features with macrophages, identify phagocytic receptors of the host immunity such as members of the scavenger receptor family dSR-C1 and Peste, peptidoglycan PGRP-LC, members of the Nimrod family of proteins Eater and Nimrod C1, and NimC4/SIMU. The dashed box represents potential secreted opsonins in mediating phagocytosis in *Drosophila*. Dashed arrows on Eater and NimC1 indicate the synergistic action of those receptors in bacterial phagocytosis. Adapted from [76].

2.3. Encapsulation

When microbial invaders are too large to be phagocytosed (greater than 10 μm in diameter) they are encapsulated; the pathogen is surrounded by a multilayered envelope of hemocytes. In fact, this response is triggered when granulocytes are attached and form a layer of cells that surrounds the foreign organism. This layer of granulocytes is then surrounded by several layers of plasmocytes and additional granulocytes [77]. Hence, after binding of hemocytes to their target, a multilayered capsule is created around the invader leading to its destruction by the local production of reactive oxygen species or by asphyxiation (deficient supply of oxygen) [78]. Lamellocytes are the major hemocytes involved in the cellular encapsulation [79]. In most cases, the capsule becomes melanized depending on the pathogen and the host [80].

2.4. Nodulation

This cellular response refers to the entrapment of invading microorganisms by the aggregation of hemocytes that surround them [81]. After infection, granulocytes exhibit a morphological and behavioral change, transform from circulating to adherent cells, and aggregate around the pathogen. Then, they release their contents enclosing the pathogen in a flocculent material. At this stage, plasmocytes aggregate and surround the nodule, leading sometimes to the melanization of the structure [82]. Nodulation is an important mechanism activated against bacterial and viral infection [83, 84]. However, this immune process remains largely unknown.

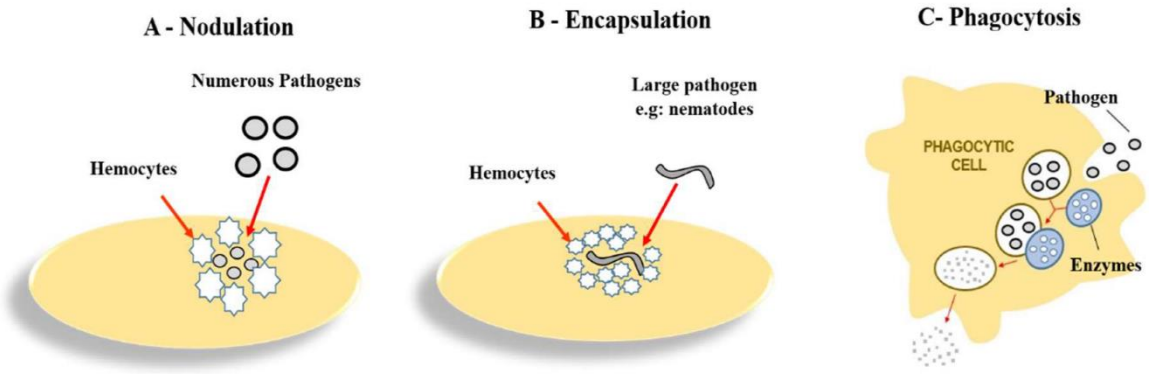


Figure 3: Schematic of the three cellular immune responses mediated by hemocytes.

This figure illustrates different cellular defense mechanisms mediated by hemocytes: A-Nodulation, hemocytes form a layer of cells around a large number of microorganism; B-Encapsulation, hemocytes (plasmocytes and granulocytes) form a capsule around large pathogen such a protozoa and nematodes [85, 86]; C-Phagocytosis, enzymes are produced by hemocytes (plasmocytes and granulocytes) to destroy the pathogen. Adapted from [87].

3. Humoral reactions of antimicrobial peptides

The response to infection in *Drosophila* is characterized by the production of different types of AMPs into the hemolymph from the fat body, which is an equivalent to mammalian liver. Two conserved signaling pathways, Toll and IMD, which are mechanistically similar to the mammalian TLR and tumor necrosis factor-α receptor signaling pathways, mediate the secretion of these AMPs [88-92].

3.1. Non-self-recognition

After microbial infection, the host needs to be alerted. The recognition of pathogen-associated molecular patterns (PAMPs) is achieved through specialized receptors known as host-derived pattern recognition receptors (PRRs). This results in activating several immune effector mechanisms that regulate in turn, the transcription of target genes encoding regulator and effector molecules.

3.1.1. Pathogen-associated molecular patterns (PAMPs)

Pathogen-associated molecular patterns (PAMPs) refer to conserved molecular structures produced by wide variety of microbial pathogens, and not present in the host. These include as example, lipopolysaccharides (LPS), peptidoglycan (PGN), dsRNA, and lipophosphoric acid [93-97]. PGN is considered the main component of bacterial cell wall, and it is classified into either Lys-type PGN found in Gram-positive bacteria, or DAP-type

PGN present in Gram-negative bacteria, depending on their difference in amino-acids residues and cross-linking methods [95].

3.1.2. Pattern recognition receptors (PRRs)

Pattern recognition receptors, or PAMPs receptors, are expressed on the surfaces of host immune cells, and are able to recognize structures that are frequently found in pathogens. They can associated to subcellular compartments, as well as extracellular [2]. Peptidoglycan recognition protein (PGRP) is an important example of PRR, which can recognize PGN. *Drosophila melanogaster* has 13 PGRP genes that have been identified, encoding 19 proteins [98, 99].

3.2. Immune signaling pathways

3.2.1. Toll pathway

The Toll pathway is activated by Lysine-type peptidoglycans (Lys-type) found in Gram-positive bacteria (GNBP1, PGRP-SA and PGRP-SD) and β-glycans (GNBP3) that are characteristics of fungal cell walls [100-103]. The recognition of these PAMPs by PRRs activate a modular serine protease known as ModSP (CG 31217) [104, 105]. This serine protease will trigger a sequential activation of the clip-SPs Grass and Spätzle-processing enzyme (SPE). SPE, then, cleaves pro-Spätzle into the active form of Spätzle (Spz), which

binds to the transmembrane Toll receptor [106]. Hence, in contrast to its mammalian counterparts, Toll is not activated by direct interaction with pathogen, but through an endogenous ligand, Spz [107]. Following the interaction of Spz-Toll, a receptor-adaptor complex is formed to transfer the signal from the cell surface to the nucleus. This complex consists of MyD88 protein, which interacts with Toll through the respective Toll/Interleukin-1 receptor domains, and connects with Tube via death domain contacts that will recruit in turn, the Kinase Pelle [108]. Pelle will then phosphorylate Cactus leading to its degradation [109]. Upon this degradation, the NF-κB homologues Dorsal or Dif are free to move to the nucleus [110]. Dorsal/Dif binds to promoters of target genes, such as *Drosomycin* and other antimicrobial peptide genes, activating their transcription [100]. Rather than the PRR arm of the Toll pathway, Persephone (Psh) arm activates SPE and Toll receptor independently of ModSP and Grass. The clip-SP Persephone is activated by a first cleavage in the bait region by virulence-associated proteases released by pathogens, followed by a second cleavage where a circulating cysteine cathepsin (26-29 p) cleaves the pre-processed form and generates the active form of Psh [111]. This second arm is regulated by the serpin protease inhibitor Necrotic (Nec) [112]. Thus, both PRR and Psh arms promote the activation of Toll pathway leading to a systemic production of certain AMPs [103, 105].

3.2.2. IMD pathway

In addition to Toll, the IMD pathway is activated by Diaminopimelic acid peptidoglycans (DAP-type) which form the cell wall of Gram-negative bacteria and some Gram-positive *Bacilli* [113]. The pathogen recognition in this pathway occurs through the transmembrane PGRP-LC, and the intracellular PGRP-LE [57, 114]. A signalling cascade is then initiated, involving Imd, Dredd and Fadd [115-117]. This signalling cascade leads to the translocation of the NF-κB transcription factors Relish, and Rel2 to the nucleus, activating the transcription of AMPs such as *Diptericin*, and other effector molecules. In some cases, Gram-positive bacteria such as *Bacillus* with DAP-type peptidoglycan can activates the IMD pathway rather than the Toll pathway [113, 118, 119]. These two pathways are presented in the figure 4 below.

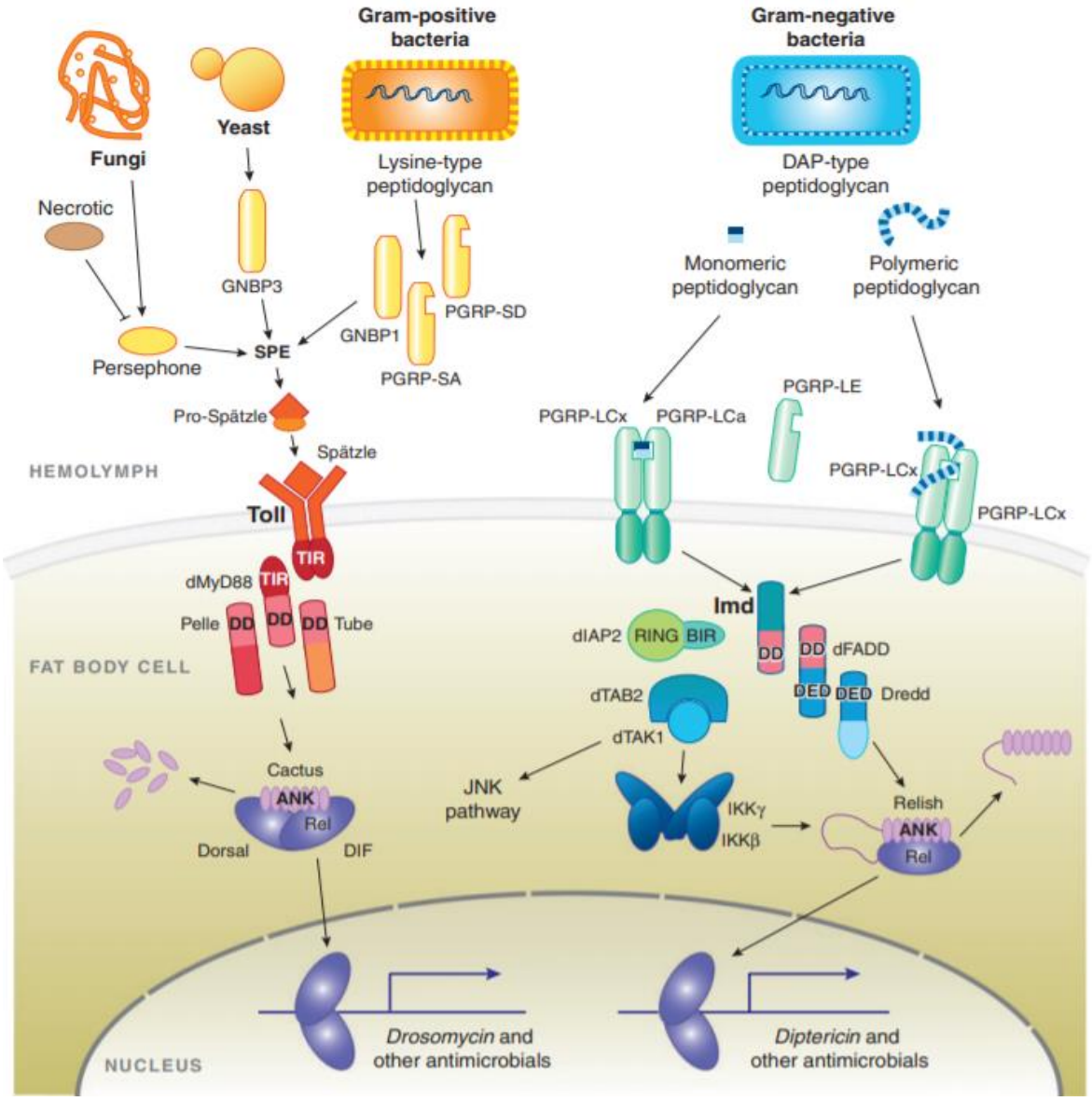


Figure 4: Activation of Toll and IMD pathways.

Two signalling pathways regulate the synthesis of antimicrobial peptides: Toll pathway, which is activated by fungi and Gram-positive bacteria, and IMD pathway that is activated by Gram-negative bacteria. In Toll pathway: distinct PAMPs such as PGRP-SA, PGRP-SD, GNBP1 for Gram-positive bacteria, and Glycan (GNBP3) for entomopathogenic fungi, stimulate a proteolytic cascade leading to the cleavage form of Spätzle. The mature Spätzle binds as a dimer to Toll inducing its dimerization at the plasma membrane [120, 121]. This interaction leads to the recruitment of three intracellular Death domain-containing proteins, MyD88, Tube, and Pelle [122]. Cactus is then phosphorylated by an unknown mechanism, and degraded by the proteasome [123]. As a consequence, the Rel transcription factors Dif and Dorsal are released and move from the cytoplasm to the nucleus [124]. In IMD

pathway: the respective PAMPs of the Gram-negative bacteria, monomeric or polymeric DAP-type PGN, and PGRP-LC, recuits the adoptor Imd [56, 57]. Imd interacts with dFADD, which itself binds the apical caspase Dredd [125]. It has been proposed that this caspase associates with Relish and cleaves it directly if Relish is phosphorylated [126, 127]. Upon the cleavage of Relish, the Rel domain translocates to the nucleus, and the inhibitory domain remains in the cytoplasm. The phosphorylation of Relish is accomplished by the IKK signaling complex [128], which is thought to be activated by TAK1 and its adaptor TAB2 through an Imd- and possibly dFADD-dependent manner. At present, the link between Imd and the IKK complex, either the precise role of TAK1 and DIAP2 (which is thought to activates dTAK1) are still unknown. TIR (Toll-IL1 receptor domain), DD (death domain), DED (death-effector domain), ANK (ANKyrin repeats), Rel (Rel homology domain), RING (RING finger domain), BIR (Baculovirus IAP repeat), SPE (Spatzle¨ processing enzyme). (Bottom) Differential expression of *Drosomycin* (Toll target) and *Diptericin* (IMD target) genes in response to injection of *E. coli* (Gram-negative bacteria) or *M. luteus* (Gram-positive bacteria) (83). *Diptericin* shows an acute phase profile, whereas *Drosomycin* exhibits a late and sustained expression pattern. Adapted from [129].

3.2.3. JAK/STAT pathway

The Janus kinase (JAK)/signal transducers and activators of transcription (STAT) signalling pathway has been identified first in fruit flies for its role in embryonic segmentation [130, 131]. The first evidence for its involvement in immune response origins from a study performed on *Anopheles* mosquitoes, by an accumulation of the protein STAT in the nucleus after immune challenge [132]. Similar to Toll pathway, JAK/STAT pathway is involved in both development and immunity [130, 133-135]. In fact, this pathway is activated when the extracellular cytokine unpaired (Upd) binds to the cellular receptor Domeless (Dome) [136-139]. Then, Dome will be phosphorylated by Hopscotch (Hop) [140, 141], and the protein STAT is recruited, dimerized and translocated to the nucleus [130, 142]. In *Drosophila*, this pathway has been shown to be involved in

antibacterial and antiviral responses, and to regulate the expression of certain AMPs. Furthermore, a subset of *Drosophila* immune-responsive genes has been shown to be regulated by the JAK/STAT pathway. These are the complement like-protein *Tep2* and the *Turandot* stress genes [143-145]. These latter genes are of unknown function, but they are induced by stress conditions, especially through septic injury [146, 147].

3.3. Inducible antimicrobial peptides

3.3.1. Classification of AMPs

Antimicrobial peptides participate strongly in the first line of defence against invading pathogens. They are produced by the cells of the fat body (an organ that is functionally equivalent to mammalian liver) and released into the hemolymph. However, the reproductive tract [148, 149], the gut [150-153], mucosal epithelia [16, 154-157], the trachea [148], and the salivary glands are also capable to produce AMPs [158]. More than 100 insect AMPs have been identified and described since the purification of the first AMP from the pupae of the moth *Hyalophora cecropia* in 1980 [159, 160]. They can be grouped into four families: (1) several cecropin isoforms; (2) the cysteine-containing peptides; (3) the proline-rich peptides; and (4) the glycine-rich polypeptides.

3.3.1.1. Several Cecropin isoforms

Cecropins are the first AMP to be purified and isolated from the immunized hemolymph of *H. cecropia* pupae [161], but later they have been found in Lepidopteran, Dipteran and Coleopteran insects. Cecropins consist of 31-39 residue cationic peptides, devoid of cysteine residues with two α-helix structures joined by a short flexible hinge [162]. They are active against Gram-positive and Gram-negative bacteria [163, 164], and some of them also act against fungi [165-168]. This type of AMP can acts through lysis of bacterial cellular membranes, through inhibiting proline uptake, or through inducing leakiness of membranes [169, 170].

3.3.1.2. Cysteine-containing peptides

These peptides contain 2-8 cysteine residues forming an intramolecular disulfide bridges. Three types of cysteine-containing peptides have been described in details; Defensin, Drosomycin, and Thanatin. Defensins are a small cationic peptide containing six cysteine residues that form three intramolecular disulfide bridges [171]. They are active against Gram-positive and Gram-negative bacteria, but they are efficiently active against Gram-positive bacteria [172]. They promote cell lysis by forming membrane channels in the cells [173]. In parallel, Drosomycins contain eight cysteine residues engaged in the formation of four intramolecular disulfide bridges [174]. They can be induced by bacterial and fungal infection, but it is predominantly active against fungi by inhibiting their spore germination or delaying the growth of hyphae resulting in abnormal morphology [175,

176]. Finally, Thanatin is a 21 residue cationic peptide, with a single disulfide bridge [177]. It is active against both Gram-positive and Gram-negative bacteria, and against filamentous fungi. This AMP has been found only in the bug *Podisus maculiventris* (Hemiptera) [178].

3.3.1.3.Proline-rich peptides

Proline-rich peptides contain at least 25% of proline with no cysteine residues. They are active against both Gram-positive and Gram-negative bacteria (predominantly active against Gram-negative bacteria), as well as fungi [171]. Some of these peptides carry an 0-glycosylated substitution, like Drosocin [179], Pyrrhocoricin [180], and Lebocin [180], which is necessary for their full activity.

3.3.1.4.Glycine-rich polypeptides

These are immune-inducible insect polypeptides (8-30KDa), that have as an average 10-20% of glycine residues [181, 182]. They are mainly active against Gram-negative bacteria. Attacin is the prototype of this family, and have been initially isolated from the moth *Hyalophora cecropia* [183]. Glycine-rich antibacterial polypeptides are present in many insect orders, and their structural homologues in vertebrates have not been reported yet.

3.3.2. AMPs found in *Drosophila*

To date in *Drosophila*, seven different inducible AMPs have been identified [184]. These are several Cecropin isoforms [185, 186], the cysteine-containing peptides Defensin and Drosomycin [174, 187]; the proline-rich peptides Drosocin and Metchnikowin [188, 189]; and the glycine-rich polypeptides Attacin and Diptericin [190, 191]. They have different activity spectrum. Drosomycin and Metchnikowin show antifungal activity [174, 189], Cecropin and Defensin have both antibacterial and antifungal activities [192-195], and finally Drosocin, Attacin, and Diptericin primarily exhibit antibacterial activity [190, 196-198]. After microbial infection, these AMPs are mainly regulated by the two by NF-κB signaling pathways Toll and IMD. Hence, these AMPs are often used as readouts to monitor the expression and the activity of these different immune pathways.

On the other hand, Bomanins are a group of peptides discovered 15 years ago [199], and have been shown to be regulated by the Toll pathway [200]. Conducted studies reveal that they play an essential role against Gram-positive and fungal infection. While these peptides promote microbial killing in the fly hemolymph, their microbicidal activity *in vitro* has not been known yet [201].

Dpt

Unknown

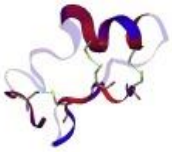
Att

Unknown

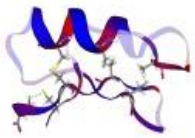
Dro

Cec

Def



Drs



Mtk

Unknown

AMP family	Gene	Location	[in vivo]	Size (AA)	Characteristics	Immune Expression
Diptericin	DptA	2R (55F)	0.5 µM	83	P-rich and G-rich domains, C-terminus amidated, O-glycosylated Thr[8], Asp[52]	Imd
	DptB	2R (55F)		32, 67	Furin cleavage produces two mature peptides: one uncharacterized P-rich peptide and one G-rich with C-terminus presumably amidated	Imd, other
Attacin	AttA	2R (51C)		190	Furin cleavage produces two mature peptides: uncharacterized 9AA short peptide and Attacin G-rich peptide, C-terminus amidated	Imd, Toll, other
	AttB	2R (51C)		190	Furin cleavage produces two mature peptides: uncharacterized 9AA short peptide and Attacin G-rich peptide, C-terminus amidated	Imd, Toll, other
	AttC	2R (50A)		23, 195	Furin cleavage produces two mature peptides: MPAC (P-rich) and Attacin (G-rich) , C-terminus amidated	Imd, other
	AttD	3R (90B)		115	Attacin G-rich domain, lacks a signal peptide	Imd
Drosocin	Dro	2R (51C)	40 µM	19	Furin cleavage, P-rich, O-glycosylated Thr[11], and also a 22AA uncharacterized C-terminal peptide	Imd, other
Cecropin	CecA1	3R (99E)	20 µM	39	Alpha-helical, C-terminus amidated	Imd
	CecA2	3R (99E)		39	Alpha-helical, C-terminus amidated	Imd
	CecB	3R (99E)		39	Alpha-helical, C-terminus amidated	Imd
	CecC	3R (99E)		39	Alpha-helical, C-terminus amidated	Imd
	Anp	3R (99E)		34	Alpha-helical	ejaculatory duct (males)
Defensin	Def	2R (46D)	1 µM	40	Furin cleavage produces two peptides: 30AA uncharacterized peptide and Defensin peptide with disulfide bonds mediated by cysteine bridges	Imd
Drosomycin	Drs	3L (63D)	100 µM	44	Disulfide bonds mediated by cysteine bridges	Toll, Imd
	Drs-like1	3L (63D)		44	Disulfide bonds mediated by cysteine bridges	
	Drs-like2	3L (63D)		50	Disulfide bonds mediated by cysteine bridges	JAK-STAT
	Drs-like3	3L (63D)		45	Disulfide bonds mediated by cysteine bridges	JAK-STAT
	Drs-like4	3L (63D)		44	Disulfide bonds mediated by cysteine bridges	
	Drs-like5	3L (63D)		44	Disulfide bonds mediated by cysteine bridges	
	Drs-like6	3L (63D)		46	Disulfide bonds mediated by cysteine bridges	
Metchnikowin	Mtk	2R (52A)	10 µM	26	P-rich	Toll, other
Bomanin *(AMP-like)*	IM1-type (6 genes)	2R (55C)	10-100 µM	16	16-residue Bomanin domain	Toll
	CG5778-type (3 genes)	2R (55C), 3R (94A)		41-97	Bomanin domain with C-terminal tail	Toll
	IM23-type (3 genes)	2R (55C), 3R (94A)		78-134	2 Bomanin domain repeats with intermediate linker	Toll

Figure 5: A summary of *Drosophila* AMPs.

(Left) The 3D structure of certain AMPs that are analyzed and known [129, 202]. (Right) A summary of AMPs and AMP-like genes (Bomanins) describing their gene family members,

genomic location, concentration *in vivo* upon immune activation, size, and specific characteristics. Adapted from [203].

4. Melanization

4.1. Mode of action

While the synthesis and the production of AMPs take few hours or days to be induced by the Toll pathway, the melanization response is induced within few minutes after infection or injury. Melanization is an immediate immune response against pathogens that breaches the cuticle and invades through septic injury. It involves a rapid synthesis of melanin (black pigment) at wound or infection sites, which can encapsulate and sequester pathogens and facilitate wound healing [204-206].

Figure 6: Melanization reaction induced by microbial infection in *Drosophila*.

The white arrow in the photo indicates the melanization (black pigment) at the site of injury, induced within few minutes after infection by *E. coli* and *M. luteus* through piercing the lateral side of the thorax. Adapted from [207].

This response relies on the enzyme Phenoloxidase (PO), an oxidoreductase that catalyzes the oxidation of phenols to quinones that will then be polymerized into melanin. A detailed illustration of melanin biosynthesis is shown in figure 7.

In *Drosophila* and other arthropods, PO is synthesized and released in the hemolymph as inactive precursor called prophenoloxidase (PPO), and is activated by the action of prophenoloxidase-activating enzyme (PPAE) [208, 209]. During the system of PO activation, the reactive intermediates produced such as quinones-like, nitrogen, and reactive oxygen species (ROS), present a cytotoxic activity against microorganisms, assist in wound healing process, and also prevent the entering of another pathogen to the host [210, 211].

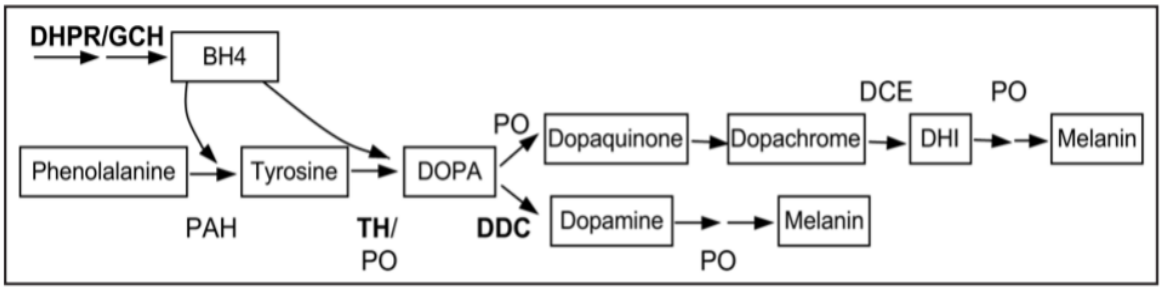


Figure 7: Schematic of melanin biosynthesis in *Drosophila*.

Phenylalanine is first hydroxylated to tyrosine by phenylalanine hydroxylase (PAH) in the presence of tetrahydrobiopterin (BH4), which is synthesized by specific enzymes including dihydropterine reductase (DHPR) and GTP cyclohydrolase (GCH). Then, tyrosine is

converted to DOPA by phenoloxidase (PO) or tyrosine hydroxylase (TH). DOPA is in turn catalyzed to dopaquinone by PO or to dopamine by dopa decarboxylase (DDC). Through non-enzymatically recation, dopaquinone is converted to dopachrome, which is also decarboxylated to 5,6-dihydroxyindole (DHI) by dopachrome conversion enzyme (DCE). Dopamine and DHI are eventually converted to melanin after some reactions involving PO. The enzymes shown in bold are upregulated upon septic injury. Adapted from [207].

4.2. Role of serine proteases and serpins in melanization

The recognition of pathogens promotes the activation of a serine protease (SPs) cascade involved in the cleavage of PPO to generate the active form of PO [212]. Serine protease inhibitors or serpins also play a role in regulating melanization by regulating certain members of the serine protease cascade [213]. *Drosophila melanogaster* genome encodes three PPO genes. PPO1 (CG 5779) is involved in the immediate delivery of PO activity, PPO2 (CG 8193) provides a storage form in crystal cells that can be used in a later phase [214], and PPO3 (CG 2952) is involved in the melanization reaction during the encapsulation response [215]. While PPO1 and PPO2 are synthesized as zymogens and required a proteolytic cleavage to be activated, PPO3 was shown to be produced in its active form [215]. Three SPs showed to be involved in the activation of PPOs in the hemolymph; MP1 (CG 1102), MP2/Sp7 (CG 3066) [213], and Hayan (CG 6361), but their positions and their roles are not fully established [216]. In addition, some serpins control melanization by inhibiting the PPO activation cascade. Spn27A [217] and Spn28D [218] both regulate hemolymph PO activity; while Spn27A prevents excessive melanization to the wound site, Spn28D controls the release of PO, probably in crystal cells. Moreover,

Spn77Ba regulates melanization in the tracheal epithelium by inhibiting the same MP1/MP2 cascade [219].

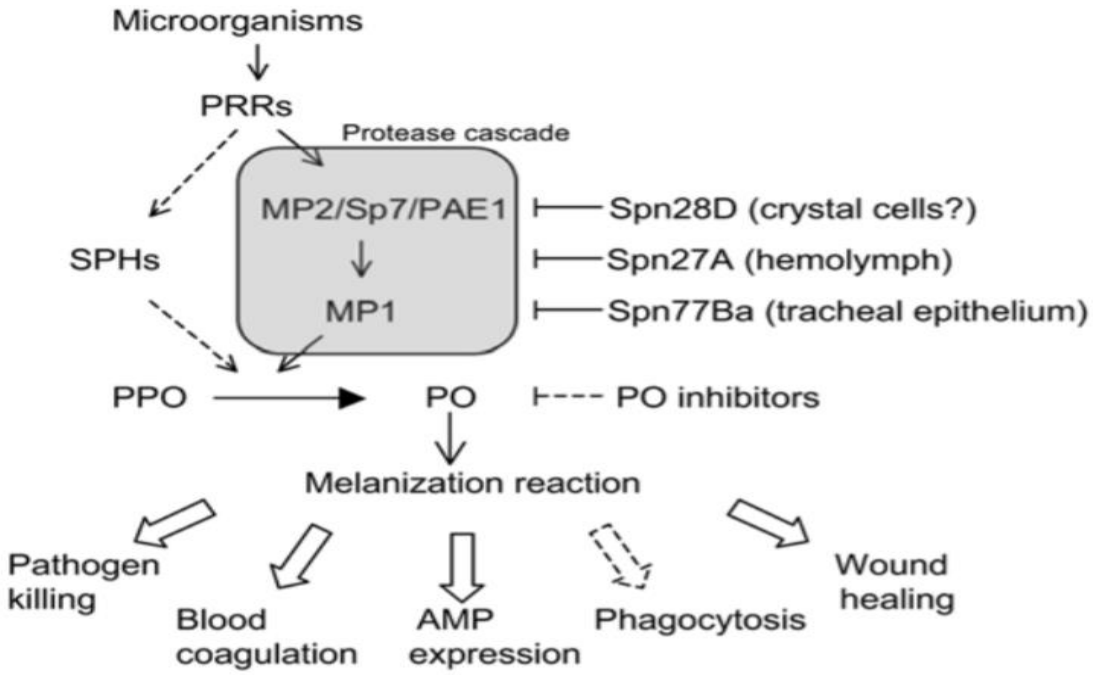


Figure 8: Schematic of melanization activation upon microbial infection in *Drosophila*.

After the recognition of a microorganism, a pattern recognition receptor (PRR) triggers a protease cascade that involves the proteases MP1 and MP2/Sp7/PAE1, which results the cleavage of PPO to PO. Some serpins are found to regulate the activation of PO. In fact, the serpin Spn28D inhibits this activation possibly in crystal cells, Spn27A inhibits the MP1/MP2 cascade in the hemolymph and prevents the excessive melanization after infection, and the serpin Spn77Ba inhibits the same protease cascade in the tracheal epithelium. Some PO inhibitors limit the melanization reaction by directly inhibiting the enzymatic activity of PO, while some serine protease homologues (SPHs) are also involved in activating PO in other insect species. At the end, the resulted melanization reaction not directly kill and clear pathogens, but also cooperates with other immune responses such as blood coagulation, AMP expression, and wound healing. Melanization facilitates also phagocytosis in other arthropod species, but this biological process has not yet been identified in *Drosophila*. Adapted from [207].

4.3. Cross-talk between melanization and Toll pathway

In the distant past, it has been thought that the PPO activation is an independent pathway. However, recent studies highlight the existence of a certain cross-talk between melanization reaction and the Toll pathway. In the beetle model *Tenebrio molitor*, results described that the terminal serine protease, SPE, involved in the Toll signaling pathway, participates also in the regulation of the proPO activation in this insect [220]. However, in the *Drosophila melanogaster* model, the results are not so evident but promising. First, a research on melanization cascade shows that after fungal infection (*Beauveria Bassiana*), MP1 and MP2 RNAi flies exhibited lower levels of expression of *Drosomycin* compared to wild-type flies, indicating the existence of a relationship between the melanization and the Toll pathway protease cascades [221]. Similarly, another research presents the fact that the serpin Spn77Ba acts as a negative regulator of melanization in the respiratory system, and that this local immune response in the trachea induces a systemic expression of *Drosomycin* via a Toll pathway activation [219]. In addition, the serine protease inhibitor Spn5 has been found to play a role in both Toll pathway and melanization reaction. While it suppresses the melanization reaction induced by mutant CHMP2B in the drosophila eye, it also acts as a negative regulator of the Toll pathway by blocking the proteolytic activation of Spätzle [222]. Thus, these studies indicate that the Toll pathway and the melanization response are regulated and controlled by the same shared SPs and serpins but diverting at the last steps of activation. Second, an important study reveals a prominent genetic link between melanization cascade and Toll pathway activation. In response to infection, the serpin

Spn27A is depleted during the melanization process, and this depletion is controlled by a certain secreted SP controlled by the Toll pathway at transcriptional levels [223].

More recently, a conducted study reveals a strong interaction between these two innate immune responses. The results present the important role of Hayan in the local blackening of wound sites, but also, Hayan possesses the same bait region motif of the Psh serine protease, providing a similar mechanism where the microbial proteases cleave the bait region leading to the activation of Toll pathway. Moreover, Sp7, which regulates the activation of the melanization reaction, is in turn regulated by Toll PRR signaling to control septic infection via an Sp7-dependent melanization response. This suggests an unknown mechanism where PRR-Toll pathway diverges to PPO activation downstream of Grass by activating Sp7 and PPO1 [224].

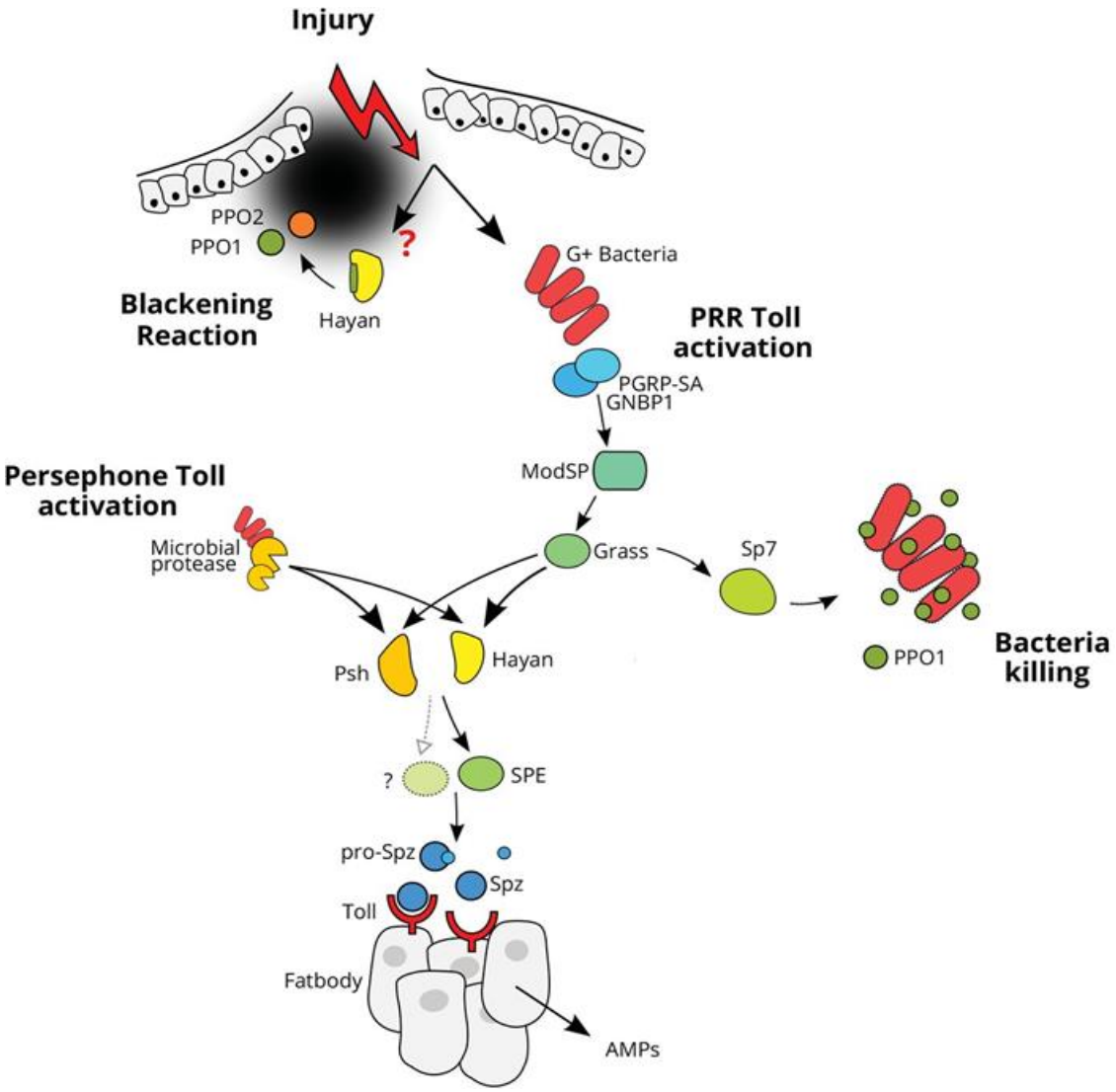


Figure 9: Summarized model of SPs regulating both the Toll pathway and the melanization reaction.

After injury, Hayan is activated by an unknown mechanism resulting in the deposition of melanin around the wound area (top left). This Hayan-dependent blackening reaction can be achieved through both PPO1 and PPO2. In parallel, upon infection with Gram-positive bacteria, peptidoglycan is recognized by specific PRRs such as PGRP-SA and GNBP1, which initiates the sequential activation of the SPs ModSPs, Grass, Psh/Hayan, and SPE, leading to the cleavage of Spz and to the activation of Toll signaling in the fat body (middle). This extracellular SP pathway likely branches at the position of Hayan and Psh to Sp7, activating PPO1 and resulting in the production of cytotoxic intermediates (not necessarily melanin itself) to combat the invading bacteria (right). Microbial proteases can activate the Toll pathway through Psh-SPE-Spz extracellular pathway, but is still unclear if these proteases can also activate Hayan. However, both Hayan and Psh regulate the Toll

pathway downstream of Grass, ModSP and PRRs. A previous study [225] reveals the existence of another SP capable of cleaving Spz beyond SPE. Adapted from [226].

Finally, it has been reported that PO activation is not essential for the activation of Toll pathway, however several studies present the fact that the Toll pathway regulates many SPs and SPHs involved in the melanization cascade, and that some SPHs regulate both melanization and Toll activation [219, 222, 223]. Furthermore, two models have been proposed recently; PRR pathway upstream of Toll branches to PPO activation downstream of Grass to activate Sp7, and microbial proteases (independently of PRRs) cleave Psh and Hayan differently and activate a common extracellular pathway upstream of Toll [224]. Hence, all the studies presented reveal the presence of a clear cross-talk between Toll pathway and PPO activation in *Drosophila*. Yet, advanced studies must be done to discover all the SPs and SPHs players involved in the interaction between melanizaion and Toll pathway activation and to complete and understand the signaling pathway connecting these two immune responses.

B. Serine proteases: role and function

Proteolytic enzymes are broadly distributed in nature and existed in all organisms, from prokaryotes, to eukaryotes, to viruses. They act as positive or negative effectors of different biological processes. Over one third of these enzymes are serine proteases, which

form a large and functionally diverse group [227]. They are a family of protein-cleaving enzymes that have an essential role in numerous biological processes like digestion, blood coagulation, fibrinolysis, immunity, and others. In humans, these serine proteases are widespread in many normal and disease-related physiological functions. Another group of proteases, called serine protease inhibitors or serpins, has been developed by nature to limit and to regulate the activity of the serine protease enzymes.

1. Role and structure of serine proteases

SPs constitute one of the largest family of genes in the *Drosophila melanogaster* genome, and are involved in several biological pathways. Among this family, serine proteases that contain a regulatory domain known as CLIP-domain (Clip-SP) have been found to be involved in embryonic development and humoral immunity [228]. In general, proteases are enzymes that cleave a peptide bond joining amino acids together in proteins. They are synthesized and secreted as inactive forms called zymogens with a regulatory N-terminal pro-domain connected to the catalytic domain by a 23-92 amino acid linker. These zymogens need to be cleaved upstream of the catalytic domain to become active and functional. Hence, the new N-terminus folds into the enzymatic active site and triggers the catalytic activity [228]. In the context of a specific protein-protein interaction, many SPs zymogens can form an efficient cascade pathway in which one protease activates the zymogen of another to mediate a rapid and local response. During evolution, proteases has evolved several times reflecting a change in the biochemical properties, thus in the

biological function of each peptidase structure [229]. Depending on the catalytic mechanism, proteases are classified into five classes: aspartic, metallo-, cysteine, threonine, and serine proteases. For the first two classes, proteases use an activated water molecule as nucleophile to attack the peptide bond of a substrate, while for the remaining ones, the nucleophile is defined by the amino-acid residues (Cys, Thr, and Ser) located in the active site from which the class names derive [230]. These different classes can be also divided into families depending on the similarities in the amino-acid sequences (common ancestor), and these families can be further grouped into clans based on significant similarities in structure (3D-fold of the catalytic domain) [231]. Nevertheless, there is a significant difference in the distribution of each clan across species. The global classification of proteases is available in the MEROPS [232] and ExPASy [233] websites.

2. Serine proteases and serpins involved in Drosophila innate immunity

SPs and proteolytically inactive serine protease homologs (SPHs) are a large family comprising approximately 200 members [234]. Several SPs have been identified for participating in the innate immune response: the Clip-SP Persephone (Psh) activates the Toll pathway by fungi [235], the modular serine protease, ModSP, activates Toll after Gram-positive and fungal infections [104], which in turn activates also the serine protease Grass [236]. Three other serine proteases homologues were identified for defense against both fungal and gram-positive bacterial infection: Spirit, Spheroid and Sphinx1/2 [237, 238]. The proteolytic cascade activates the Clip-SP SPE (Spätzle-processing-Enzyme) that

cleaves pro-spätzle protein to generate the active form of Spz, essential for the activation of antimicrobial response [106]. The latter SPHs are found to be downstream of Grass and upstream of SPE. In addition to AMPs production, the systemic immune response includes the activation of phenoloxydase enzymes leading to melanization reaction [239]. In this process, three Clip-SPs, MP1, MP2, and Hayan are required for the activation of PO during the melanization reaction [204, 216, 221, 240]. A little bit far from a direct immune response, three SPs, Gastrulation defective, Snake and Easter, lead to the cleavage of the cytokine Spz and the activation of the Toll pathway during the establishment of the embryonic dorso-ventral axis [241]. Nevertheless, these SPs are not required for Toll activation during the immune response. A large number of SPs (approximately 100) however, remain uncharacterized.

In addition to the 200 SPs, the *Drosophila* genome encodes 29 serine protease inhibitors. Studies have demonstrated that a few SPNs have a role in the regulation of physiological and immune responses (Toll pathway and melanization) [242]. For instance, the two serpins 28D and 27A (Spn28Dc and Spn27A) control melanization by inhibiting the PPO activation cascade [243]. They regulate both hemolymph PO activity; while Spn27A prevents excessive melanization to the wound site [217], Spn28Dc controls the release of PO, probably in crystal cells [218]. Moreover, the serpin Spn77Ba regulates melanization in the tracheal epithelium by inhibiting the same MP1/MP2 cascade [219], and the serpin Spn88Ea has been found to be involved in the activation of Toll pathway and the systematic *Drosomycin* expression [244]. Finally, the necrotic serpin Spn43ac (Nec), encodes a protease inhibitor controlling a proteolytic cascade that activates the immune

response against fungal and Gram-positive bacterial infections [245, 246]. However, several serpins remain uncharacterized. Therefore, further genetic analysis on the remaining clip-SPs and serpins will be essential to complete our understanding on the host defense systems.

C. RNA interference

1. Important tool to study gene function

RNA interference mechanism (RNAi) is a natural occurring mechanism of silencing gene expression in a wide range of organisms. It's an endogenous cellular mechanism initiated by double-stranded RNA (dsRNA), which leads to the degradation of RNAs into short RNAs that activate ribonucleases to target homologous mRNA [247]. Hence, it becomes an important biological method used to inhibit ("knockdown") or to reduce the expression of target genes in a sequence-specific manner.

RNAi was first identified in *Caenorhabditis elegans* model, where the injection of long dsRNA or feeding the worms with bacteria expressing dsRNA is able to silence gene expression with clear resulting phenotypes [248]. In the fruit fly *Drosophila melanogaster*, the RNAi mechanism was first used by the injection of dsRNA into early embryos to study genes involved in development [249]. This study demonstrates that dsRNA interference can be used to analyze many aspects of gene function. However, limited number of screens

were performed for large injection of dsRNA revealing that injection-based methods are challenging and not efficient for a larger scale [250, 251].

For *in vivo* screens, the use of long or short dsRNAs to generate transgenic libraries proved to be a powerful method and allow the expression of dsRNA in a tissue-specific manner. This is feasible by the availability of transgenic *Drosophila* lines. In fact, *Drosophila* genome-wide RNAi libraries have been developed and RNAi fly stock collections are available from different public stock centers such as the Bloomington Drosophila stock center (BDSC), the Vienna Drosophila Resource Center (VDRC), the National Institute of Genetics (NIG-Japan), and the Transgenic RNAi Project (TRiP) [252]. Furthermore, online websites are also available to evaluate the efficiency of knockdown (KD) such as the UP-TORR, RSVP and FlyBase by proving information about perfect match to RNAi sequence, mismatches and off-target genes [253]. In addition, negative and positive control RNAi transgenic flies should also be included in the screen.

2. UAS/Gal4 system

In this study, an RNAi approach is used to screen for new SPs acting upstream of the Toll pathway and to investigate the role of candidate SPNs in the immune response. This RNAi *in vivo* screen is done using the Gal4-UAS system to study the unexplored SPs and SPNs genes. The system is composed of two parts: the Gal4 gene, encoding the yeast transcription activator protein Gal4, and the UAS (Upstream Activation Sequence), an

enhancer to which Gal4 binds in order to activate gene transcription [254]. Indeed, a parental fly carries the inverted repeat (IR) construct of the SP or SPN gene of interest (transgenic expression of RNAi) under the control of the UAS element, and another parental fly harbors the construct for gene expression of the Gal4 protein. The RNAi is under the control of a minimal promotor that needs the transcription activator Gal4 bound to the enhancer sequence UAS allowing the transgene expression. Thus, to activate RNAi of the candidate SPs in the progeny (F1 flies), virgin females carrying Gal4 driver are crossed to males carrying the IR construct of the SP leading to the knockdown of the target SP or SPN gene [254, 255].

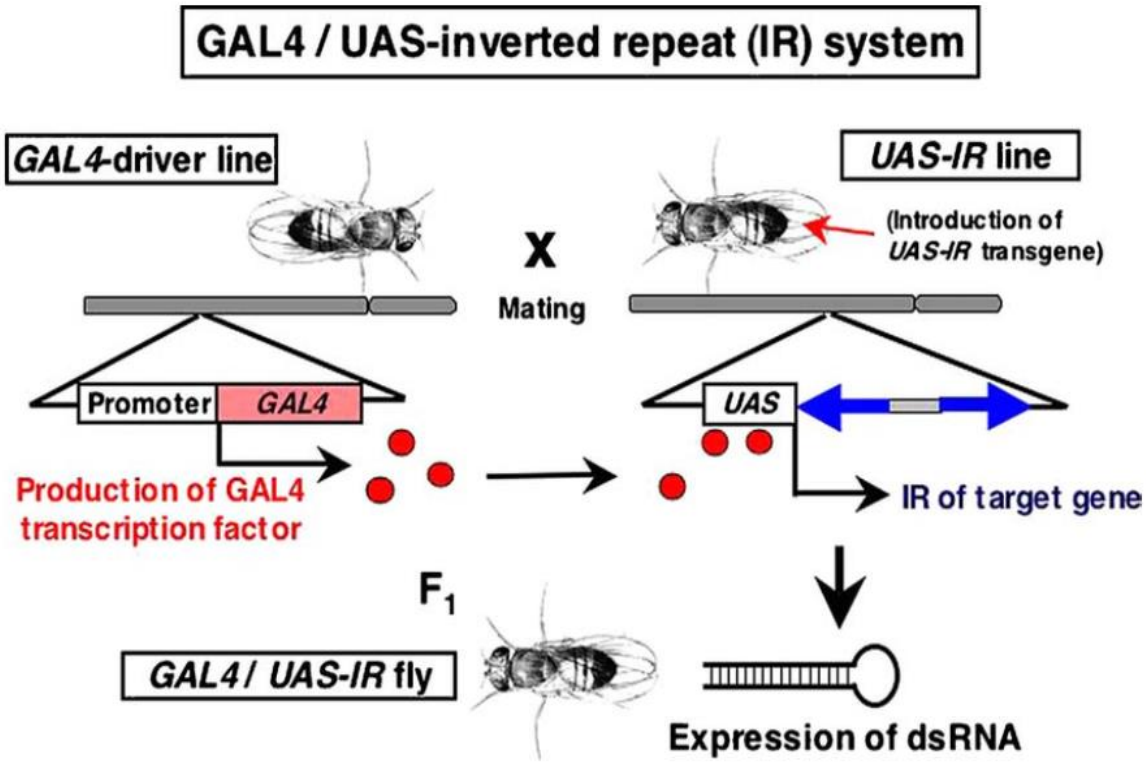


Figure 10: UAS/Gal4 system used for gene targeting in *Drosophila*.

This system is based on two transgenic fly stocks, Gal4-driver and UAS-IR. The first fly has a transgene containing the yeast transcription factor Gal4, which its expression is under the control of a tissue-specific promotor. The second fly has an IR of the target gene ligated to the UAS promotor, a target of Gal4. In the F1 progeny of these flies, the dsRNA of the target gene is expressed in specific tissue to induce gene silencing. Adapted from [256].

D. *Drosophila* as a model for studying pathogens that can be used as potential pest control

Insects (order Insecta or Hexapoda) dominate the world of fauna with about one million described species [257]. They have an essential role in many ecosystem processes. While some insects are considered beneficial by pollinating plants, acting as scavengers and serving as predators for others, many are classified to be disease-vectors such as mosquitos, sandflies, ticks, tsetse flies, triatomine bugs, mites and lice.

1. Mosquitoes as disease vectors

Mosquitoes are vectors of several deadly human diseases like malaria, dengue fever, chikungunya, Japanese encephalitis and more recently emerging the Zika virus [258]. They are a diverse group of insects, with over 3000 species spread over the world. The most common and most dangerous mosquitoes are *Culex*, *Anopheles* and *Aedes* as they constitute vectors of human and animal diseases. In fact, some pathogens, like parasites and viruses, succeed to evade the mosquito immune system and are transmitted by the mosquito

vector. Hence, mosquitoes can transmit infectious diseases from animals to humans or between humans (blood meal from infected host) and cause a wide range of diseases, some of which are deadly [259]. Facing this fact, the development of various control methods is a need, in order to limit the spread of mosquito-borne diseases.

2. Mosquitoes species in Lebanon

It has been reported in Lebanon the existence of around twelve mosquito species. These are *Culex pipiens*, *Cx. laticinctus*, *Cx. mimeticus*, *Cx. hortensis*, *Cx. judaicus*, *Aedes aegypti*, *Ae. cretinus*, *Ochlerotatus caspius*, *Oc. geniculatus*, *Oc. pulchritarsis*, *Culiseta longiareolata* and *Anopheles claviger* [260]. Some of these mosquitoes are known be vectors of certain diseases like the mosquitoes of the *Culex* group, and the Asian tiger mosquito, *Aedes albopictus*. *C. pipiens* are the most widely distributed species that can be found in rural and urban regions. A recent study showed that this predominant strain are efficient vectors of the West Nile Virus, and a lower extent of the Rift Valley fever virus [261]. In addition, *A. albopictus* was first identified in Lebanon in 2003, and its population size and geographical distribution has considerably increased since then [262]. This local species has also been reported to transmit chikungunya, dengue, and West Nile viruses, classifying it as a potential risk for being vectors of arboviruses [263]. So far, no case of mosquito-borne disease outbreak has been reported in Lebanon. However, climate warning, the frequent travel and massive mobility of people associated with modern life, and the presence of endogenous mosquito vectors places some areas such as the Mediterranean

countries at high risk of epidemics. Hence, it is important to anticipate the development of new control strategies.

3. Mosquitoes control strategies

3.1. Chemical methods

Chemical insecticides played an important role in controlling vectors of diseases and have been used to reduce the risk of insect-borne diseases, especially in the case of malaria. Malaria has been controlled by the use of residual house-spraying of DDT and prophylaxis with chloroquine, which together form a powerful combination for limiting malaria transmission [264, 265]. However, the problem of resistance to all classes of insecticides becomes to rise [266-268]. In Sri Lanka, for example, resistance to malathion occurs in *Anopheles culicifacies* species B [269]. In addition, resistance to organophosphorus insecticide is widespread in all the *Culex* vectors [270] and pyrethroid resistance was noted in *An. Albimanus* [271], *An. Stephensi* [272] and *An. Gambiae* [273]. For *Aedes aegypti*, resistance to pyrethroid [274], organophosphate and carbamate was also recorded in this species [275]. The major factors that influence the degree of insecticide resistance depend on the volume and the frequency of applications of insecticides used against insect vectors and the inherent characteristics of the insect species [266]. Thus, the use of chemical insecticides has been limited by the emergence of resistance problems, but also because of the potential human health risk (relationship between pesticides and

mutations and cancer) [276-278], of environmental contamination [279, 280], and of being prohibitively expensive. Therefore, alternative biocontrol strategies are required to control insects. One proposed approach is the use of the endosymbiotic bacterium Wolbachia [281-285]. Moreover, control strategies based on naturally occurring microbial pathogens emerged as promising method to control insects [286].

3.2. Biological methods

Biological pest control agents are based on naturally occurring microorganisms, including viruses, bacteria and fungi.

3.2.1. Viruses and bacteria

Viral insecticide production increases the production costs 4 to 5 folds because the purification of the viral preparation is labor-intensive and time consuming [287, 288]. Concerning bacteria, different groups have been identified and used for their pathogenicity to insects. The most powerful and the best bacterial insecticide is one of the member of genus *Bacillus*. *Bacillus thuringiensis* (*Bti*) is a Gram-positive, facultative anaerobic sporulating bacterium. It can be found naturally in soils, and it produces endospores, so it can remain as dormant cells in the environment in time of extreme stress until favorable conditions allow again for its development [289]. During sporulation, *Bti* produces

parasporal crystalline inclusions containing Cry and Cyt proteins. These δ-endotoxines have insecticidal action [290-292]. In fact, studies demonstrate that each crystal protein is characterized by a highly specific to a very restricted insect host spectrum [293-295]. Furthermore, these toxins are biodegradable, and harmless to humans and plants [296]. So insecticidal crystal protein increases the value of *B. thuringiensis* as an important biological control agent [297, 298]. Lastly, fungal preparation offers a potential control towards insects and diseases [299].

3.2.2.Fungi

Fungi are the most studied source of insect disease in nature. A conducted research reported that approximately a thousand of known entomopathogenic fungi have narrow host range, but together, they target all known insect species including sucking insects and several coleopteran and orthopteran pests [300]. The majority of research focused on *Beauveria Bassiana* and *Metarhizium anisopliae* strains [301-306]. These two fungal strains have a broad host range of insect orders (Lepidoptera, Coleoptera, Diptera, and others) but also extend their specificity to ticks and mites (subclass Acari) [307]. They are the most common applied mycoinsecticides for different insect controls reasons.

3.2.2.1. Mode of infection

Unlike viral and bacterial pathogens, fungi infect the insect with contact and do not require to be consumed by their host to cause infection. In fact, when fungal spores land on the cuticle of susceptible insects, they germinate and produce a germ tube. Then, they grow directly through the cuticle by producing an appresorium and enter the insect body through mechanical pressure and enzymatic degradation of the cuticle. At this level, the fungus proliferates throughout the insect's body by producing toxins and draining the host of nutrients, which will lead to its death. Upon the death of the insect, fungus emerges to cover the cadaver and the sporulation occurs. The massive new spores produced are released to the environment in order to infect new hosts [308].

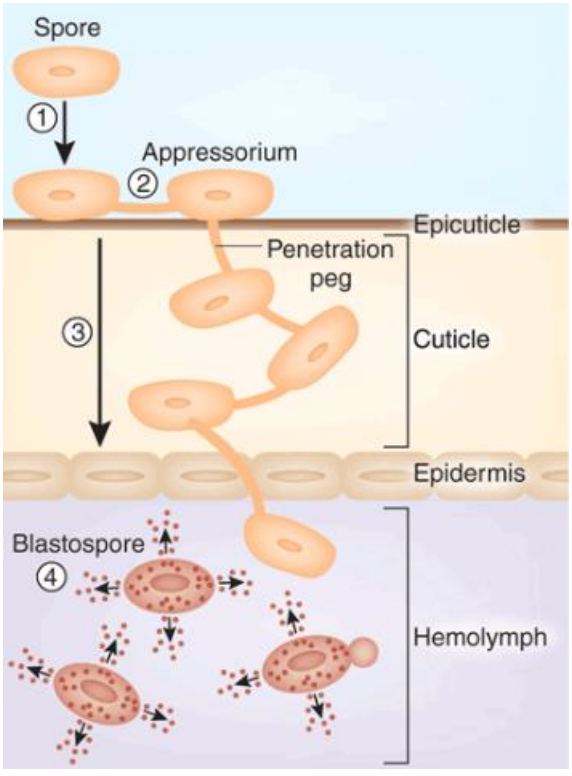


Figure 11: The mode of action of entomopathogenic fungus in insects.

When the fungal spore came into contact with the insect cuticle (1), the recognition of the host by the fungus leads to spore germination and production of a penetration structure called appressorium (2). This latter structure grows a penetration peg, and a series of hyphal bodies to cross the cuticle and the epidermis (3). Once inside the insect, the fungus produces blastospores, which bud and spread through the hemolymph (4). This expression restricts the release of an insect-specific toxin to the period after infection. Adapted from [309].

3.2.2.2. Advantages

The use of fungi for controlling insect pests is considered a good biological control agent for different reasons. First, they are safe to use as insecticide since they do not affect people or other mammals. Second, the massive production of fungal spores is easy, which reflect a comparable price of the fungal product compared to other biological control agents. The commercial fungal product is also easy to handle; the spores are extracted and made into a sprayable form, which is similar to existing application methods. Concerning host specificity, several fungi have a broad host range meaning that one same product could be used for multiple pests. Finally, the fast spreading of spores to infect new hosts lead to high rate of persistence within a growing season, even if for some fungi, the persistence tends to be low between season [310].

3.2.2.3. Limitations of use

Despite the advantages of the fungal pathogens used as biological control, the fungal product notes some disadvantages. The killing time of most of the fungi is relatively long (takes 4-5 days), while the pathogens selected for commercial products are best to kill much faster. Their broad host range could sometimes affect beneficial insects leading to a possible negative impact. In addition, environmental factors such as fluctuation in temperature, humidity and moist conditions, influence the efficacy of the fungi. Fungal spores are damaged and inactivated by solar radiation (UV light), so it's better to spray the product in cool to moderate temperatures (morning or late afternoon) [311].

In general, the success of any biological control agents relies on certain criteria, which are defined by being easy and cheap, commercially viable, sustainable and safe. The use of fungi as biological control agents is environmentally friendly and cost-effective offering a safer alternative of use than chemical insecticides. However, future efforts in genetic engineering could be focused on developing recombinant strains of insect pathogenic fungi to produce target-specific and marker-free products [312, 313]. This established strategy will improve the efficacy and the safety of entomopathogenic fungi as a powerful insect biological control agent.

CHAPTER II

SIGNIFICANCE AND AIMS OF THE PROJECT

Innate immunity is the first line of host defense mechanism to restrict pathogen infections. Toll-like receptors (TLRs) count among the key molecules that alert the immune system, and are named for their similarity to toll, a receptor first identified in the fruit fly *Drosophila melanogaster*. This reflects a conserved signaling pathway that governs an evolutionarily ancient immune response in both insects and vertebrates, and presents *Drosophila melanogaster* as a powerful model to decipher innate immune responses and to study host-pathogens interactions. Antimicrobial peptides are a major component of insects' immunity and their production is mainly controlled by two pathways: Toll and IMD. Since Toll pathway is regulated by different SPs and SPNs, it is important to investigate the role of the unexplored ones for a better understanding of the resulted pathogenesis. In response to fungal or Gram-positive bacterial infections, the Toll signaling pathway is activated by a cascade of Serine Proteases (SPs) leading to the cleavage of the cytokine Spz, the Toll ligand. The *Drosophila* genome encodes 200 SPs and around 29 serine protease inhibitors (SPNs). Several SPs have been shown to act upstream of Toll, linking microbial recognition to the cleavage of Spz. Other SPs have also been shown to mediate the flies' melanization reaction. However, a large number of SPs and SPNs remain unexplored and the complexity of proteolytic cascades operating upstream of Toll during the flies' immune response is not completely understood.

In this project, we aimed to identify new SPs acting upstream of the Toll pathway. We used an *in vivo* RNAi approach to knockdown (KD) the expression of different unexplored SP genes. At the functional level, we tested the SP-KD flies for their ability to survive different microbial infections. At the molecular level, we determined the level of antimicrobial peptides produced by the SP-KD flies with compromised survival. We aimed also to analyze the effect of SP-KD flies on melanization reaction.

In parallel, billions of human lives are threatened by mosquito-borne diseases especially in tropical and sub-tropical zones, and climate warming will lead to their spread in the near future. In Lebanon, the *Culex* group and the Asian tiger mosquito *Aedes albopictus*, were found. The use of insecticides to control these pests is limited by the issues of environmental contamination, risks for human health and by the emergence of resistance problems. Therefore, alternative biocontrol strategies based on naturally occurring microbial pathogens emerged as promising method to control insects. Fungi are the most common and the most studied cause of insect disease in nature, and the fungus *Beauveria Bassiana* has been approved by the United States Environmental Protection Agency as a pest biological control method. Hence, we collected different naturally occurring fungi from dead arthropods and tested their pathogenicity on lab insect models (*D. melanogaster*, *C. pipiens* and *A. albopictus*) in the aim of a potential utilization as new biological pest control agents.

SPECIFIC AIMS

<u>Specific aim 1:</u> Identification of Serine Proteases involved in *Drosophila* immunity.

Several SPs have been shown to play a role in the Toll pathway activation. A large number of SPs (about 90) remain unexplored. We planned to determine if some of these SPs are acting upstream of Toll. For this, we will:

1. Use an *in vivo* RNAi approach to knockdown (KD) the expression of different SPs genes.
2. Test the SP-KD flies for their ability to survive different infections.
3. Determine if the SP-KD flies with compromised survival have lower levels of AMPs.

<u>Specific aim 2:</u> Identification of fungi that can be used as potential insect biocontrol agents.

The use of insect natural pathogens is a promising pest control strategy. We planned to identify fungi that may be used as potential biocontrol agents. For this, we will:

1. Collect dead arthropods and identify them.
2. Isolate and identify fungi from the cadavers.
3. Test the pathogenicity of isolated fungi using *Drosophila melanogaster*.
4. Test the fungi that are pathogenic to *Drosophila* on mosquitoes.
5. Test the fungi by natural infection on mosquitoes in order to mimic the natural route of infection (spraying a suspension of fungal spores).
6. Test the pathogenicity of the fungus *A. nomius* using another type of mosquito.

CHAPTER III

RESULTS

A. Identification of Serine Proteases involved in *Drosophila* immunity

1. Use an in vivo RNAi approach to knockdown (KD) the expression of different SPs genes

An RNAi approach is used to screen for new SPs acting upstream of the Toll pathway. This RNAi *in vivo* screen is done using the Gal4-UAS system, where each transgenic fly possesses an inverted repeat construct of the SP of interest under the control of the UAS element, which is recognized by a Gal4 transcriptional activator. Thus, to activate RNAi of the candidate SPs in the progeny (F1 flies), virgin females carrying Gal4 driver are crossed to males carrying the IR construct of the SP of interest. Hence, the inverted repeats of the target gene are transcribed and bind to the specific mRNA inducing its degradation, thus gene silencing of the target SP.

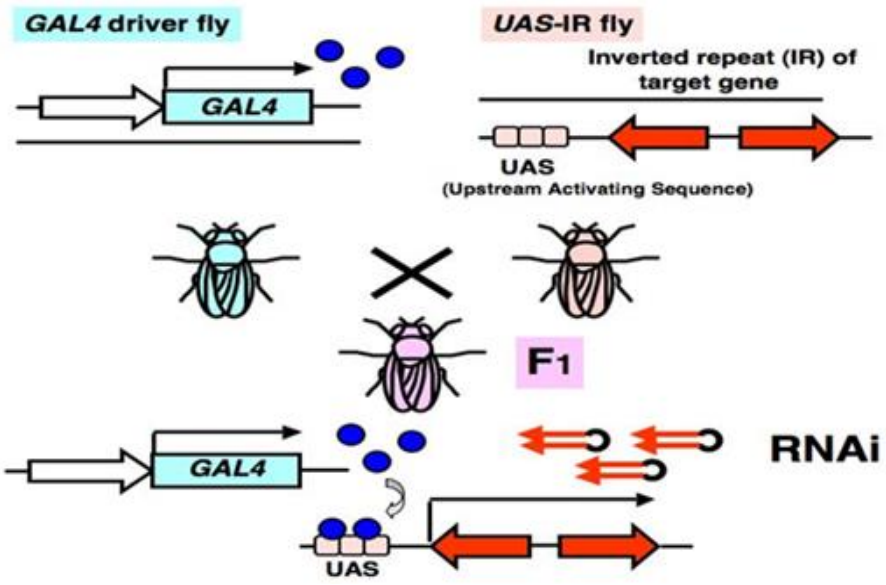


Figure 12: UAS/Gal4 system used for gene silencing with RNAi in *Drosophila.*

The parental fly carrying Gal4 driver will mate another parental fly harboring the UAS-IR. In the progeny, Gal4 is expressed and binds to UAS leading to the synthesis of dsRNAs. Adapted from [253].

In this study, we analyzed 44 transgenic strains of fruit flies targeting different SP genes. These flies are shown in the table below.

Table 1: List of the analyzed 44 strains of fruit flies and their corresponding names and synonyms.

This table below shows the list of the CG number of the 44 analyzed SPs, their name and their synonyms. "c-" refers that the SP gene contains a clip-domain. This information is provided according to the FlyBase online tool (https://flybase.org/).

CG number	Name synonym(s)	Synonym(s)
CG6361	Hayan	c-SP31
CG6462	-	SP127
CG5909	-	c-SP8
CG10477	NF-YB-like	SP51
CG3795	-	SP160/EG:9D2.4
CG10405	-	SP155
CG4053	-	SP185
CG11668	-	c-SP115
CG3088	-	SPH188
CG11529	-	SP84
CG4653	-	SPH179
CG17477	Chymotrypsin-type peptidase	SPH174
CG17571	Anon-38C.22	SP83
CG14892	-	SP175
CG11670	-	SP27
CG10232	-	c-SP14
CG12256	-	SP133
CG9897	-	SPH195
CG9733	Pro-phenoloxidase AE	c-SP10/proAE/proPO-AE
CG11664	-	SP193/EG:BACR7A4.3
CG12951	-	SP87
CG8464	HTRA2-related serine protease	DmHtrA2/Omi/domi
CG9631	-	SP60/BcDNA:GH08420
CG11842	-	SP68
CG15002	Masquerade	c-SPH79/mas
CG15873	-	SPH184
CG1304	-	SP46
CG4386	Tracheal-prostasin	SP17/tyr
CG14088	-	SPH199
CG3700	-	c-SP48
CG18420	-	SPH111/SPH205
CG7754	ιTrypsin/Trypsin iota	SP129/IotaTry/ιTry
CG15046	-	c-SPH
CG3650	-	SP159
CG1773	-	SP74
CG8213	Filzig	c-SP44/flz/lint
CG4650	-	SPH197
CG7432	-	c-SP19
CG6865	-	SP63
CG4927	-	c-SP42
CG6592	-	SP105/lincRNA.S4213
CG18681	εTrypsin/epsilonTrypsin	SP88/epsilonTry/εTry
CG12133	-	SP15
CG16710	-	c-SP16

We performed Blast analysis of these 44 SP genes in order to check if their IR constructs could inactivate other genes due to homology with their nucleotide sequences. These off-targets analysis is done based on VDRC and UP-TORR websites, and the results are shown in Table S1.

Furthermore, we checked for lethality after ubiquitously knowing-down genes using the Actin-Gal4 driver. We have performed at least three independent repeats, and the results showed that ten out of thirty-three genes were found to be lethal. These genes correspond to: CG6462, CG11668, CG11529, CG17571, CG14892, CG12256, CG9897, CG9733, CG1304, and CG1773. Thus, we will have to use a more restricted driver, the C564-Gal4 that expresses Gal4 in the fat body, in order to achieve gene KD. With this latter one, no lethality was observed after SP-KD.

2. Test the SP-KD flies for their ability to survive different infections

After gene KD, the first generation (F1) progeny was collected three to seven days post-eclosure from pupae. The tubes contained fifteen flies and each batch of these flies was infected with four different microbes: *Erwinia carotovora carotovora 15* (Gram-nagative bacteria), *Staphylococcus aureus* and *Enterococcus faecalis* (Gram-positive bacteria), and the entomopathogenic fungus *Beauveria Bassiana*. After infection, the dead flies were counted at frequent intervals, and survival graphs were plotted, representing the percentage of surviving flies in function of time (hours) for each microbial infection.

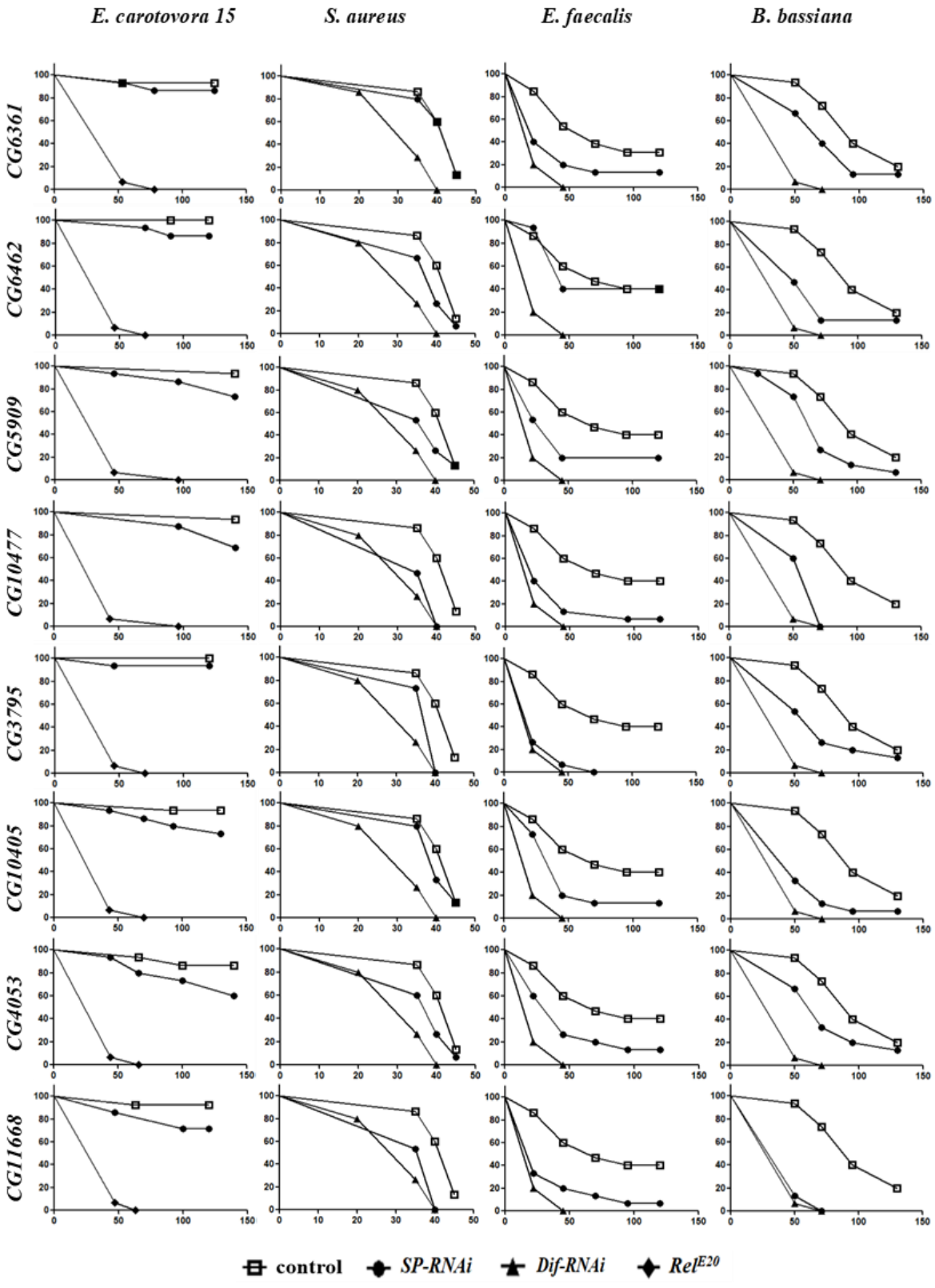
After infection with the Gram-positive bacterium, *S. aureus* (OD 0.05). Only 8 SP-KD flies out of forty-four showed compromised survival and these correspond to: CG10477, CG3795, CG11668, CG3088, CG11670, CG10232, CG9897, and CG9733.

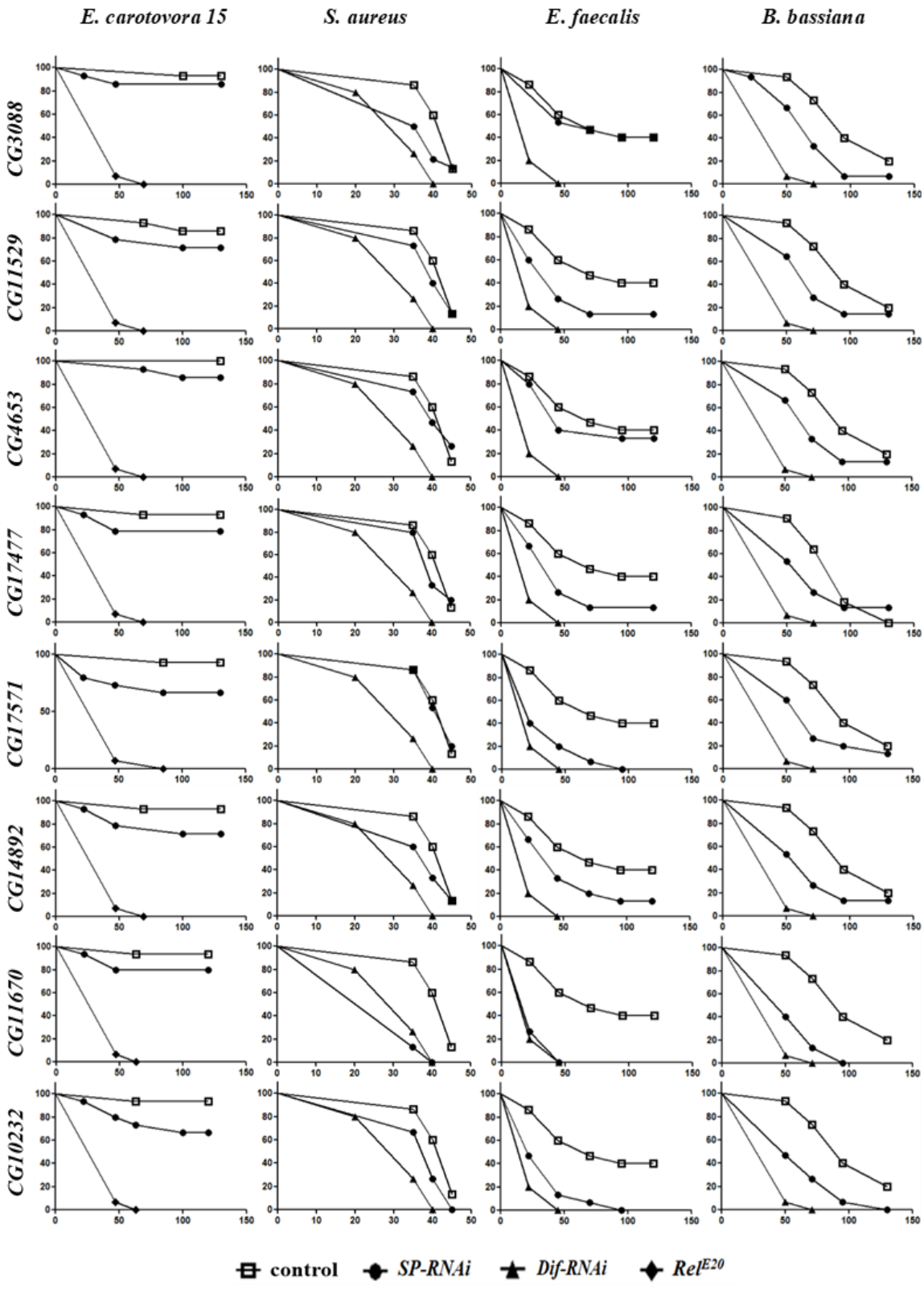
Another Gram-positive bacterium is used, *E. faecalis* (OD 0.05), and 19 SP-RNAi flies showed compromised survival as compared to the wild-type flies, and to the other KD genes. These SP-KD flies are: CG6361 (Hayan), CG5909, CG10477, CG3795, CG10405, CG4053, CG11668, CG11529, CG17477, CG17571, CG11670, CG10232, CG12256, CG9897, CG9733, CG12951, CG8464, CG9631, and CG11842.

Survival assays with the fungus *B. bassiana* (100 fungal spores) reveals that 22 SP-KD flies sowed compromised survival as compared to the wild-type and *Dif*-KD flies which constitute the positive control for both Gram-positive and fungal infection. These twenty-one SP-RNAi flies are CG6361, CG6462, CG5909, CG10477, CG3795, CG10405, CG4053, CG11668, CG3088, CG11529, CG4653, CG17571, CG14892, CG11670, CG10232, CG12256, CG9897, CG9733, CG11664, CG12951, CG8464, and CG11842.

On the other hand, none of the SP-KD flies infected with the Gram-negative bacterium *E. carotovora 15* (OD 0.1) showed compromised survival as compared to the wild-type flies and to the positive control Rel^{E20} that succumbed fast after microbial infection.

As a result, 24 out of the 44 analyzed SP-KD flies showed compromised survival after at least one microbial infection (p-value less than 0.05 compared to wild-type control flies). All the results were confirmed at least three times (different biological replicates) and the representative graphs of the twenty-four SP-KD flies with compromised survival are shown in figure 13.


E. carotovora 15
S. aureus
E. faecalis
B. bassiana
CG6361
CG6462
CG5909
CG10477
CG3795
CG10405
CG4053
CG11668
control
SP-RNAi
Dif-RNAi
Rel^E20


E. carotovora 15
S. aureus
E. faecalis
B. bassiana
CG3088
CG11529
CG4653
CG17477
CG17571
CG14892
CG11670
CG10232
control
SP-RNAi
Dif-RNAi
RelE20

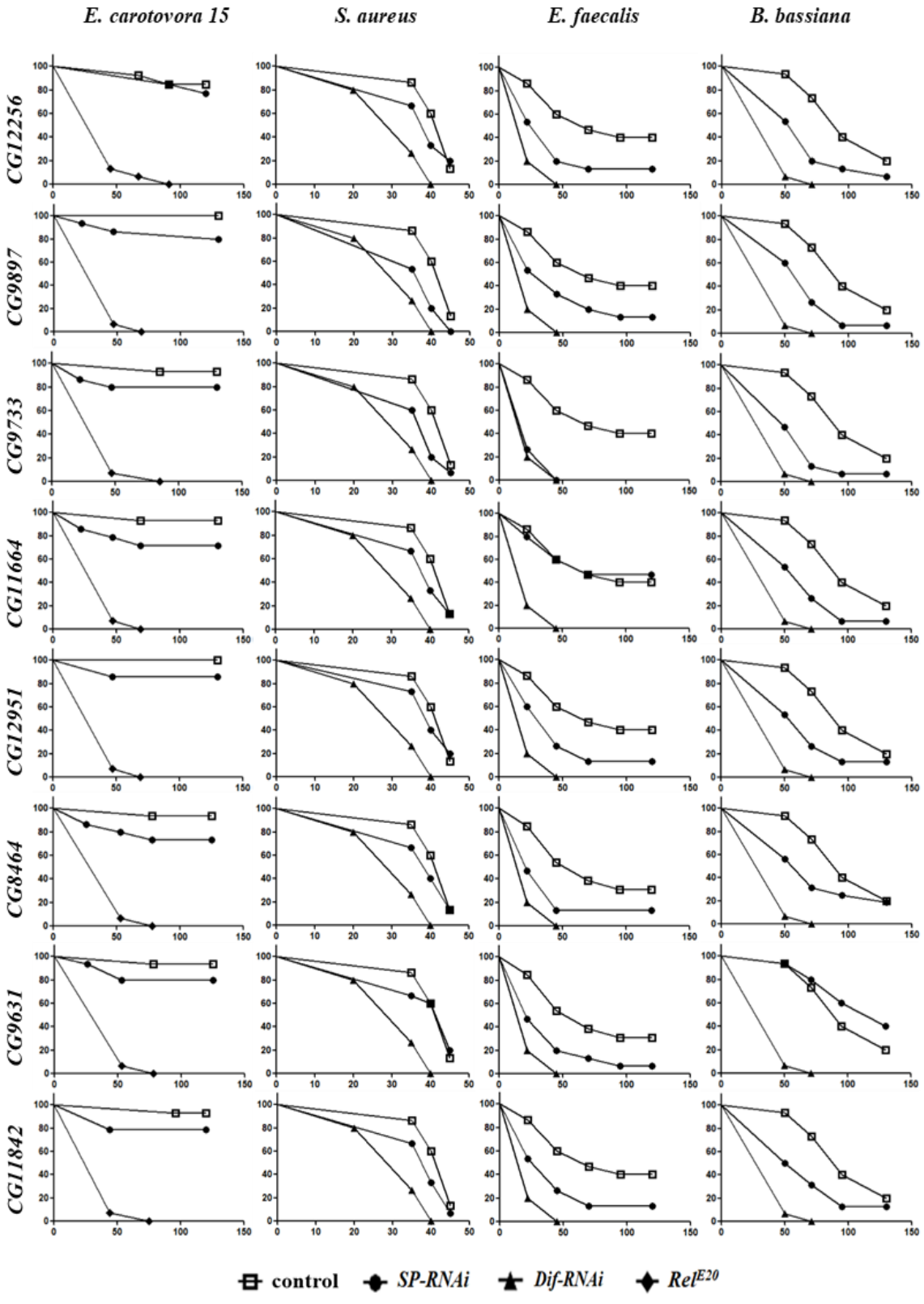
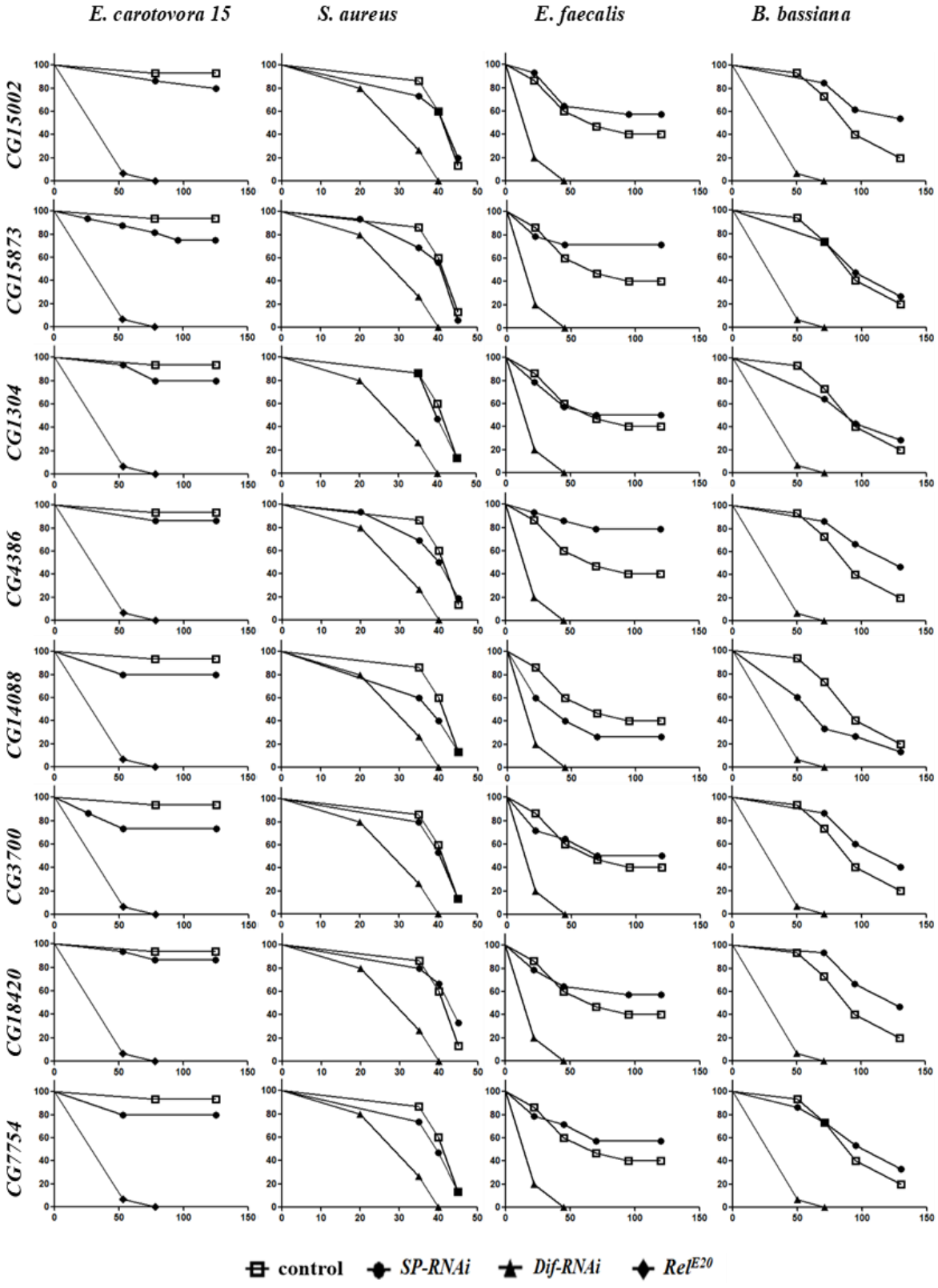
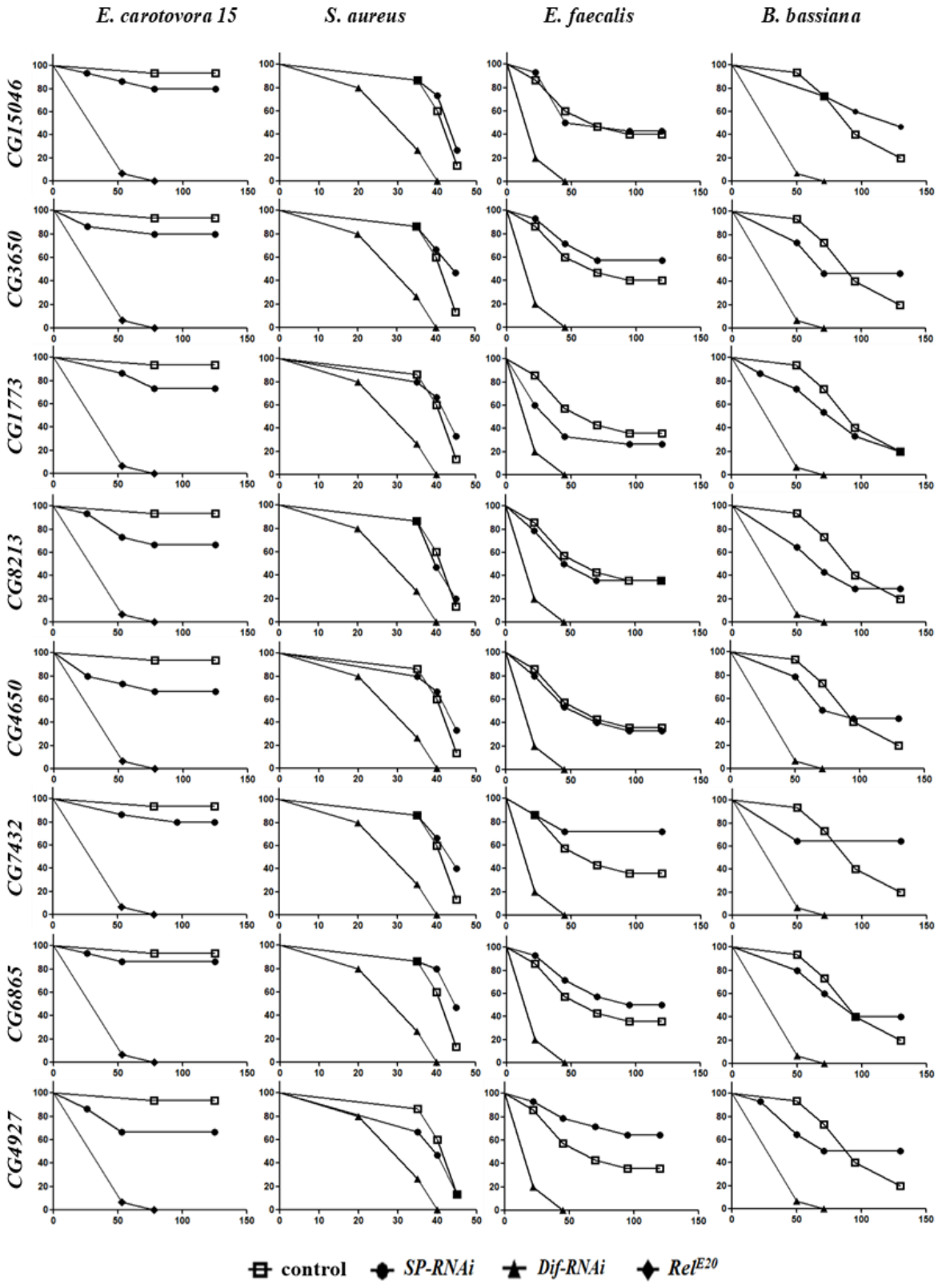

E. carotovora 15
S. aureus
E. faecalis
B. bassiana
CG12256
CG9897
CG9733
CG11664
CG12951
CG8464
CG9631
CG11842
control
SP-RNAi
Dif-RNAi
Rel^E20

Figure 13: Twenty-four SPs genes required for resistance to microbial infections.

These survival graphs represent the percentage of surviving flies in function of time (hours) for each microbial infection. Wild-type flies (plain line with squares) are used as a control for all the microbial infections. *Dif-RNAi* flies (plain line with triangles) are used as a control for Gram-positive bacteria, and $Relish^{E20}$ (plain line with rhombus) as a control for Gram-negative bacteria.

Since twenty-four SP-KD flies out of the forty-four showed compromised survival after infection with different pathogens, the remaining analyzed 20 SP-KD flies showed no statistically significant results compared to the wild-type control flies ($p>0.05$). These SP-RNAi flies with no compromised survival are CG15002, CG15873, CG1304, CG4386, CG14088, CG3700, CG18420, CG7754, CG15046, CG3650, CG1773, CG8213, CG4650, CG7432, CG6865, CG4927, CG6592, CG18681, CG12133, and CG16710. The survival graphs of these SP-RNAi flies are shown in the Figure 14.


E. carotovora 15
S. aureus
E. faecalis
B. bassiana
CG15002
CG15873
CG1304
CG4386
CG14088
CG3700
CG18420
CG7754
control
SP-RNAi
Dif-RNAi
Rel^E20


E. carotovora 15
S. aureus
E. faecalis
B. bassiana
CG15046
CG3650
CG1773
CG8213
CG4650
CG7432
CG6865
CG4927
control
SP-RNAi
Dif-RNAi
Rel^E20

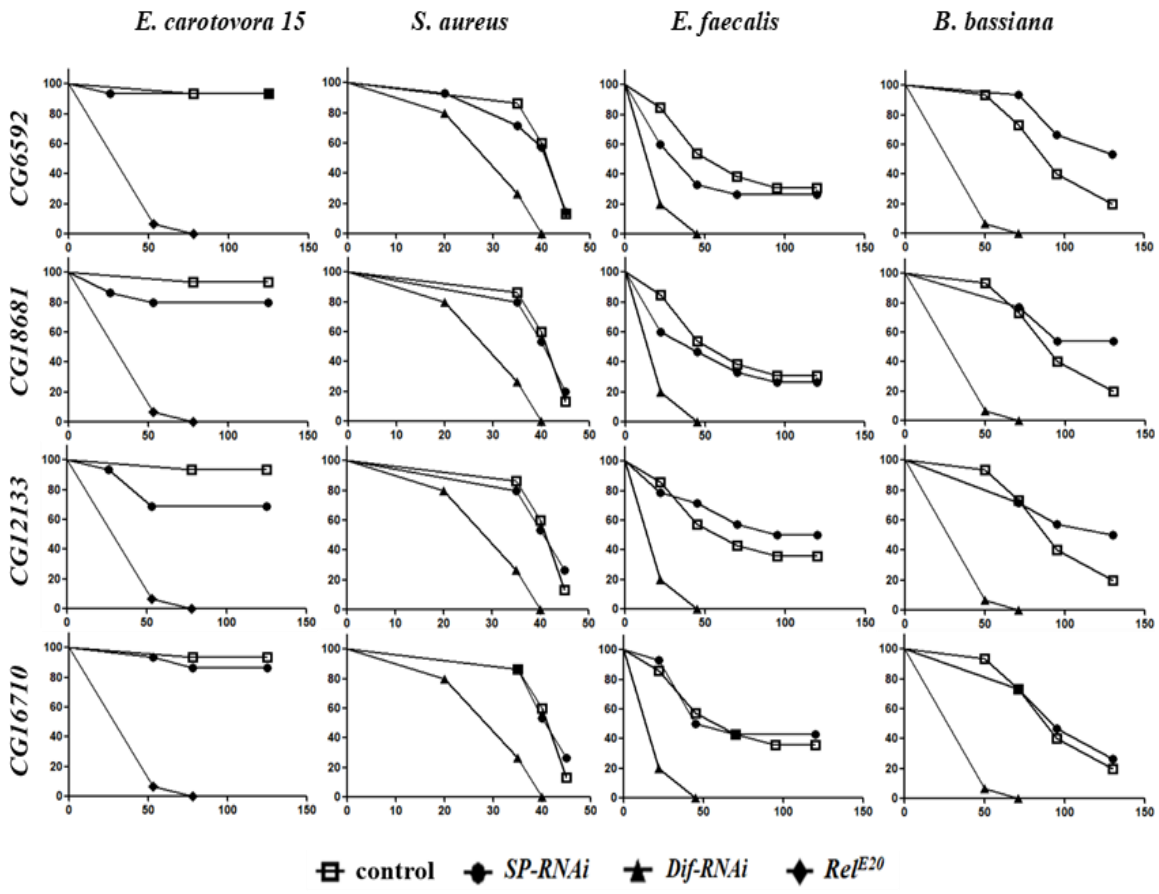


Figure 14: Twenty SP-KD flies with no compromised survival after different microbial challenge.

These survival graphs represent the percentage of surviving flies in function of time (hours) for each microbial infection. Wild-type flies (plain line with squares) are used as a control for all the microbial infections. *Dif-RNAi* flies (plain line with triangles) are used as a control for Gram-positive bacteria, and *Relish*^{E20} (plain line with rhombus) as a control for Gram-negative bacteria.

Since survival experiments reflect the ability of the SP-KD flies to tolerate certain infection, but they do not show the flies' ability to resist and eliminate the microbes, bacterial proliferation assay were performed in order to count the bacterial number that resides within these flies.

The 24 SP-KD flies that showed compromised survival after at least one microbial infection, were injected with the bacteria *E. Coli* and *S. aureus*, which are both antibiotic resistance (Ampicillin and Tetracycline, respectively). Then, CFU (colony forming units) were counted to see how the bacteria proliferate within these SP-RNAi flies after infection.

After *E. Coli* infection, the bacterial CFU were detected after 24 to 48 hours post infection. The CFU count was very low within these 24 analyzed flies, except for CG9733, CG11842, and CG17571 that showed the higher CFU count after Gram-negative bacterial infection. The obtained results are shown in the Figure S1; however, this experiment should be repeated for further confirmation and analysis of the results.

In parallel, it was hard to count the bacterial CFU after infection with the Gram-positive *S. aureus*. In fact, the CFU count was very high for wild-type infected control flies, but also within the non-infected wild-type control flies. Therefore, these results reveal the existence of another type of bacteria, which is also Tetracycline resistant, within these flies (data not shown).

3. Determine if the SP-KD flies with compromised survival have lower levels of AMPs

The 24 SP-KD flies that showed compromised survival after microbial challenge with different pathogens were chosen to look for antimicrobial peptides activation after infection. To do so, male flies were selected and infected with either *Micrococcus luteus* (*M. luteus*) or *B. bassiana*. Note that *M. luteus* is a Gram-positive bacterial infection much milder than *E. fecalis* and *S. aureus*. When infected with this bacterium, the flies were frozen after 24 hours, while they were frozen after 48 hours after fungus infection. RNA extraction was then performed, followed by Real-time PCR, in order to measure *Drosomycin* (*Drs*) induction levels after infection. *Drosomycin* is used as a readout that reflects the activation of Toll pathway, and *Rp49* is a reference gene used for normalization.

The controls used in AMPs quantification are infected *Dif-RNAi* flies (C564*CG6794), infected wild-type flies (C564*W^{1118}), and non-infected wild-type flies.

After infection with *M. luteus*, 6 SP-RNAi flies, CG3795, CG11668, CG11670, CG10232, CG12256, and CG11842, show impaired *Drs* expression with at least 50% reduction of the expression of this antimicrobial peptide (47%, 40%, 35%, 20%, 33%, and 40% respectively). This confirms the involvement of these SPs in the recognition of gram-positive bacteria. Note that only CG11842 was used with Actin-Gal4 driver since this latter showed only compromised survival with Actin-Gal4 driver. The results are shown in figure 15.

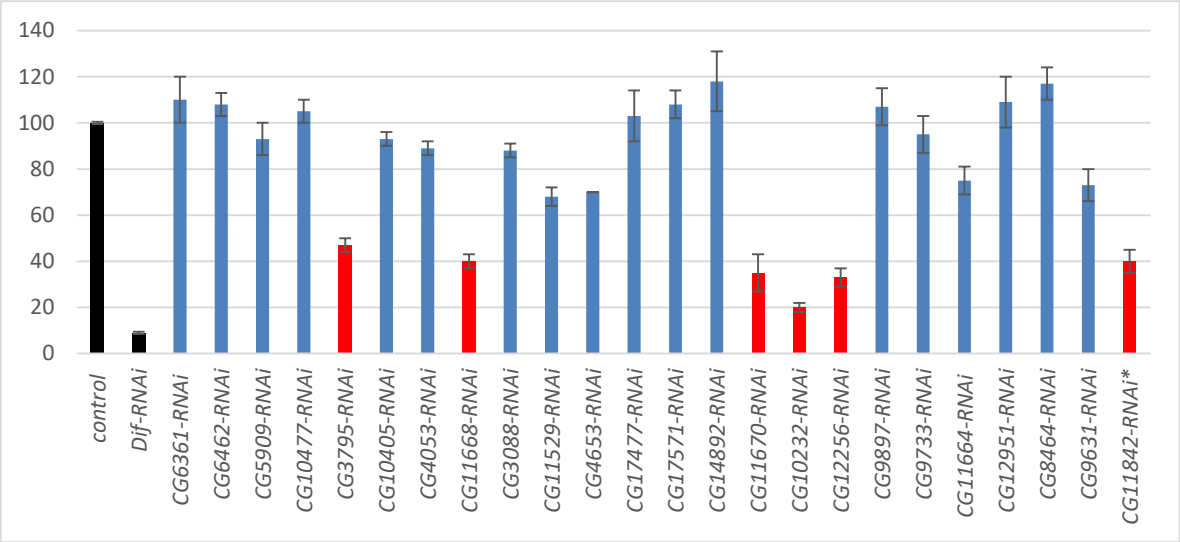


Figure 15 : Analysis of antimicrobial peptide gene expression, *Drosomycin*, after infection with the Gram-positive bacteria *M. luteus*.

Drosomycin gene expression was monitored by RT-qPCR with total extraction of RNA from wild-type control and *RNAi* males that were collected 20 h after *M. luteus* infection. In the control *Dif-RNAi*, the level of *Drs* was reduced significantly after infection. Each bar represents the mean of three to five independent experiments with standard deviation.

After infection with *B. bassiana*, only 4 SP-RNAi flies impaired *Drs* expression. In fact, the two SPs genes, CG6361 and CG8464 that failed to induce *Drs* after gram-positive bacterial infection, impaired *Drs* expression after injection with fungal spores (36% and 32% reduction of *Drs* expression, respectively). In addition, CG11670 and CG10232 that impaired *Drs* expression after *M. luteus* infection, impaired also successfully *Drs* production after fungal infection (39% and 43% reduction of *Drs* expression, respectively). However, the other SP-RNAi flies failed to induce *Drs* expression. The results are shown in figure 16.

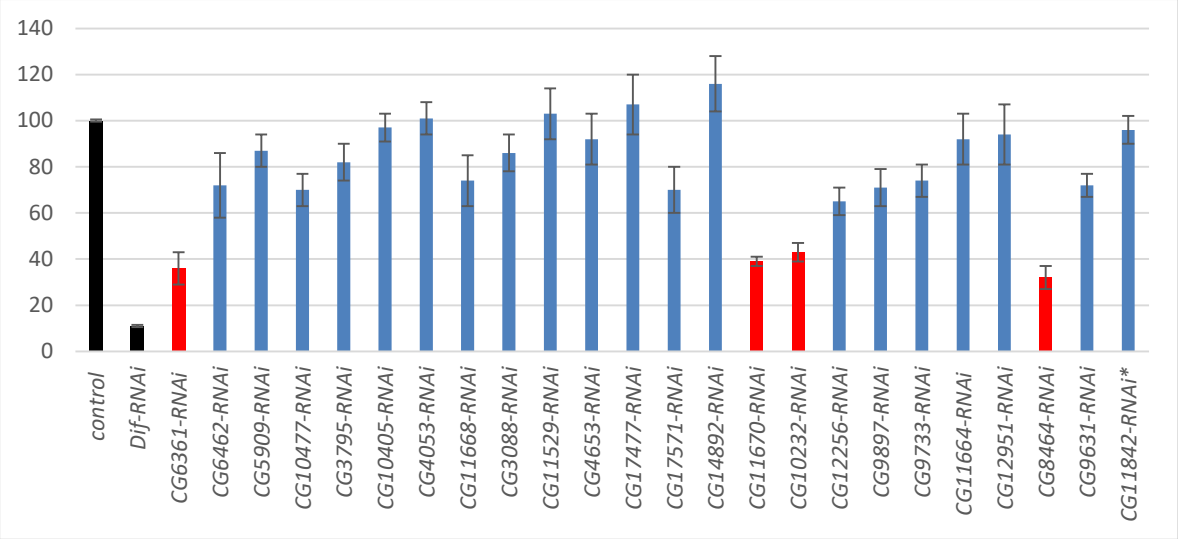


Figure 16: Analysis of antimicrobial peptide gene expression, *Drosomycin*, after infection with the fungus *B. bassiana*.

Drosomycin gene expression was monitored by RT-qPCR with total extraction of RNA from wild-type control and *RNAi* males that were collected 20 h after *M. luteus* infection. In the control *Dif-RNAi*, the level of *Drs* was reduced significantly after infection. Each bar represents the mean of three to five independent experiments with standard deviation.

The overall obtained data are summarized in the table below in order to simplify and organize the results.

Table 2: Summarized analysis of the 44 SP genes.

This table summarized the analysis of the 44 unexplored SP genes concerning their lethality with Act-Gal4 driver, their susceptibility against different microbial infection; *E. carotovora* (*ECC*), *S. aureus* (*SA*), *B. bassiana* (*BB*) and *M. Luteus* (*ML*) and their potential of *Drosomycin* production after *B. bassiana* and *M. luteus* infection.

CG number	Lethality with Act	ECC	SA	EF	BB	Drs expression
CG6361, GD28410	-	-	-	+	+	*BB* (36%)
CG6462, GD38180	+	-	-	-	+	
CG5909, GD16777	-	-	-	+	+	
CG10477, GD38177	-	-	+	+	+	
CG3795, KK105053	-	-	+	+	+	*ML* (47%)
CG10405, GD43171	-	-	-	+	+	
CG4053, GD51551	-	-	-	+	+	
CG11668, KK105073	+	-	+	+	+	*ML* (40%)
CG3088, GD14206	-	-	+	-	+	
CG11529, KK103207	+	-	-	+	+	
CG4653, GD42909	-	-	-	-	+	
CG17477, GD22932	-	-	-	+	-	
CG17571, GD46987	+	-	-	+	+	
CG14892, KK100443	+	-	-	-	+	
CG11670, GD16433	-	-	+	+	+	*BB* (39%) – *ML* (35%)
CG10232, KK100033	-	-	+	+	+	*BB* (43%) - *ML* (20%)
CG12256, GD5638	+	-	-	+	+	*ML* (33%)
CG9897, GD16841	+	-	+	+	+	
CG9733, GD16546	+	-	+	+	+	
CG11664, GD43300	-	-	-	-	+	
CG12951, GD16569	-	-	-	+	+	
CG8464, GD24104	-	-	-	+	+	*BB* (32%)
CG9631, KK108696	-	-	-	+	-	
CG11842, GD14853	-	-	-	+	+	*ML* (40%)
CG15002, GD32263		-	-	-	-	
CG15873, KK101822		-	-	-	-	
CG1304, KK109378	+	-	-	-	-	
CG4386, KK109488		-	-	-	-	
CG14088, GD5361	-	-	-	-	-	
CG3700, KK108237		-	-	-	-	
CG18420, GD50208		-	-	-	-	
CG7754, GD43205		-	-	-	-	
CG15046, KK102693		-	-	-	-	
CG3650, GD18830		-	-	-	-	
CG1773, KK110112	+	-	-	-	-	
CG8213, GD7372	-	-	-	-	-	
CG4650, GD101073	-	-	-	-	-	
CG7432, GD31091		-	-	-	-	
CG6865, GD35128		-	-	-	-	
CG4927, KK102088		-	-	-	-	
CG6592, KK101721	-	-	-	-	-	
CG18681, GD30808	-	-	-	-	-	
CG12133, KK108984	-	-	-	-	-	
CG16710, GD32700	-	-	-	-	-	

B. Identification of fungi that can be used as potential insect biocontrol agents

1. Dead arthropod collection and identification

Sixteen different dead animals were collected from two areas. The first series was collected in July 2014 from the American University of Beirut campus, and the second series was obtained in May 2015 from Nabatieh area (south of Lebanon). Areas where insecticides may have been used were avoided and cadavers near spider nets or incandescent lights were also disregarded.

The identification of these dead arthropods was based on morphological criteria and determined to lowest taxonomic rank possible. Furthermore, this identification depends also on the preservation of the specimen, the size of its group, and the presence of distinctive features. Specimens collected were from the orders Coleoptera, Lepidoptera, Hemiptera, Hymenoptera, Thysanura, Isopoda, Aranea, Polydesmida and Diptera. (Table 3 and Table S3).

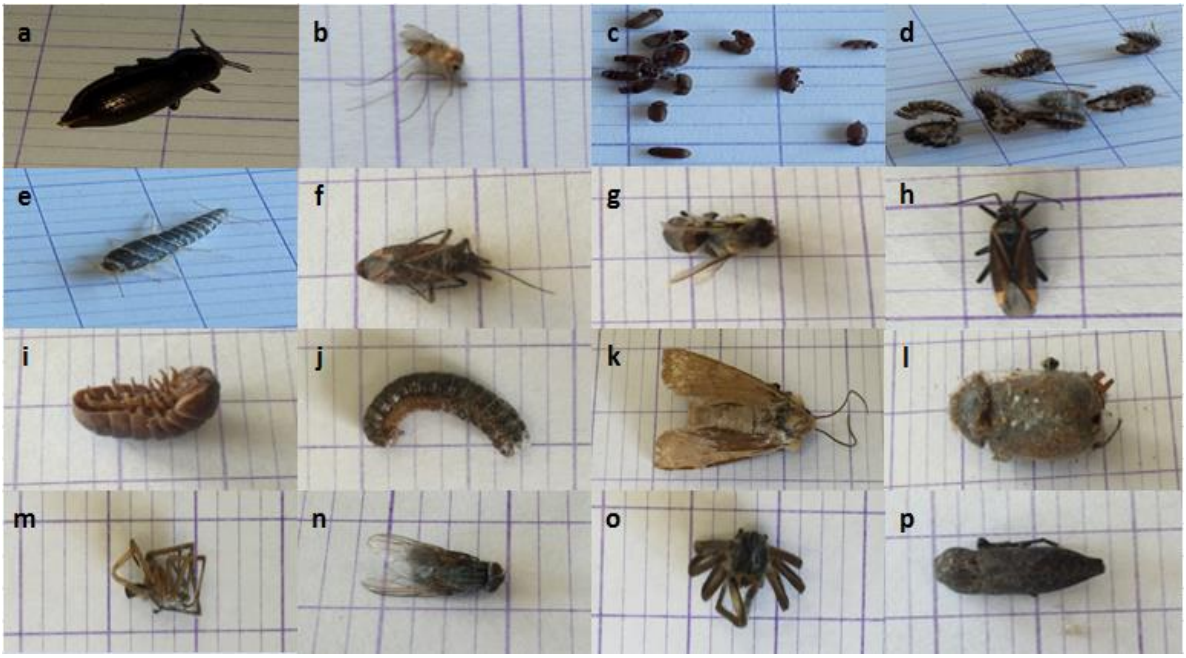


Figure 17: Photos of the dead arthropods from which fungi were isolated.

a Buprestidae (Coleoptera); b *Culex sp*. Culicidae (Diptera); c Curculionidae (Coleoptera); d Dermestidae (Coleoptera); e Lepismatidae (Thysanura); f Miridae (Hemiptera); g *Apis mellifera*, Apidae (Hymenoptera); h Pyrrhocoridae (Hemiptera); i *Armadillidium vulgare*, Armadillidae (Isopoda); j Polydesmidae (Polydesmida); k Pyralidae (Lepidoptera); l *Aphodius Sp*. Scarabaeida (Coleoptera); m Araneidae (Araneae); n Sarcophagidae (Diptera); o Araneidae (Araneae); p *Capnodis tenebrionis*, Buprestidae (Coleoptera).

2. Isolation and identification of fungi from the cadavers

From 17 different dead animals, 130 fungal germinations were isolated and purified on PDA plates. The precise identity of fungal species isolated from dead arthropods was determined by sequencing PCR-amplified Internal Transcribed Spacers

(ITSs) and comparing the results to the Genbank database. Obtained ITS sequences have been deposited in the GenBank database (accession numbers KX394525–KX394566).

In a first step, fungi were clustered according to the morphology of their mycelium. Two morphologies were overrepresented and present on several cadavers. The decision was made to sequence one isolate per cadaver for the overrepresented fungi. The genus *Cladosporium* represented 46 isolates and was found on 12 cadavers (Table S3). The two other most represented genera were *Penicillium* and *Talaromyces*, two very close genera belonging to the order Eurotiales. *Talaromyces* was isolated 20 times and from four different arthropods. One isolate per insect was sequenced and only one species, *Talaromyces amestolkiae*, was identified. Four morphological groups of *Penicillium* were identified; sequencing revealed that they belong to four different species. *Penicillium commune* was isolated from seven cadavers, *P. digitatum* and *P. frei* from two dead animals each. All the fungi that were isolated belong to the *Dikaria* group. Two isolates were basidiomycetes, *Fomes fomentarius* and *Wallemia* sp. The remaining species were ascomycete fungi belonging to the most prevalent phyla Dothideomycetes, Eurotiomycetes, Leotiomycetes and Sordariomycetes. Many of the isolated fungi were saprophytes; others had a life style depending on plants. Interestingly, two fungi, *Simplicillium sympodiophorum* isolated from dead woodlice *Armadillidium vulgare*, and *Engyodontium album* isolated from *Aphodius* sp. (Coleoptera) belong to the *Cordycipitaceae*, a family comprising the genera of the best-studied entomopathogens *Metharizium*, *Chordyceps* and *Beauveria*.

In order to summarize and simplify the results, the list of collected insects, the corresponding fungi, and their pathogenicity are reported in this table below.

Table 3: List of the collected dead arthropods and the corresponding fungi.

The fungus # refers to the arthropod it was isolated from and letters correspond to different fungal isolates. Arthropod order is given between parentheses. Dead arthropods 1 to 6 were collected in Beirut, and cadavers 7 to 17 were sampled from a more rural area in the south of Lebanon. The last column summarizes the results of spore microinjection: + denotes a pathogenic fungus (killing *Drosophila* with no statistically significant difference than *B. bassiana*, $p>0.05$), - denotes a mildly pathogenic or non-pathogenic fungus (killing at statistically significant different rate compared to *B. bassiana*, $p<0.05$), nt = not tested.

Fungus #	Fungus Species	Carrier insect	Pathogenicity by injection
1a	*Aspergillus ustus*	Buprestidae (Coleoptera)	+
1b	*Aspergillus candidus*	Buprestidae (Coleoptera)	-
1c	*Aspergillus sclerotium*	Buprestidae (Coleoptera)	+
1d	*Aspergillus candidus*	Buprestidae (Coleoptera)	-
1e	*Aspergillus nomius*	Buprestidae (Coleoptera)	+
1f	*Aspergillus sclerotium*	Buprestidae (Coleoptera)	+
2a	*Wallemia sp.*	Culex sp. Culicidae (Diptera)	+
3a	*Aspergillus sclerotium*	Curculionidae (Coleoptera)	nt
3b	*Scopulariopsis brevicaulis*	Curculionidae (Coleoptera)	-
3c	*Aspergillus sclerotium*	Curculionidae (Coleoptera)	nt
4a	*Aspergillus fumigatus*	Dermestidae (Coleoptera)	nt
4b	*Aspergillus ruber*	Dermestidae (Coleoptera)	nt
4c	*Aspergillus ruber*	Dermestidae (Coleoptera)	+
4d	*Aspergillus glaucus*	Dermestidae (Coleoptera)	-
5a	*Chaetomium globosum*	Lepismatidae (Thysanura)	-
6a	*Pyrenophora dictyoides*	Miridae (Hemiptera)	nt
6b	*Fusarim tricinctum*	Miridae (Hemiptera)	nt
7a	*Botrytis cinerea*	Apidae (Hymenoptera)	nt

7b	*Alternaria alternata*	Apidae (Hymenoptera)	+
7c	*Fomes fomentarius*	Apidae (Hymenoptera)	-
8a	*Talaromyces amestolkiae*	Pyrrhocoridae (Hemiptera)	nt
8b	*Cladosporium cladosporioides*	Pyrrhocoridae (Hemiptera)	-
8c	*Stachybotrys chartarum*	Pyrrhocoridae (Hemiptera)	nt
8d	*Ascomycota sp.*	Pyrrhocoridae (Hemiptera)	nt
9a	*Alternaria infectoria*	Armadillidium vulgare, Armadillidae (Isopoda)	-
9b	*Cladosporium cladosporoides*	Armadillidium vulgare, Armadillidae (Isopoda)	nt
9c	*Simplicillium sympodiophorum*	Armadillidium vulgare, Armadillidae (Isopoda)	nt
10a	*Penicillium digitatum*	Polydesmidae (Polydesmida)	-
10b	*Periconia sp.*	Polydesmidae (Polydesmida)	-
11a	*Penicillium freii*	Pyralidae (Lepidoptera)	-
11b	*Talaromyces amestolkiae*	Pyralidae (Lepidoptera)	nt
12a	*Chaetomium nigricolor*	Aphodius Sp. Scarabaeida (Coleoptera)	nt
12b	*Chaetomium bostrychodes*	Aphodius Sp. Scarabaeida (Coleoptera)	nt
12c	*Engyodontium album*	Aphodius Sp. Scarabaeida (Coleoptera)	-
13a	*Penicillim commune*	Araneidae (Araneae)	+
13b	*Phoma herbarum*	Araneidae (Araneae)	-
14a	*Alternaria infectoria*	Sarcophagidae (Diptera)	nt
14b	*Botrytis cinerea*	Sarcophagidae (Diptera)	nt
15a	*Embellisia abundans*	Araneidae (Araneae)	-
16a	*Talaromyces amestolkiae*	Capnodis tenebrionis, Buprestidae (Coleoptera)	nt
17a	*Penicillium polonicum*	Culex sp. Culicidae (Diptera)	-
17b	*Talaromyces amestolkiae*	Culex sp. Culicidae (Diptera)	-

3. Test the pathogenicity of isolated fungi using Drosophila melanogaster

After identification, 24 fungal isolates were grown in the laboratory and were able to induce sporulation. Spores were collected, washed, counted and microinjected into wild-type *Drosophila* to determine the pathogenic potential of each isolate. In parallel, for each experiment, the same number of spores obtained from the well characterized entomopathogen *Beauveria bassiana* was microinjected as a reference.

Fungi that significantly differed from *B. bassiana* in the rate at which they kill the flies ($p < 0.05$) were considered negatives; these were 16 isolates corresponding to *Aspergillus candidus* (2 isolates), *Scopulariopsis brevicaulis, Aspergillus glaucus, Chaetomium globosum, Fomes fomentarius, Cladosporium cladosporioides, Alternaria infectoria, Penicillium digitatum, Periconia sp., Penicillium freii, Engyodontium album, Phoma herbarum, Embellisia abundans, Penicillium polonicum, Talaromyces amestolkiae*. Among these isolates 13 did not kill more than 25 % of the injected flies while two isolates (*P. herbarum* and *P. polonicum*) killed about 30 % and one isolate (*A. candidus*) killed about 50 %. The remaining 8 isolates that killed with a rate that is not statistically different than that observed with *B. bassiana* ($p > 0.05$) were considered positives. These fungi correspond to this category: *Aspergillus ustus, Aspergillus sclerotium* (2 distinct isolates tested), *Aspergillus nomius, Wallemia sp., Aspergillus ruber, Alternaria alternata and Penicillium commune*. Among these, five isolates (*A. ustus, Wallemia sp. A. ruber, A. alternata* and *P. commune*) killed between 50 and 75 % of the injected animals, while only three isolates (*A. nomius* and *A. sclerotium*) were able to kill 100 % of the injected flies. It

was noted that *A. nomius* was the only fungus that was able to kill injected *Drosophila* at an even faster rate than *B. bassiana*. These results are shown in the graphs below and summarized in Table 3.

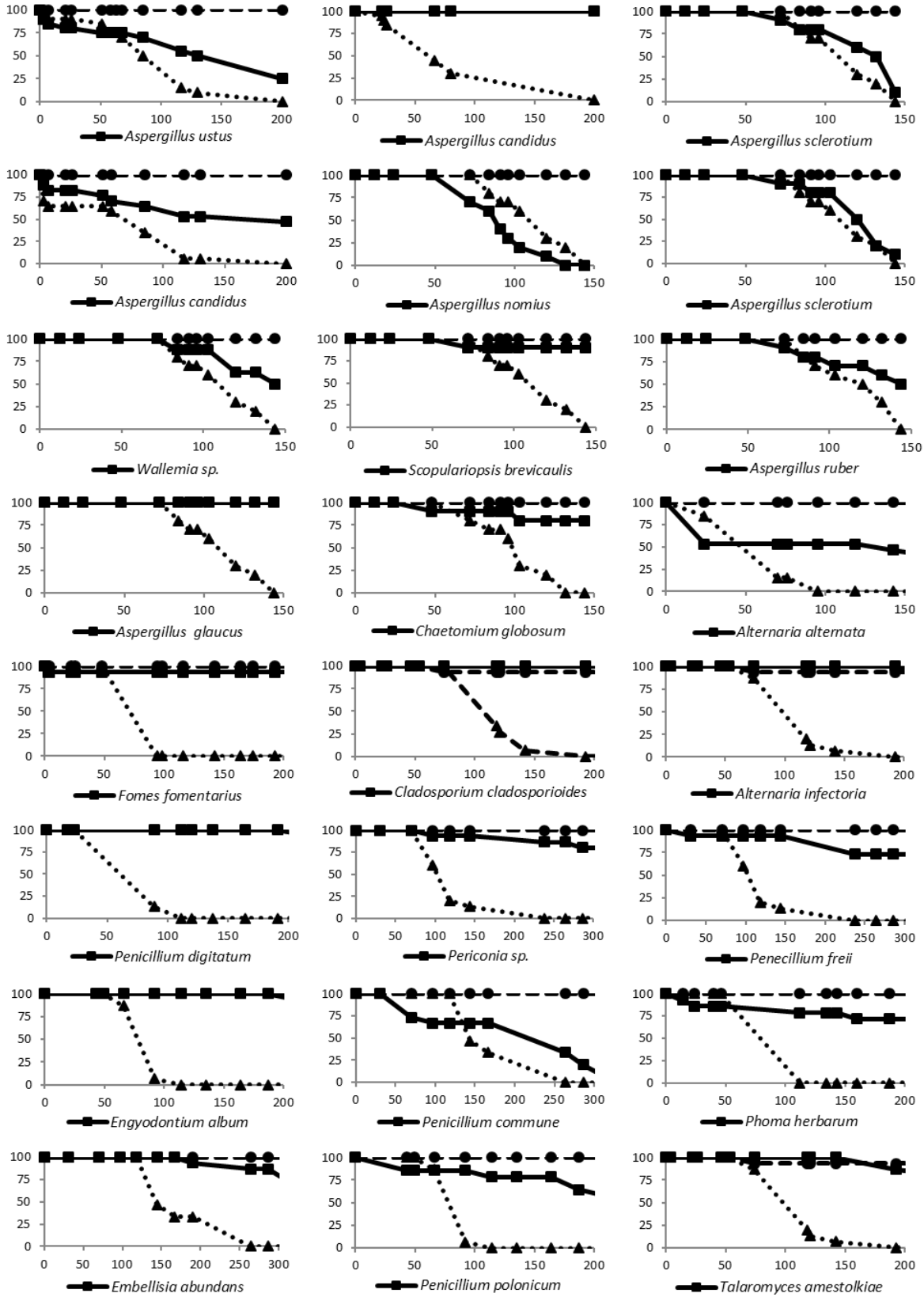

Aspergillus ustus
Aspergillus candidus
Aspergillus sclerotium
Aspergillus candidus
Aspergillus nomius
Aspergillus sclerotium
Wallemia sp.
Scopulariopsis brevicaulis
Aspergillus ruber
Aspergillus glaucus
Chaetomium globosum
Alternaria alternata
Fomes fomentarius
Cladosporium cladosporioides
Alternaria infectoria
Penicillium digitatum
Periconia sp.
Penecillium freii
Engyodontium album
Penicillium commune
Phoma herbarum
Embellisia abundans
Penicillium polonicum
Talaromyces amestolkiae

Figure 18: *Drosophila* susceptibility to the microinjection of spores obtained from the different isolated fungi.

Drosophila susceptibility to the microinjection of spores obtained from the different isolated fungi. Survival of *Drosophila* following microinjection of fungal spores (plain line with squares) is shown as percentage of flies alive plotted versus time in hours. In each experiment flies microinjected with the same number of *B. bassiana* spores were used as a reference (dotted line with triangles). In parallel flies microinjected with water are included as control (dashed line with circles). Seven fungi (*A. ustus*, *A. sclerotium*, *A. nomius, Wallemia* sp., *A. ruber*, *A. alternata* and *P. commune*) showed pathogenicity levels that were not statistically different compared to those triggered by *B. bassiana* ($P > 0.05$).

4. Test the fungi that were pathogenic to Drosophila on mosquitoes (Aedes albopictus)

Based on the results obtained, a subset of the fungal isolates (including the eight that were considered positive and four of the isolates that were not highly pathogenic to *Drosophila*) was used to microinject *Aedes albopictus* mosquitoes under similar conditions. *Aspergillus nomius, A. sclerotium* (2 isolates) and *A. ruber* showed pathogenicity levels that were not statistically different compared to those triggered by *B. bassiana* ($P > 0.05$) corroborating the results obtained using *Drosophila* and indicating that these four isolates are highly pathogenic to *Aedes* spp. Indeed, *A. ruber* killed about 75% of injected mosquitoes and *A. nomius, A. sclerotium* led to a 100% lethality in *Aedes* spp. On the other hand, *Periconia sp., P. herbarum, P. polonicum* and *T. amestolkiae* were not highly pathogenic to mosquitoes in agreement with what has been observed in Figure 19. However, although *A. ustus, Wallemia sp., A. alternata* and *P. commune* injections led to the death of some injected mosquitoes, these isolates were not as pathogenic for *Aedes* spp. as they were for *Drosophila*.

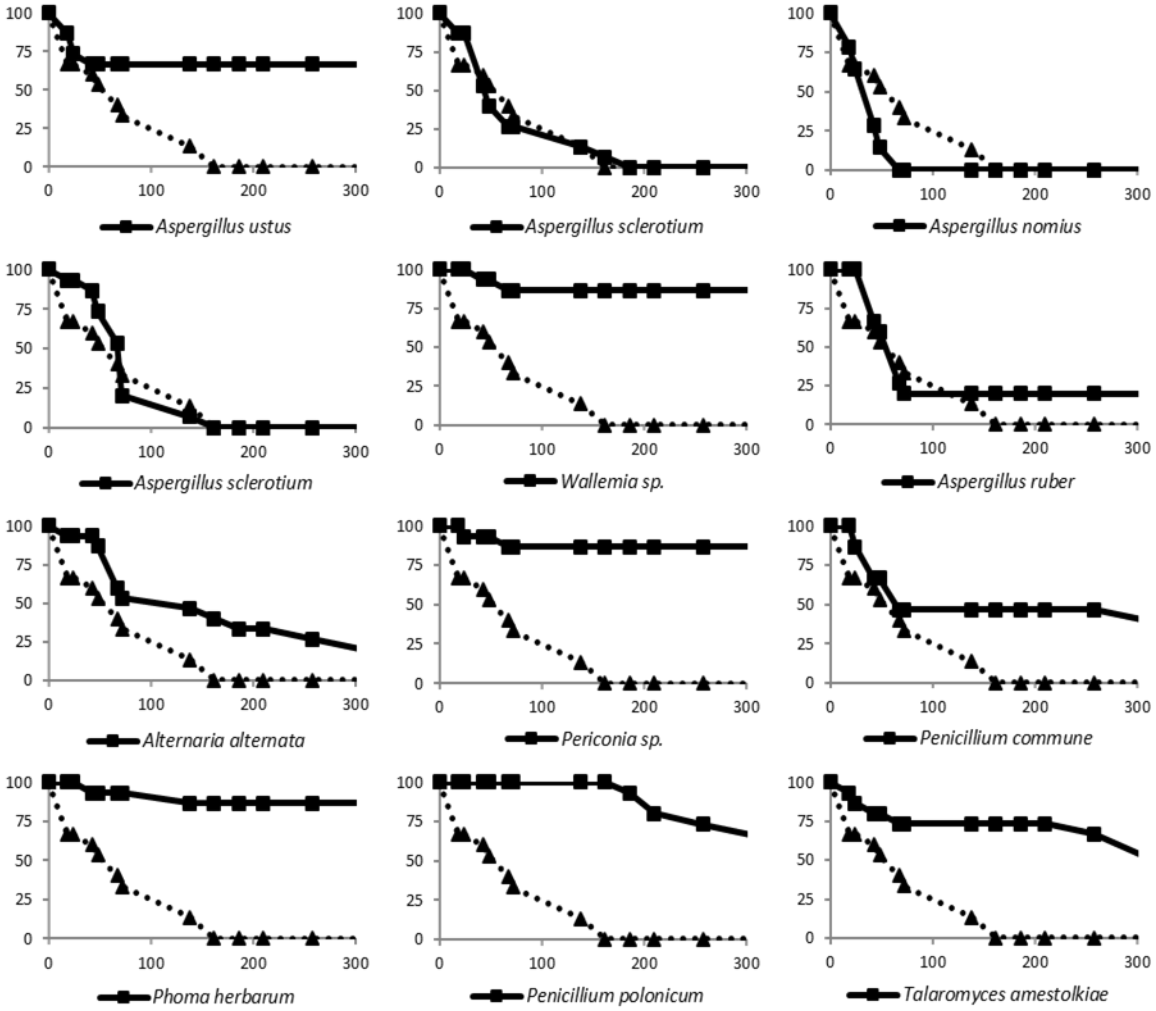


Figure 19: *Aedes* susceptibility to the microinjection of fungal spores.

Aedes susceptibility to the microinjection of fungal spores**.** Survival of *Aedes* spp. following microinjection of fungal spores is shown. In each experiment flies microinjected with *B. bassiana* spores were used as a reference (dotted line with triangles). *Aedes nomius, A. sclerotium* (2 isolates) and *A. ruber* showed death rates that were not statistically different compared to those triggered by *B. bassiana* ($P > 0.05$) indicating that these four isolates are highly pathogenic to *Aedes* spp. Although *A. ustus, Wallemia* sp., *A. alternata* and *P. commune* injections led to the death of some injected mosquitoes, the results were statistically different when compared to *B. bassiana* ($P < 0.05$) reflecting low pathogenicity. *Periconia* sp., *P. herbarum, P. polonicum* and *T. amestolkiae* were not highly pathogenic to mosquitoes.

5. *Test the fungi by natural infection on mosquitoes in order to mimic the natural route of infection*

The fact that an isolate showed high virulence in the microinjection experiment does not imply that it is a natural pathogen of insects. Indeed, the insect cuticle is an important barrier that needs to be breached by the germinating fungal spores. Therefore, before concluding that a fungus is a real entomopathogen, it is important to test it in a system that is close to natural infection setting. This can be achieved by spraying spores of the selected pathogenic fungi on the mosquitoes without injuring the cuticle. For this reason, we wanted to assay the pathogenicity of *A. nomius* - along with a selection of other isolates - in comparison to *B. bassiana* after natural spore spraying.

In this experiment, *A. albopictus* mosquitoes were used as model. Among nine isolates tested in this mode of infection (including *A. nomius, Wallemia sp., A. ruber, A. alternata and P. commune* of the fungi that were pathogenic by microinjection and *P. digitatum, Periconia sp., P. freii,* and *T. amestolkiae* of the ones that were not highly pathogenic by microinjection). *A. nomius* was the only fungus that killed at a very similar rate compared to *B. bassiana*.

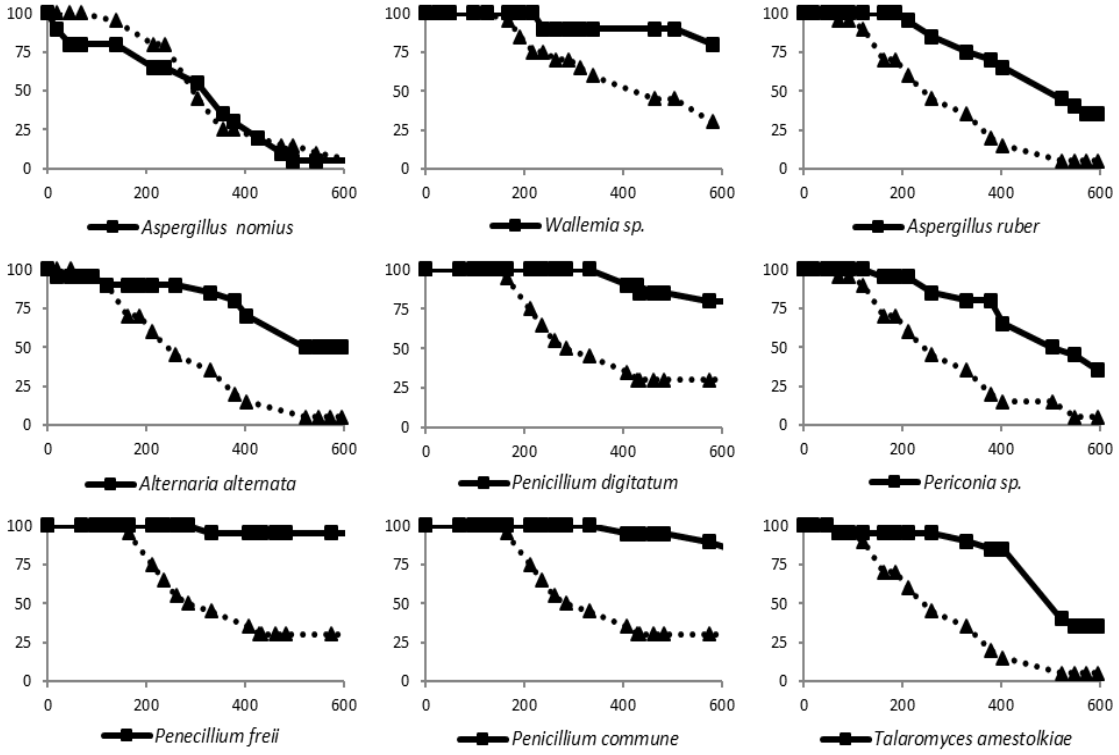


Figure 20: Survival of *A. albopictus* mosquitoes after natural infection.

Survival of *Aedes albopictus* mosquitoes after infection by spraying the insects with a suspension of fungal spores (plain line with squares) is shown as the percentage of mosquitoes alive plotted versus time in hours. In each experiment, the same number of *B. bassiana* spores was sprayed on control mosquitoes as a reference (dotted line with triangles). Only *A. nomius* was able to kill the mosquitoes at a very similar rate compared to *B. bassiana*. None of the mosquitoes that were mock-sprayed with water under the same conditions succumbed to the treatment (not shown).

Interestingly, only in the case of *A. nomius* (in addition to *B. bassiana),* irrespectively of whether the exposure to the spores was by microinjection or via natural infection, the dead flies were completely covered by fungal mycelia. This confirm that the

cause of death is due to the development of the spores in the insect and that the spores were able to germinate and probably pierce the mosquito cuticle.

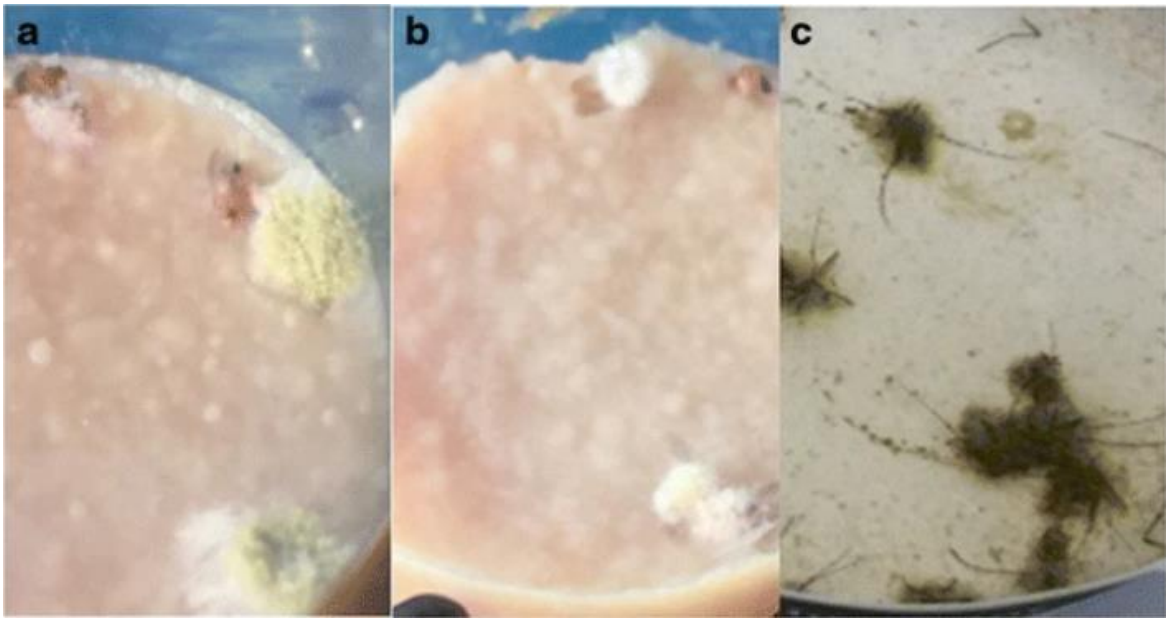


Figure 21: Photos of dead insects after microinjection or spraying with *A. nomius* spores.

a *Drosophila* cadavers following *A. nomius* spores microinjection. **c** *Aedes* mosquitoes after spraying with the same fungus. The dead insects are completely covered by fungal growth indicating that the cause of death is due to the development of the spores within the animal. *Drosophila* cadavers after *B. bassiana* spores microinjection are shown for comparison (**b**).

6. *Test the pathogenicity of the fungus A. nomius using another type of mosquito: Culex pipiens*

The experiment natural infection was also performed with the pathogenic fungus *A. nomius* using another type of mosquito, *Culex pipiens,* and the results confirmed that this isolate is as pathogenic as *B. bassiana* to mosquitoes.

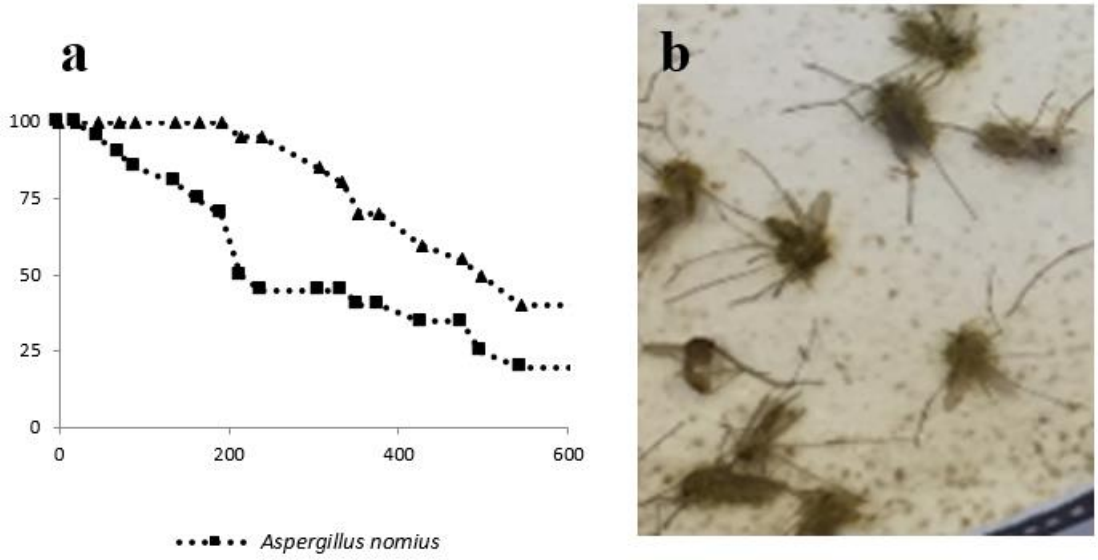


Figure 22: Effects of *A. nomius* on another species of mosquitoes: *Culex pipiens*.

a- Survival of *Culex* mosquitoes after spraying the insects with a suspension of *A. nomius* spores is compared to that of mosquitoes sprayed with the same concentration of *B. bassiana* spores (red line). *A. nomius* was able to kill the mosquitoes at a very similar rate compared to *B. bassiana*.
b- Photo of *Culex* mosquitoes after they succumbed to *A. nomius* spore exposure. The dead insects are completely covered by fungal growth.

CHAPTER IV

GENERAL DISCUSSION

Due to their crucial roles in various physiological processes, like digestion, blood coagulation, developmental process and immunity, serine proteases (SPs) and their non-catalytic homologs (SPHs) have been investigated in several species that are considered research models, especially *Drosophila melanogaster*. Many members of these SPs and SPHs possess a clip-domain, which is found in the N-terminal position to the catalytic domain. This domain is only found in invertebrates, and it plays an important role in the sequential activation of SPs [228, 241, 314]. The *Drosophila* genome encodes around 204 serine proteases [234], and until recently, few of them have been shown to be involved in the immune responses.

Persephone, was the first clip-serine protease that have been recognized to act upstream of the Toll pathway and in response to fungi [235]. Later, a previous study has performed the first *in vivo* RNAi screen of a large number of SPs genes (75 *Drosophila* SPs genes), and has identified five new serine proteases that are required for Toll activation. Spirit, Grass, and SPE (Spätzle-processing enzyme) are functional chymotrypsin-like SPs containing a clip-domain, while Spheroide and Sphinx1/2 are two SP-homologs that lacks the catalytic serine residue [237].

In our study, we performed a large-scale RNAi screen to investigate the role of the 44 of the remaining unexplored SP genes and their involvement in the immune responses of

Drosophila. Transgenic lines, carrying IR targeting these SPs were first individually crossed to flies carrying the Actin-Gal4 driver in order to induce RNAi in the F1 progeny. The knockdown of approximately a third of these genes led to lethality with this ubiquitous driver, which indicates the importance of these SP genes in certain vital functions of *Drosophila*. Hence, we used a more restricted driver, the C564-Gal4 that expresses Gal4 in the fat body, which is the major site of antimicrobial peptides production.

The flies knocked-down for different SP genes were tested for their susceptibility to survive different microbial infection (Gram-positive and Gram-negative bacteria, and the fungus *B. bassiana*). Interestingly, the obtained results allow us to divide these genes into three categories. Some of the SP-KD flies were susceptible to infection with the Gram-positive bacterium *E. feacalis*, such as CG17477 and CG9631. In contrast, other SP genes KD led to susceptibility towards infection with the fungus *B. bassiana*, like CG6462, CG4653, CG14892 and CG11664. The third category is the largest one, and includes 18 SP genes for which the KD led to compromised survival after infection with both Gram-positive and fungal infection. These genes are CG6361, CG5909, CG10477, CG3795, CG10405, CG4053, CG11668, CG 3088, CG11529, CG17571, CG11670, CG10232, CG12256, CG9897, CG9733, CG12951, CG8464, and CG11842. Note that none of the analyzed SPs genes showed compromised survival after infection with the Gram-negative *E. carotovora 15*, indicating that the IMD pathway is functional and that the injection process was not the cause of death for any of the candidates. Nevertheless, some variability in the resistance of SP-RNAi flies was observed in the preliminary CFU assays.

Furthermore, we monitored the expression of the antimicrobial peptide gene *Drosomycin*, a target of the Toll humoral response, produced by these SP-RNAi flies after infection with the Gram-positive bacterium *Micrococcus luteus* or the entomopathogenic fungus *B. bassiana*. After *M. luteus* infection, 6 SP-RNAi flies found to be significantly impaired in *Drosomycin* expression: CG3795, CG11668, CG11670, CG10232, CG12256 and CG11842. For these SP-RNAi flies, at least 50% reduction of *Drosomycin* expression was observed, confirming the requirement of these SPs in the signaling cascade after the entry of Gram-positive bacteria. On the other hand, only 4 SP-RNAi flies were impaired *Drs* expression after infection with the fungus *B. bassiana*. These are CG6361 and CG8464 that had normal *Drs* induction after gram-positive bacterial infection, and CG11670 and CG10232 that were also impaired in *Drs* expression after gram-positive bacterial infection.

Hence, after screening different 44 SP genes, 8 SP-RNAi flies proved to be involved in the extracellular cascade that activates the Toll pathway. Among these, four SPs seem to be specific in the signaling of Gram-positive bacteria. These are CG3795, CG11668, CG12256, and CG11842. Only two of these 8 SP-RNAi, CG6361 and CG8464, seem to be required for signaling after fungal infection. Two SP genes, CG11670 and CG10232, are involved in *Drs* induction after both Gram-positive and fungal infections. Note that CG11842, CG6361, and CG11670 were previously shown to be upregulated after fungal infection or septic injury, while CG10232 has been shown to be downregulated after the same condition [234, 315, 316]. Furthermore, these 8 SPs genes all belong to chymotrypsin-like serine protease family with potential endopeptidase activity, and only

three of them contain at least one clip-domain: CG6361 or Hayan (1 clip-domain), CG11668 (1 clip-domain), and CG10232 (2 clip-domains) [241].

For further investigation about the function of these candidates SPs, epistatical analysis remains to be done in order to determine their hierarchy in the proteolytic cascade upstream of the Toll pathway, and the precise function of these genes can be elucidated by generating real mutants as these induced SP-RNAi flies are hypomorphs. Moreover, it is important to determine whether they are also involved in the melanization response. In addition, our preliminary results reveal the feasibility of this investigation using a very simple method (Figure S2).

To date, several SPs are well characterized and known to be involved in the activation of Toll pathway: ModSP, Grass, SPE, and Psh. However, the precise role and contribution of other SPs remain unclear. The identification of the clip-SP Persephone revealed that the Toll pathway could be triggered by virulence proteases released not only by the fungus *B. bassiana*, but also by certain Gram-positive bacteria like *E. faecalis*, and *Bacillus subtillis*[103]. Also, recent evidence suggests that Grass, which was first thought to be associated only in signaling against Gram-positive bacterial infection, could be also activated by fungal infection [317]. Although the main players of the activation of Toll pathway are known, the proteolytic cascade appears to still have several missing links, since ModSP is unable to cleave Grass directly [236], which suggest the existence of undefined SPs at this level. Furthermore, the exact role of the three SPHs, Spheroide, Spirit, and Sphynx1/2, is still elusive. Therefore, the investigation of new SPs and SPHs involved

in *Drosophila melanogaster* immunity is valuable for a better understanding of the sequential activation, organization, and regulation of these proteolytic cascades.

In parallel, and in the context of recovering novel entomopathogens, we isolated fungi from dead arthropods and a subset of isolates per cadaver underwent ITS-sequencing and identification as well as pathogenicity testing. Several isolated fungi are likely to be airborne contaminant and/or saprotrophic fungi that may have developed on the arthropod carcass after the death of the animal has occurred. Examples of such possible contaminants are *Penicillium*, *Talaromyces* and *Cladosporium* isolates that have been oversampled in the course of the survey. *Fomes fomentarius* is known for its role in wood decay and for causing white-rot in plants according to some reports [318]; it has also been used in traditional medicine mostly for its anti-inflammatory and pain-killing properties [319-321]. *Wallemia* sp. has a saprophyte life-style and has been shown in some cases to be involved in food spoilage [322, 323].

Noteworthy, two human-related fungi were isolated in our study: *Aspergillus fumigatus* and *S. brevicaulis. Aspergillus fumigatus* is considered an opportunistic human-pathogen. However, it is primarily a ubiquitous saprophyte fungus present in many natural environments [324]. Although aspergilli are well-known airborne contaminants or soil inhabitants, *A. nomius* proved to be of considerable interest in our survey. Indeed, this fungus was as pathogenic as *B. bassiana* both by microinjection into *Drosophila* and *A. albopictus* or by infection via spore spraying onto *A. albopictus* and *C. pipiens*. Moreover, a study focusing on stonebrood, a fungi-caused disease that affects honey bee larvae, has detected the presence of *A. nomius* in affected hives. Indeed, among the

ten *Aspergillus* species identified in honey bee hives, *A. flavus*, *A. phoenicis* and *A. nomius* were shown to be pathogenic to the larvae [325].

In the present study, *A. nomius* was isolated from a dead beetle (Coleoptera: Buprestidae) and was able to develop on and kill both *Drosophila* and mosquitoes (Diptera) indicating that it is a general entomopathogen with a broad host range. Targeting different insects can be considered an advantage, since the same fungus can be used to target several pests. However, fungi with a broad range of target insects can lead to the undesirable killing of non-target species and they should be used with caution [312, 326]. In contrast, bacteria can be used to kill insects in a very specific manner, due to the presence of toxin receptors on their epithelial cells in the target species [298].

Host-range specificity could also be correlated to differences in the immune systems of the target insects. Therefore, in addition to a potential use as biocontrol agent, *A. nomius* could be used as elicitor of insect immune responses in model organisms to decipher the pathways involved in the recognition of fungal infections. Indeed, although the major antifungal players have been characterized such as GNBP3 which plays different roles, both activating Toll pathway and assembling effector complexes that directly attack fungi, some aspects of insect antifungal responses remain unknown [327, 328].

Differences in the immune system between *Aedes* and *Drosopila* could explain the fact that from the eight fungal isolates that were pathogenic to *Drosophila*, only four (including *A. nomius*) were pathogenic to the mosquito, while *A. ustus*, *Wallemia* sp., *A. alternata* and *P. commune* were not as pathogenic to the mosquitoes as *B. bassiana*. It is

worth mentioning that these four isolates were relatively “mild” in *Drosophila* (killing between 50 and 75 % of injected flies) as compared to the four that killed both *Drosophila* and *Aedes* (killing 75–100 % of injected flies). However, these differences are not surprising if we take into consideration that even between *Drosphila* species there are differences in antifungal defenses [329].

The mildly pathogenic fungi too can be interesting as biocontrol agent if they show more restricted host range as compared to the virulent ones. Also, the slow killing rate can allow more time for the infected animals to spread the spores within a population, especially because it has been reported that *Anopheles* female mosquito are attracted to dead insect carrying *B. bassiana* spores [330] and because transmission of *B. bassiana* from male to female *Aedes* mosquitoes has been observed [331].

CHAPTER V

CONCLUSION

This study represents an extension of *in vivo* RNAi screen of the remaining unexplored SP genes in *Drosophila*. This adopted strategy offers an important advantage by the fact that the genes are inactivated in a tissue- and temporal-specific manner. The results reveal the role of some prominent candidates SPs in the innate immune response, especially in the activation of Toll pathway. However, a lot remains to be discovered about their precise role and their mechanisms of function. In addition, the existence of possible functional redundancies, and the fact that one gene could be involved in more than one biological pathway or process, makes this mission more challenging.

Furthermore, the identification of *A. nomius* as a new natural insect pathogen and a potential disease-vector control agent is encouraging. This demonstrates the feasibility of a simple approach for the identification of potential mosquito killers especially that this may provide a solution to pest control within the ecosystem rather than utilizing toxic substances. Indeed, it is essential to anticipate and prepare biocontrol methods to fight the expansion of mosquitoes' habitat predicted in certain geographical areas in association with the occurring climatic changes. A larger scale screen could be conducted in the aim of identifying more entomopathogens with perhaps some fungi that are specific to certain host families and to give a more precise idea about saprophyte fungi that decompose arthropod cadavers in nature.

CHAPTER VI

MATERIALS AND METHODS

A. Ethics Statement

All animal procedures were carried according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) at the American University of Beirut, and all methods were carried out in accordance with relevant IACUC guidelines and regulations.

B. Materials

1. Drosophila stock and maintenance

Drosophila W^{1118} strain was used in infection experiments as wild-type flies. All *Drosophila melanogaster* IR lines are purchased from the Vienna Drosophila Melanogaster Resource Center (VDRC). Stocks were reared in 50 mL vials containing standard cornmeal agar food prepared according to the Drosophila Bloomington Stock Center recipe. The main stocks were kept at 18°C, while the crosses were kept at 25°C. To maintain the fitness of the flies, the humidity is set at 45% and on a 12:12 light/dark photocycle.

2. Mosquito rearing

Aedes albopictus (Sarba strain) a local mosquito strain was reared in the insectary at 28°C, 70% humidity on a 12:12 light/dark cycle. Mosquito cages were supplemented with a cup of tap water and a cotton pad soaked in 10% sucrose. Eggs were collected 4 days after a blood meal and allowed to air dry for two weeks before hatching. Dried eggs were hatched by immersion into deoxygenated water. Larvae were reared in pans containing tap water and fed on beer-brewing yeast for the first day after hatching then on fish pellets till pupation.

3. Bacterial strains

Enterococcus fecalis, *Staphylococcus aureus*, *Erwinia carotovora carotovora 15*, *Echerichia Coli* (DH5 alpha laboratory strain), and *Micrococcus luteus* were either cultured in Luria-Bertani (LB) broth or plated on LB plates, with addition of certain antibiotics (LB-Ampicillin for *E. Coli* and *E. carotovora*, and *S. aureus* for LB-Tetracycline).

4. Fungus strain

Beauveria bassiana strain 80.2 (a gift from Dominique Ferrandon) was used as a control in all survival experiments.

C. Methods

1. Drosophila crosses

Approximately, 10 virgin females containing the driver were crossed to 6 males carrying the IR construct in vials with fresh medium. The crosses were maintained at 25°C until the progeny reaches the late larval stage. Then, the F1 progeny carrying both the UAS-construct and the GAL4 driver were transferred to 29°C for optimal efficiency of the UAS/Gal4 system.

2. *Microbe microinjection of Drosophila flies*

SP-KD flies were injected with 32 nL of different bacterial suspension such as *Enterococcus faecalis* (*E. faecalis*; OD=0.05), *Erwinia carotovora carotovora 15* (*E. carotovora 15*; OD=0.1), *Staphylococcus aureus* (*S. aureus*; OD=0.05) or 4 spores/nL of the fungus *Beauveria bassiana,* using a NanodropII microinjector (Nanoject II apparatus, Drummond Scientific, CA). For survival assays, dead flies were counted at regular intervals. Survival graphs were then plotted as percentage of surviving flies in function of time. For RNA extraction and Real-Time PCR, the microbe used for infection are *E. faecalis* (OD=0.1), *Micrococcus luteus* (*M. luteus*; OD=0.01) and *B. bassiana* (32 nl of water containing 100 fungal spores*).* The flies were frozen at -20°C for a specific time depending on the pathogen injected (24 h after bacterial infection and 48h after fungal

infection). Each experiment was performed at least 4 times using a minimum of 15 flies, and a representative result is shown.

3. RNA isolation and Real-time PCR

Whole SP-KD and SPN-KD flies were directly placed and homogenized in TRIzol®. Total RNA was extracted using choloroform and precipitated with isopropanol according to the manufacturer's instructions (Invitrogen). The extracted RNAs were quantified using a nanodrop spectrophotometer (Thermo) and 500 ng were retrotranscribed into cDNA (iScript Biorad) for each sample. Real-time PCR was performed in presence of SYBR green (Qiagen) on 1/20 dilutions of the RT reactions using a BIO RAD thermocycler (CFX 96 Real-time System, C1000). Ct values for target genes were normalized to *Rp49* and compared to controls using the delta Ct method. The expression level of *Drosomycin* is used as a read-out for the Toll pathway. The results shown were repeated and confirmed at least 3 times. Specific primers were used:

Drs Forward: 5'- CATTTACCAAGCTCCGTGAGAACC-3'

Drs Reverse: 5'- GATTTAGCATCCTTCGCACCAGCA-3'

Rp49 Forward: 5'-AGTCGGACCGCTATGACAAG-3'

Rp49 Reverse: 5'-GACGTTGTGGACCAGGAACT-3'

4. Arthropod cadaver collection

Two series of dead animals were analyzed: the first series was collected in July 2014 from the American University of Beirut campus; the temperature range was 27°C - 32°C and humidity 70%-80%. The second series was obtained in May 2015 from Nabatieh area (south of Lebanon); the temperature range was 20°C - 26°C and humidity around 70%. Areas where insecticides may have been used were avoided and cadavers that were close to spider nets or incandescent lights were also disregarded.

5. Fungus isolation from cadavers

Carcasses were suspended in water containing 5% Tween and shaken vigorously to resuspend spores or mycelium fragments present on the cuticle surface. Ten µL of different dilutions of this suspension was plated on standard PDA/chloramphenicol medium. After one or two days of incubation at 27°C, individual germinations (or mycelium regeneration) were transferred to a new plate. For each insect, only one isolate per group of morphologically identical thalli was selected. These isolates were submitted to several rounds of purification in order to follow morphological stability after the successive transfers. Conidial species like *Aspergillus sp*. and *Penicillium sp.* were submitted to single spore purification.

6. DNA extraction and sequencing

Fungal isolates were grown on cellophane/PDA for two to four days at 27°C. DNA was extracted as in [332]. ITS sequences were PCR amplified with the following universal primers ITS1: TCCGTAGGTGAACCTGCGG, ITS4: TCCTCCGCTTATTGATATGC. Amplification and direct sequencing of fungal ribosomal RNA genes was as in [333]. Sequences were blasted against NCBI Genbank for identification purposes [334].

7. Spore purification

Fungal spores were extracted from 4 weeks old PDA plates by adding 25 mL sterile distilled water to each plate and scrapping the surface. A sterile funnel containing autoclaved glass wool was used to separate the spores from other mycelia structures. The collected spore suspension was centrifuged at 4000 g and washed three times with distilled water and finally resuspended in 0.5 mL water. Spores were then counted using a hemocytometer and diluted to the desired concentration. Freshly prepared fungal spore solutions were used for the microinjection of all *Drosophila* and mosquito challenges.

8. Infection of mosquitoes and survival assays

Survival experiments were performed on batches of 15 wild-type flies or 20 mosquitoes. In all experiments, 3 to 7 day-old females were used. Mosquitoes were only sugar fed (no blood meal). Two types of infection were performed: microinjections and natural infection. For microinjections, flies were anesthetized on a CO_2 flow bed and 32 nL of water containing 100 fungal spores were injected into the thorax using a NanodropII microinjector (Drummond Scientific, California, US). For natural infections, a suspension of 50 x 10^6 spores/mL was sprayed on anesthetized mosquitoes. Vials (for *Drosophila*) or cups (for *Aedes*) containing the challenged animals were then put in an incubator at 29^{o}C and the surviving flies counted every few hours. Flies that died within the first 2 hours after injection were disregarded since their death is considered to be due to the needle injury. Each experiment was repeated at least 3 independent times and a representative result is shown.

9. CFU assays

SP-KD flies were injected with Ampicillin-resistant DH5 alpha *E. Coli* (OD=0.05). After 24 hours post-infection, the flies maintained at 25^{o}C were collected, and serial dilutions in sterile LB of approximately 150 µl of hemolymph were plated on LB plates supplemented with Ampicillin (100µg/mL). The colonies were counted to estimate the approximate CFUs per SP-KD fly.

10. Melanization Spot Test

Third instar larvae of the analyzed SP genes were pricked at their posterior part (close to their spiracles) with a pointed needle dipped in a concentrated bacterial suspension of *E. Coli* (OD=20). The injected larvae were maintained at 25^{o}C for 2 hours. Then, 2 larvae from each cross were torn apart using forceps, and their hemolymph was dipped on a filter paper soaked with L-DOPA (2 mg/mL). L-DOPA is light sensitive, so the filter paper should be covered with aluminum foil during the whole experiment to avoid light exposure. The darkening of the obtained spots was monitored every 10 min for 1 hour at room temperature.

11. ***Statistical analysis***

For statistical analysis of the survival data, Gehan-Breslow-Wilcoxon test was performed. Results with a p value of less than 0.05 were considered as significant.

CHAPTER VII

APPENDICES

A. Identification of Serine Proteases involved in *Drosophila* immunity

Table S1: Off-targets analysis of the 44 analyzed SP genes.

For efficiency of gene knockdown, and in order to check if these SPs have some off-target genes, VDRC (Vienna Drosophila Resource Center) UP-TORR websites are used to perform this analysis. This table shows the sequence of each SP gene and its length, but also the off-targets for each SP gene based on 19-mer hits.

CG number	Sequence	Length (bp)	Off-targets	19-mer hits
CG6361	ACGGCGAACG GGTGGATAGG GGCGTCTATC CCCACATGGC GGCCATTGCG TACAACTCCT TCGGGAGCGC AGCGTTCCGA TGTGGTGGAT CGCTCATCGC CAGTCGATTC GTCCTCACAG CGGCTCACTG CGTCAATAGT GACGATAGCA CACCCAGTTT CGTCCGTCTG GGTGCGTTGA ACATCGAAAA TCCCGAGCCG GGCTACCAGG ACATCAATGT GATTGATGTT CAAATTCATC CGGACTATTC CGGCAGTAGT AAGTACTACG ATATCGCCAT TCTGCAGCTG GCCGAGGATG CCAAGGAGTC CGATGTCATC CGTCCCGCCT GTCTC	335	CG17778	2
CG6462	GTGCTTTCTT CCTGCTACTT CTATCATCCA CACTAGTTAA ATCAAGTGAG CCTTGGCTAG ATACTTTTGA GCACCCCAAA GAGGAGACAC CGGACGATGA CGATGCAATC ATGGAAAGAC GTTGGCAGCT TGGCTACGAA AACTTTCGAC TTCGCTGCGA AAAATTCGAG ATGGAAGGTA ATCAAACCGC TGCTGTGAGA ACTCGAATAG CTGGAGGAGA ATTGGCCACG CGCGGCATGT TTCCCTATCA AGTGGGTCTG GTGATTCAGC TGAGTGGCGC AGATCTGGTC AAGTGCGGTG GTTCCCT	307	CG6298	4
CG5909	GACCTGGAAT CGGAGGAGGA CTGCCACTAT TTGGGCGGTA CGAACCGCGT ATGCATTCCG CCCTACGAGG AGTACGGCAT AGAGCAGATA CGAGTACATC CCAACTACGT GCATGGCAAG ATCAGCCATG ACGTTGCCAT CATCAAGCTG	365	CG6988	1

	GATCGCGTGG TCAAGGAAAA ATCGCATATT AAGCCCGTTT GCCTGCCAAT CGACCAGAAG TCACAGGAAC TGGACTTCGA TCAGAGCTTC TTCGTCGCCG GCTGGGGCGG CACCGAGAAG GAGACTGTGG CCACCAAGCT CCAACAGGCG CTGATCACCA GGAAGAGCCT GAACGAGTGT CGCCAGTACT ACAACAAGGG TGAGGTGAGC GACAA			
CG10477	**AGCTACTCCA CCTACGCCGG ACAGACTGCA GTGGCTTCCG GATGGGGCAG GACCTCCGAT TCCAGCATTG CCGTCGCCAC GAACCTTCAA TACGCACAAT TTCAGGTCAT TACCAATGCT GTGTGTCAGA AGACCTTTGG GTCATCTGTG GTCACCAGTG GAGTTATCTG CGTGGAGTCC ATCAACAAGA AGTCGACCTG TCAGGGCGAT TCTGGCGGTC CGTTGGCTTT GAACAATAGA CTAATTGGTG TGACCTCGTT TGTGTCCTCC AAGGGATGTG AGAAAAATGC GCCTGCTGGT TTCACCCGCG TCACCAGC**	**318**	**CG7432 CG43110**	**5 3**
CG3795	**TACTTCTGCG ACTTGCCCTT CTTGTACTTG GGATGCGGCA ATAGCTCCTC GGCCTCAATA ATCTGTGTTG TACTGCTCTT GAGCAGCCTC CTTGGCGTCC CGGCAACGAC CATCAACTTC T**	**121**	**-**	**-**
CG10405	**ATTGCATCGA CGGGCATGAG CAGCAGCCGC GGGAATTCAC ACTCCGCCAG GGCAGCATCA TGCGTACCTC GGGCGGCACT GTTCAGCCGG TGAAAGCCAT CTACAAGCAT CCGGCCTACG ATCGGGCGGA CATGAACTTC GACGTGGCCC TTCTACGAAC AGCGGACGGA GCACTCAGCC TGCCGTTGGG CAAAGTGGCG CCCATTCGAT TGCCCACCGT TGGCGAGGCC ATCTCGGAAA GCATGCCCGC CGTCGTCTCC GGCTGGGGAC ACATGAGCAC CTCCAATCCC GTCCTGTCGT C**	**301**	**-**	**-**
CG4053	**AGGAGGCGGA GGACGGAGTG GCACCTTATC AGGTGTCCAT TCAGACCATC TGGAAAACCC ACATCTGCAG CGGAGTCATT CTCAACGAAC AGTGGATCCT CACCGCGGGT CATTGTGCAC TGGACTTTAG CATCGAGGAT CTAAGGATTA TAGTGGGCAC GAATGATCGT CTGGAGCCAG GACAGACCTT ATTTCCGGAC GAGGCCCTAG TCCATTGCTT GTACGACATA CCCTATGTCT ACAATAATGA CATAGCGTTG ATCCATGTCA ACGAGTCGAT TATATTCAAT GATCGCACCC AGATCGTTGA GTTGAGCCGA GAGCA**	**325**	**CG5246**	**2**
CG11668	**TTGTTGCTCC ACCAACACAT GCGCCATCAT CGCCATACGA TACCCTATGT GGTGGGTATA ACGTCCTTTG GAGGAGCCTG TGCATCCGGT CAACCTGGAG TTTATGTGCG AATAGCGCAC**	**120**	**-**	**-**
CG3088	**CGCAGCAGGA AGTGCTAAGA AGGATTCCGA GGATCCTGAT CACATTATAA CCAATGGAAG CCCCGCTTAT GAAGGTCAGG CACCCTATGT GGTGGGCATG GCCTTTGGAC AGAGCAACAT CTGGTGCAGT GGCACTATTA TAGGCGACAC**	**400**	**-**	**-**

	CTGGATCCTT ACATCCGCTC AGTGTCTAAC GGGCAGTTCC GGAGTGACCA TCTACTTTGG AGCCACCCGG CTGAGTCAGG CCCAGTTTAC GGTGACAGTG GGAACTAGTG AGTACGTTAC GGGTAATCAA CATCTCGCCC TGGTTCGAGT TCCTCGAGTC GGATTCAGCA ACCGGGTCAA CCGGGTGGCC CTTCCATCAC TGAGAAATCG ATCCCAGCGC TACGAGAACT GGTGGGCAAA TGTCTGTGGA			
CG11529	**CTCGGACTCG ATGCAGTACA CCGAGCTGAA GGTGATCTCA AATGCGGAGT GCGCCCAGGA GTACGATGTG GTCACGTCGG GAGTGATCTG TGCCAAGGGC CTGAAGGATG AGACAGTGTG CACTGGTGAC TCTGGCGGTC CACTCGTTCT CAAGGACACT CAAATAGTGG TGGGCATAAC CAGTTTCGGG CCAGCCGATG GTTGTGAGAC CAATATTCCC GGAGGCTTCA CCCGCGTCAC ACACTATCTG GACTGGATCG AGAGCAAGAT TGGTAGCC**	**278**	**-**	**-**
CG4653	**GTGCGAGTGG GCAGCATTCA GCGACTGACC GGTGGTCAAC TGGTGCCTCT GTCCAAGATC ATAATCCACA CGAACTACTC CAGTTCGGAT GCAGTTGGCT CTAATGACTT GGCCTTGCTG GAGCTGGAAA CGTCGGTGGT CCTGAATGCG AATACGAATC CGATTGATTT GGCCACCGAG CGACCGGCGG CGGGCTCCCA GATTATCTTC TCCGGCTGGG GATCCAGCCA GGTGGACGGA TCTCTCAGTC ATGTCCTCCA GGTGGCCACC AGACAGAGCC TGAGTGCGTC CGATTGCCAA ACGGAGCTGT ACCTGCAGCA GGAGGATCTG CTCTGTTTGT CCCCGGTGGA CGAGGACTTC GCCGGACTCT GTTCCGGTGA TGCCGG**	**386**	**-**	**-**
CG17477	**GCTTGCACGT TTCCTATTTT ATATTCTCGT GTTCAGTTCA CTCTACTGTG ACTTATTGGC ATTGGAGCAC TTCATTGTGG GTGGCCAGAA TGCAGCTGAA GGAGATGCCC CCTACCAGGT GTCGCTCCAA ACTCTTTTGG GTAGTCACCT ATGCGGTGGT GCCATCATAT CGGACCGATG GATAATTACG GCTGGTCACT GTGTCAAAGG ATACCCGACT AGCAGACTTC AAGTGGCCAC TGGTACAATT CGCTATGCGG AACCAGGAGC TGTTTATTAC CCAGACGCCA TCTACCTGCA CTGCAACTAT GACAGTCCCA AGTACCAGAA TGATATTGGC CTGCTCCACC TGAACGAGAG CATTACCTTT AACGC**	**375**	**CG33141**	**1**
CG17571	**TTGTGAACGG CGAGGACGTG GATATCGAAA ACTACCCCTA CCAGGTGTCC GTCCAGACGA CCAAGGGCTC CCACTTCTGT GGCGGAAGTC TGATCGATTC GGAGACCGTC CTGACCGCCG CCCATTGCAT GCAATCCTAC GCCGCCAGCG AGCTGCAGGT GCGAGTGGGT TCCACTTCCA GGAGCTCCGG TGGTGAGGTG GTCACCGTCC GCGCCTTCAA GTACCACGAG GGCTACAACA**	**301**	**CG31190**	**1**

	GCAAGTTGAT GATCAACGAT GTGGCCATCA TCAAGCTGAG CTCTCCCGTT CGCCAGACCT C			
CG14892	**CGCTACCACA ACTTCAAGCA CGACGTGGTG CTGATGAAGC TTTCAAAGCC AGCGGATCTC ACCAGGGCCT CCAATATCCG GCGGATCTGT TTGCCCTTTC TCCTAGCCGA ATCGCCGGAT CAGGCCCAGT CAGAGACAGT GTCTCCACCT TCATCGGCCG ATGAGGATGT GCTAATCCAG CAGCTGGAGC TAGAAGATGT GCCCGAAAAG ATCGATAACT TTCTGCGCAG CGTCCAGAGT CGTCGGCGCT ATCGAAATGT CACGGCCCCC AGCATGAAGG AACTGATGAA CATGAAGATC CTCAGCAGGA TGCGTCAAGC GCTGGCGCAA CGCTCCCCGC GCAGCCACAA GCGTTCGCGA AGACGCAACG ATAAGCTAAT GAAGCTGGGT CCTCGCCGGG ATTCGGATGA TTCTGCTGAG CAGAAGCACC CAAAAGTCAG TGATGAGCCA AAGGAGATTG CCTTTGTGGA CTGCGTTGCC ACGGGATGGG GCAAGGCCAA CATTAGTGGT**	**510**	**-**	**-**
CG11670	**CACCTCGCCC GACATCGTAA AAATCGGAGA CATTAAGCTC AAGGAATGGG AGCTCAACGT GGCGCCCCAA AGACGACGTG TTGCCCAGAT CTATCTGCAT CCACTGTACA ATGCGAGTCT TAACTACCAT GACATCGGGC TCATCCAGCT GAATCGACCG GTGGAATACA CCTGGTTCGT GAGGCCAGTT CGCCTGTGGC CCATGAACGA CATACCATAC GGCAAGCTGC ACACCATGGG CTATGGCTCG ACGGGATTCG CCCAGCCGCA GACGAACATA CTCACGGAAC TGGATCTCTC GGTGGTGCCC**	**310**	**-**	**-**
CG10232	**ATCTCTACAA AGGGGGCAGC GCAGTTTCCT GTAAAGTCGC AATCCGGATT GGTGGTAATA TCATGCTCGC CCAGCCGCAC TCTCCTGAGC ACCAAATCTG TGTTCACCAT TTTATCCTTG ACCACACAGT GTGCAGCGGT GAGGACGTAG CGTTTGTTAA TCAGGGAGCC ACTACAGTTG TTGGTCATGG TCGATAGTCT GCGATTCTCG TAGATAAGCA TGGCCATCCA AGGATATTCA TTTGGCCTCG CCGCTGTGCC ATATGCCATT CGATAAAGAG GCGGAGCTTG TCCACAGGAC GTTGGTAGAA CGTTACCTGG TTCCGGGCAG CAGATGTAGT GGCGCTTATC CGAATCTATT CTTCTGGTAT CGATGGCACA CTGTCTGTTA TCCATTAAAT TGGCCCCA**	**408**	**-**	**-**
CG12256	**GCGAGTTGTT GGCGGTTACG ATGTGCCAGA GGATGAATAT GTTCCCTACC AGGTGTCCAT GCAGTTTCTT ACCCGCAGTG GAAAGATGCG ACACTTCTGT GGTGGATCCC TGATAGCCCC AAATCGCGTC CTTACCGCTG CCCACTGCGT CAATGGCCAG AATGCCAGTC GAATTAGCGT AGTTGCTGGG ATTAGGGATC TCAACGATAG CTCCGGCTTC CGATCGCAGG TGCAGTCGTA CGAGATGAAC GAGAACTACC AGGAGCTGGT**	**327**	**CG32271 CG10042**	**2 1**

	GACTAGTGAC ATTGCCATCC TCAAGATCGA TCCGCCCTTT GAGCTGGACG AGAAGCG			
CG9897	**TTGTGGCTCT GCCTTGGTTG GCTCTCGGAG ATCAGCGGAT CATAAACGGA AACACTGTCA ACATTAAGGA TGCACCGTGG TATGCCTCCA TCATAGTCAA TTCGAAGCTC AAGTGCGGTG GTGCCATTAT CTCGAAGAAC TACATCCTGA CTGCCGCCAA GTGTGTGGAT GGCTACAGCG CCAGGAGCAT ACAAGTCAGG TTGGGAACCA GCAGCTGCGG CACTAGTGGA TCAATTGCTG GAATCTGTAA GGTCAAAGTT CATAGCCAGT ACTCCAGCTG GCGCTTTGAC AATAACTTGG CTCTCTTGAA AACCTGCGAG CTACTCAACA CCACCGATGA AATAAAGCCA**	**350**	**-**	**-**
CG9733	**CCCCTGGACT TTGTGTGCTC ATTAATGAAT GCCAGACACT CTACTCCGTG CTGAAGCGGG CCACTTTGAC TGATCAGGAG AAGAGCTTCA TCAAGTCCTC GGCCTGTGGA AGGGGCAGCA ATAATCAGCC CTATGTTTGC TGCACCCAGG ATACCGGCTA TGTGAGGATC CAACGCCAGG ATCGCACCTT TCCGGACTAC GGTGCATTTG GCGGTGATTG GGAGGAGGAG CGGCCACAGA GTTTCGTTTT TCCCAGACAA GAAAGACGTC CCTGGAGCTT TGGCAACCAG CCAGCCACCA GCAG**	**304**	**-**	**-**
CG11664	**CGCCATCCGA AGTTTTCACC TCTAACCCTG CGAAACGACA TCGCTGTGCT GAGGGTCAAG GCGGCCATAT CGCATTCCCA CATGATCAAC TACATCGGCC TCTGCTCGCG GCCCTTGACC CCTCTTAACA TGTTCGCACC GCCGCAGGAG CTTGCAGGCT GGAATTTGAT GCATATCGCT CAGCCCCTGA AATCTATGAG TGTTCAAGTG GAACCCGAGA AAAACTGTCG TCAATGGTTT CCCCAGATCT CGGGCGGCGT GATCTGCGCC TCAGCCACGA TGGGAGAGGG GCTGTGCTAT GGGG**	**304**	**-**	**-**
CG12951	**CTCGCATTCC TGCGGTGGTT CTATTATTTC AAAACATTTT GTGATGACCG CTGCTCATTG CACCAATGGT CGACCTGCGG ATACCCTATC AATTCAGTTT GGAGTGACCA ATATTAGTGC CATGGGTCCG AATGTGGTGG GCATAAAGAA GATAATCCAG CACGAAGACT TTGATCCCAC TCGCCAAAAT GCAAATGACA TCTCGCTGCT GATGGTGGAG GAACCTTTTG AGTTCGATGG CGTCTCTGTG GCCCCGGTGG AACTGCCAGC TCTGGCTTTT GCTGTGCCTC AATCGGATGC TGGAGTCGAA GGAGTGCTCA TCGG**	**324**	**-**	**-**
CG8464	**TGGTAACAGT GGGCAATATC AGCAAAATGG TGAACAAAAA GAAAAAGGCT GGAGACGATT GGTTCGATTC TTTGTGCCCT TCTCCCTGGG CGCTGTGGTC AGTGCGGCGA TCATACAGCG GGAAGACTTA ACGCCAACGA TCGCCGCTTC CAAAATGACC GGTCGTCGGC GGGACTTCAA**	**340**	**-**	**-**

	CTTTATAGCT GATGTGGTGG CAGGTTGTGC GGATTCGGTG GTCTACATTG AGATTAAGGA CACCCGCCAC TTTGACTACT TCAGCGGCCA ACCGATTACG GCATCGAATG GTTCCGGCTT CATTATTGAG CAGAACGGTC TCATCCTTAC CAACGCCCAT			
CG9631	**GCTACTGCTC CAGTATCGCC CTCATTCGGT GGCAATCCCA TATTGGAGCC CCAAAGGCAG AGGGGTCGAA TGTACTTGGT GTACACCATC GGAGAGGTCA GCACCAGCAG ACCAACGTCC GCATCCGGAA CTGGATTGCC CTCGTAGGCG GACGGAGTCA GCACGCTGGT CACGCTAACC AGGGAGGCTC CATTCTCAGG ATTCTCGTTG CGATCATGCC TGCCCAAATA GACCCAAAGC TGGCTGGCGC TCTTTCCATA AATGCAGTGA GCTGCCGTGA TCACAGTCCG TTTGGAGATA ACCGACACCA CGCATTTGTA TGTAGCCGTG CCAACACCTT CGTAAAGGGC CGCGAGCCAG GGGTACTGAC CGCGGGTCAC CAGGTCGCCT CCGATTTGAA GAGGCGAAAA GCCCTCAACG CCGCATTCCT CAAAGTCCGA ATCTATTCTG GGCATGATTG AAGGAAGGAA AGGATTGGGG TCATTGCTCG GTGTGCTCGG CGGCGGACGC CGCTGACGCA CTATTTCTCT TGGTGCTGAC TTTTGACTAA TTGGCGTTGA GGTGG**	**565**	**-**	**-**
CG11842	**CATAGCCATC GGTTGGGGTC AGCTGGAGAT CGTGCCCAGG ACGGAGAACA AAAAGCTACA GAAAGTGAAG CTCTACAACT ATGGAACGCG CTGCAGGATT ACGGCGGATA GAAATGATGA GCTACCCGAG GGATATAATG CTACCACCCA ACTGTGCATC GGGTCCAACG AGCACAAGGA CACCTGCAAC GGCGATTCCG GCGGACCGGT GCTCATCTAT CACATGGACT ACCCCTGCAT GTACCATGTG ATGGGTATCA CATCCATCGG AGTGGCCTGC GACACACCCG ATCTTCCCGC GATGTACACA CGGGTTCACT TCTACCTGGA CTGGATTAAG CAGCAGC**	**347**	**CG8299** **CG43336**	**9** **2**
CG15002	**CGGAGGAGGA TGGCGAGGAG GAGGAGCCAC CACTGAGCAA CAAGCTGAAG TCCGGACAAG GTCAGGGTCA GGTGTTGAAG GAATGCGAGG GCGAGTGCAT GAATGGAATA TTTGCGATCT TCTGTGATGA CATCGATTCC GATGCTTTCT GTCCCGGAGA AGAAAGCTGT TGTGTGACTG GAGGTGCCTC GGAGGCAACA CCCTCCTCCA AGGCTCCACC CACCAAGCCG GCCATCAAGC ATGCTCCCAA GCCAGCAGCC AAGCCCGCTC GTCCCGCGTC TCCACCGCCA GCACCACCAT CTTCGACCTC TGGAGGCGGA GGAGGTGGTG ACTTCCTCTC TCAAATCATA TCCTTTGCGG AGAGCACTCT GAACTCGCCA TCTCCACCAC CG**	**394**	**CG10663** **CG12809** **CG34113** **CG7538**	**1** **1** **1** **1**
CG15873	**TAAGGATACT CTGACCCCGC AGTCGGAAAG GGATTGGATG GTGAGAAGAA TCCAGTCCTT**	**281**	**-**	**-**

	GTAGTACAGA AAGCTGAGGA ACTTCATGGC CTTGCCGCCC GCGCATCCCA TGTGTCCGCC AATGAGCCCG AACAGAGCCC CCTTGCACAG CAGGGGTCCG CCCATGTCCC CGGCGCAGTT CATGCTTTCT CCCACGGGTT CCGTGCACAC ATTGTGGTCC GCGGTGAAGG TGTCGTAGTG CTTCTGACAG AGCGAGGGCG GCCTCAAAAT CACGTCCAGG T			
CG1304	**TCCCTCAATA CAGCTATGCC AGCATTATTT CACATCCGAG TTATTCTTAA TCCATTCGTT GTGGTAGTAC ACCCTGGCAT ATCCATCTGG ATAGCTCGTT CCACAG**	**106**	**-**	**-**
CG4386	**GATGAACCTC CGTTTCCTGA CCACCGACTA TTCTCTTCTG TATATTGGCT ATTCCGCAGA CACAGTCGCT GCAATTCCTT GGCGGATTCA AAGTGGGTGG GGCCGGAGTG GTTGCCCTTC TCGTTGTAGT GGTGCTGGAA GACGGGGCTG GTGTGGTGGT TGATGTGGTA GATGCCGCGT CCGGCATCAT AGAGCTGCTG CTCAAGGTGG CCAGAGTGGC ATTTTCCGAA TCCGAACTGC CGGGACGTTG GGGCAAAAGT GAGAGAACCC ATTGGATAAA TGTGTTCTGA GATTGTTTCA GCAGCGGACT CGCTGCCGTT TGGTTCGTTG CACGATCCTG GCTGGCTGAT TGGCTTGGAC CGCAGCTTAA GGCCAGGCAG ATCAGCAGAC ACAACCAGGC TCGATTCATT T**	**411**	**-**	**-**
CG14088	**GGAGTTATTA GAGCACGGTT GGGAGAGTAC GGAAGAATTG GTTCTGAGCT GGCAGAAGAC CACATAGTCG CTGCATTCTT CAGTAACGCC AACTTTAATC CGGAAACGCA GGCGAATAAC ATGGGCCTAA TGAAACTGCT GCGAACCGTA GTATACAAAG AGCATATAAT TCCGGTCTGC ATTCTTATGG ACTCACGGAT GCAGACGTTC GCCGATGAAC TGGACTATTT CAATGGAACT ACATGGAAGA ACTCGGACAA ATCACCCTAT GCTGAGGTCC AAAACTGTGA TCCGAATGCC**	**300**	**-**	**-**
CG3700	**GCTGGACTAC AACAGCACCA CCGATGATGC TCTGGTTCAG GACTTTCGGG TGGTCAACTA TGTGGTGCAC CCTGGATATG ATACTGAAGA CGAAGAGCAG GGCTTTAAAA ACGATATTGC CCTGGTGGAA CTGGATCGGA AGGCTGAGTT CAACGACCAT GTGGCAGCGG TATGCCTTCC GCCAGACAGC GGCAACGATG TTCAGCAGGT CACAGCCGCC GGCTGGGGAT TTACCGCGGA CGGTGTGAAG TCCTCGCACC TGCTGAAGGT CAACCTCCAG CGATTCAGTG ACGAGGTGTG CCAGAAGCGC CTGCGTTTCA GCATCGATAC ACG**	**333**	**CG4821**	**1**
CG18420	**TGTGGGATGC GTCGTGGAAA CATCATATCG ATAGCATTAA AGTGTTAACT GGTACTGGAT GGGGACGAAC TGAATCGATG CATGACAGCA GTGAGCTCAG GACACTGGAC ATTTCGCGAC AGCCTTCAAA AATGTGCGCT TTTGGCAGCG**	**400**	**-**	**-**

	TTTTGAGCAA TCAGTTTTGT GCCGGAAATT GGAACAGCAA TCTTTGCATC GGCGATACTG GTGGTCCTGT GGGAGCAATG GTTCGATATA GAAATGCATT CCGCTTTGTT CAAGTAGGTA TCGCCATTAC TAACAAAAGG TGCCAGAGAC CAAGTGTATT CACTGATGTC ATGAGCCATA TCGAATTTAT CCGTCGTATA TTTTTGACGC AAAACGGCAA CGATAGGAAC CAACCGACAC CAAAGCCAGA			
CG7754	**TTGCCGTGCT TCGTCTGTCC ACACCACTGA CCTTTGGCCT CTCAACGAGA GCCATCAATT TAGCCAGTAC GAGTCCATCG GGTGGAACAA CAGTCACTGT CACGGGTTGG GGCCACACTG ATAATGGAGC CCTCTCCGAT AGCTTGCAGA AGGCCCAGTT GCAGATCATC GATCGCGGAG AGTGTGCCTC GCAAAAGTTT GGCTACGGTG CGGATTTTGT GGGCGAGGAA ACAATTTGCG CTGCCAGCAC TGATGCAGAT GCCTGTACGG GAGACTCTGG AGGTCCTTTG GTGGCCAGTA GCCAGCTGGT GGGCATTGTA TCCTGGG**	**327**	**-**	**-**
CG15046	**AGATTGAGCG GACAGCAGAT GATCTCCTCG TGCGTTCCCT CGCGGCACGT TGTGAAATCC TCGTCCCTGA GACGACCCTG CGACAGGAGT GGCTCCACAC TGGGGCAGGC GGATACGGCA CGACACTGGC CCTCGTACAG GGGAGCCGTG CAGTCGGCGC CATCGGAGGT GTCTCTGAAG GTGATGCTCT CCCTCGTTGG CCGGTGCGTG GACCCAATGG ATTGCTGGAC GTAATCGCGA TCCGTCTTGA ATATGCTCAG CCAAGGTTCT GGCGCTGGAG TTGTGGTAGT AGTGGTGGTT GTGGTGGAAG TA**	**312**	**CG7884**	**2**
CG3650	**GTCCAGGGCG GAGTGAGCAA GCTCAGCCAG TCGGGCGTCG TGAGACGCGT GGCCAGGTAC TTTATTCCAA ACGGCTTCAG CAGCTCCAGC CTCAACTGGG ACGTGGGCGT CATCCGGCTG CAGAGTGCCT TGACTGGCAG TGGCATCACC ACCATTCCCC TCTGCCAGGT CCAGTGGAAC CCCGGCAACT ACATGCGCGT CTCCGGCTGG GGCACCACTC GCTACGGCAA CTCCAGTCCC TCCAACCAGC TGCGCACGGT GCGCATCCAG CTGATCCGCA AAAAGGTGTG CCAGAGGGCC TACCAGGGAA GGGACACGCT CACCGCCTCC ACCTTCTGC**	**339**	**CG17571**	**2**
CG1773	**AACAGGATTG CGGTGTCCTA TCGAATCTGA TTCCCGCCCA AAGGCTTCGA CGGCGGATCA CCGGCGGCAG GAAATCTTCG CTGTTGTCCC AGCCTTGGAT GGCTTTTCTC CACATTTCCG GTGATA**	**126**	**-**	**-**
CG8213	**TTCGCCGCCT CAAAACGATG ACTTCGTAAT GCAGGTGCTA AGCACCTTGC CACCTGAGCA TGCGGATGAC CATCATATTG TCTTTACCAC CGAGGTGCCC ACCAAGATCA CCAGTGGATT GCAGGATCAA ACGAGCTCTG AGTCCAACTC**	**278**	**-**	**-**

	CTTCGAAGAG GTGTCTTCGA CGCCAGCAGC CACCCAAAAA CCGAAACCCA AGCCAACTCA AATGCCCACT CAGAAGACCA CTCAGAAGGC CACGCAGAAG CCCACTCCAA AGCCCACACA GAAAGCCA			
CG4650	**TTTTGGGAGA TTTTTCACCG TTGCGGTACT GTCGCCATAC TGAAAGAATA AAATCGGTGT GACTCAGGAC ATCCGTATAA ACGCTTGCCC TTTTACATTT TTGGTTAGTG GTTGCTATGC CAATTAGGAC ATAGCGCTGA ATATTCTTGA AGGTTATAAT GGCTCCCAGA GGACTGCTAA AGTCAACATT GCACAGTTTG GAATCCGAGT CTCCAGCACA AAACTGACTG CTCAAAATTG CAGTGCCGTT TAGGGTAGAA CACATATTTG CTGGTTGACG TCTGATGTCT GTAATTCTAA ATGCATCGCT TTCATTTCGA TCATTGGGTA GACCC**	**335**	**-**	**-**
CG7432	**GACGACCACC AGCACCACCA AGGCTACGCA ACCCACGAAG AAAAGCACTG TTCGACCCAC CACGCGCCCC ACATCTGGTC TCGTGCTCAT TCCCCAGAAG AAACCGCCCA CTACCACGAC GACCACCACG ACGGAGGTGC CTCTGGAGCC GGAGGGTCTG GACGAGATCG GCAACAACAT TGTGGACCCC GATGAGTGCG GTCAGCAGGA GTACTCAACT GGTAGGATTG TGGGCGGAGT GGAGGCTCCG AATGGTCAGT GGCCATGGAT GGCAGCCATT TTTCTCCATG GACCCAAGCG CACGGAGTTC TGGTGCGGTG GTTCGTTGAT T**	**331**	**-**	**-**
CG6865	**TGACAGTTAG CAACCAGCCA TGCTCGGTTC GAAATCCAAA AATCGTGGGA GGTAGTGAGG CGGAGCGCAA CGAAATGCCC TACATGGTCA GTCTGATGCG TCGTGGTGGT CACTTTTGTG GCGGCACTAT CATCTCGGAG CGATGGATCC TCACGGCGGG ACATTGCATC TGCAATGGGC TGCAGCAGTT CATGAAACCA GCTCAAATCC AAGGAGTTGT GGGTTTGCAT AGCATCAGGG AGTACCTCAA CGGGATTGGC AACGGTCCGG ATGCACTGAG GGTGGACTTC AAGAACATTG TGCCCCATCC GCAATACGAC TGCAACGATG TGAAACACG**	**339**	**-**	**-**
CG4927	**AGTACGTGGT CAGATTGGGC GAACTGGACT ACAATAGCAC CACGGATGAT GCCCAGCCGC AGGATTTCCG GGTACTCAAC TATGTGGTGC ATCCGGCGTA CGGCGAGGAT GATGACACGG GCAGTCGCAA GAACGACATT GCCGTGGTGG AGCTGGAAAT GGAGGCGACT TTCAGCGAGT ATGTGGCACC TGCCTGCCTG CCGCTCGATG GCGGAAATGA GCAACTGCAG GTGGCAGCCG CCGGATGGGG CGCCACCTCG GAGAGTGGAC ACGCCTCGTC GCATCTGCTC AAGGTGAGTC TCGATCGATA CGATGTGGCC GAGTGCAGCC AGCGACTGGA GCACAAGATC GATGTGCGCA**	**402**	**CG14838 CG42389 CG44248 CG44249**	**2 2 1 1**

	CCCAATTGTG TGCGGGATCG CGGTCCACCA GTGCGGATAC TT			
CG6592	**ATCCAACTTC CCGCTATCCT ATCGAGGTAC AAATATATGC ACCAGTGGAA GGAATGCCCG TTCAACTTGC AATGGTGATT CGGGAGGACC TTTGGTTCTC CAAAGGAGGC ACTCGAAGAA GAGAGTCCTG GTGGGAATTA CATCCTTTGG CAGCATATAC GGTTGTGATC GAGGCTATCC GGCGGCCTTC ACCAAAGTTG CCTCATATTT GGATTGGATC AGCGATGAAA CTGGTGTAAG TGCCCACCAG GATACCACGG AGGCAATATT TTTCGACCAG TATGTGAGGG AATATGGGAA ACCACGTCAA AGCCGACGCT TGGAAACGGA GGAGCAGTTG GAAGACGATG TGCCCGACGA ACTAGATGT**	**369**	**-**	**-**
CG18681	**AGGGCTACAA CTCCCGCACC ATGGTCAACG ACATTGCTAT TATTCGCATC GAGTCCGATC TGAGCTTCCG CTCCAGCATT CGCGAGATCC GTATTGCCGA CTCCAACCCT CGTGAGGGCG CCACCGCCGT GGTTTCCGGC TGGGGCACCA CCGAGTCCGG TGGCAGCACC ATTCCCGATC ATCTGCTGGC CGTTGATCTG GAGATCATCG ATGTGTCGCG TTGCCGCTCG GATGAGTTCG GATACGGAAA GAAGATCAAG GACACCATGC TCTGCGCCTA CGCCCCACAC AAGGATGCCT GCCAGGGTGA CTCCGGTGGC CCACTTGTCT CCGGAGACCG CCTTGTCGGT GTTGTGTCCT GGGG**	**364**	**CG12351 CG30025 CG30028 CG30031 CG18444 CG18211 CG17571 CG34458 CG32755 CG7996**	**21 21 21 21 18 8 5 4 2 1**
CG12133	**CTACATCCCC GGAACATGAC AAACGATCAA AAATCACAGT ACCGGAACAA ACTATGCAAC ATTAATCCTT TTGCCCACGA GCTAGTGCAC ATGGTTTTTA CCTGCTGCCC GATGGTTGCA GGGGATAAAC TACCAGATAG CAGGGTCTGC GGACAGAGTC CTCCTTCGTC ATATATCGTA GGCGGCATGG AGGCACAGTC CAACCAGTTT CCCTGGACAG TCCTGCTGGG TTACGAAGCC TACACAGCAA AGCAGCGACC GTCTCCCATG TGTGCGGGCT CCTTGATCGC CAGTCGCTAT GTGCTGACGG CAGCCCATTG TTTGAATGTG**	**330**	**-**	**-**
CG16710	**GAGAGGAGAC GCAACCCAAC GAGTTGCCTT GGATGGCCCT GATTCTATAT GCGCATCGCA GCCGTTCGGT GTGGAATGAA AGACTCGTGT CCAGGTGCGC TGGATCCCTG ATCACAAACC GCTATGTGCT GACCGCAGCC CATTGTCTGA GGATAACGGG ATTGGATCTC AGGAGGGTGC GCCTTGGCGA GCATAACATT TTAAGCAATC CCGACTGCGT CACCCATATT AATGGTAGGG AACACTGCGC TCCAGAGCAT CTGGAAATTG ACGTCGATCT GAGTATCAAG CATAGGCACT ACATGGTCTT CGAGGAAAGG CCCTACAACG ACATCGCCCT GCTGCGATTG AAGTTTCCAG TACG**	**364**	**-**	**-**

Table S2: Statistical analysis for survival experiments of the candidates 44 SP genes.

For statistical analysis of all survival data (Figures 13 and 14), Gehan-Breslow-Wilcoxon test was applied using GraphPad Prism 5 software. Results with a p-value of less than 0.05 were considered as significant.

CG number	p-value	df	Chi square
CG6361	0.6204	1	0.2453
CG6462	0.1646	1	1.931
CG5909	0.1326	1	2.262
CG10477	0.08	1	3.065
CG3795	0.3173	1	1
CG10405	0.1414	1	2.162
CG4053	0.1156	1	2.476
CG11668	0.174	1	1.848
CG3088	0.4935	1	0.4689
CG11529	0.2898	1	1.12
CG4653	0.1498	1	2.074
CG17477	0.2413	1	1.373
CG17571	0.0684	1	3.321
CG14892	0.1297	1	2.297
CG11670	0.2463	1	1.344
CG10232	0.0651	1	3.401
CG12256	0.7371	1	0.1127
CG9897	0.073	1	3.214
CG9733	0.2749	1	1.192
CG11664	0.1207	1	2.409
CG12951	0.1495	1	2.077
CG8464	0.1244	1	2.362
CG9631	0.2463	1	1.344
CG11842	0.2408	1	1.376

Statistical analysis for Figure 13 (after infection with *E. carotovora 15*)

CG number	p-value	df	Chi square
CG6361	0.8947	1	0.01751
CG6462	0.0741	1	3.191
CG5909	0.0646	1	3.416
CG10477	0.0007	1	11.55
CG3795	0.0035	1	8.505
CG10405	0.2763	1	1.185
CG4053	0.0552	1	3.675
CG11668	0.0011	1	10.66
CG3088	0.0406	1	4.195
CG11529	0.3291	1	0.9524
CG4653	0.731	1	0.1182
CG17477	0.374	1	0.7905
CG17571	0.9653	1	0.001895
CG14892	0.1405	1	2.173
CG11670	< 0.0001	1	18.14
CG10232	0.0462	1	3.975
CG12256	0.253	1	1.307
CG9897	0.0124	1	6.257
CG9733	0.0308	1	4.666
CG11664	0.1794	1	1.803
CG12951	0.4375	1	0.6029
CG8464	0.2689	1	1.222
CG9631	0.844	1	0.03874
CG11842	0.122	1	2.391

Statistical analysis for Figure 13 (after infection with *S. aureus*)

CG number	p-value	df	Chi square
CG6361	0.0212	1	5.314
CG6462	0.7392	1	0.1108
CG5909	0.0307	1	4.671
CG10477	0.0032	1	8.717
CG3795	0.0002	1	13.87
CG10405	0.0467	1	3.957
CG4053	0.0424	1	4.12
CG11668	0.003	1	8.794
CG3088	0.8949	1	0.01744
CG11529	0.036	1	4.398
CG4653	0.4605	1	0.5447
CG17477	0.0491	1	3.871
CG17571	0.0025	1	9.173
CG14892	0.0783	1	3.099
CG11670	0.0001	1	15.01
CG10232	0.0025	1	9.151
CG12256	0.0176	1	5.635
CG9897	0.0427	1	4.106
CG9733	0.0001	1	15.01
CG11664	0.9826	1	0.000475
CG12951	0.036	1	4.398
CG8464	0.0213	1	5.305
CG9631	0.0237	1	5.113
CG11842	0.0256	1	4.983

Statistical analysis for Figure 13 (after infection with *E. faecalis*)

CG number	p-value	df	Chi square
CG6361	0.0433	1	4.083
CG6462	0.0024	1	9.178
CG5909	0.0146	1	5.964
CG10477	0.0001	1	15.1
CG3795	0.0174	1	5.654
CG10405	0.0004	1	12.71
CG4053	0.0437	1	4.067
CG11668	< 0.0001	1	22.17
CG3088	0.0116	1	6.375
CG11529	0.0226	1	5.198
CG4653	0.0281	1	4.824
CG17477	0.0688	1	3.311
CG17571	0.0223	1	5.223
CG14892	0.0105	1	6.546
CG11670	0.0002	1	14.23
CG10232	0.0019	1	9.623
CG12256	0.0041	1	8.25
CG9897	0.0055	1	7.712
CG9733	0.0008	1	11.19
CG11664	0.0041	1	8.244
CG12951	0.0105	1	6.546
CG8464	0.0453	1	4.006
CG9631	0.2862	1	1.138
CG11842	0.0106	1	6.525

Statistical analysis for Figure 13 (after infection with *B. bassiana*)

CG number	p-value	df	Chi square
CG15002	0.3437	1	0.8966
CG15873	0.1653	1	1.925
CG1304	0.2761	1	1.186
CG4386	0.5496	1	0.358
CG14088	0.2458	1	1.347
CG3700	0.1166	1	2.462
CG18420	0.5238	1	0.4063
CG7754	0.2458	1	1.347
CG15046	0.261	1	1.263
CG3650	0.2609	1	1.264
CG1773	0.1325	1	2.263
CG8213	0.0566	1	3.635
CG4650	0.0564	1	3.64
CG7432	0.2765	1	1.184
CG6865	0.4982	1	0.4587
CG4927	0.0528	1	3.752
CG6592	0.9618	1	0.002299
CG18681	0.2461	1	1.345
CG12133	0.066	1	3.38
CG16710	0.5238	1	0.4063

Statistical analysis for Figure 14 (after infection with *E. carotovora 15*)

CG number	p-value	df	Chi square
CG15002	0.9478	1	0.004289
CG15873	0.4472	1	0.5778
CG1304	0.6304	1	0.2316
CG4386	0.5511	1	0.3553
CG14088	0.2158	1	1.532
CG3700	0.7099	1	0.1384
CG18420	0.4882	1	0.4806
CG7754	0.4597	1	0.5467
CG15046	0.3777	1	0.7783
CG3650	0.2278	1	1.455
CG1773	0.4882	1	0.4806
CG8213	0.7777	1	0.07974
CG4650	0.4882	1	0.4806
CG7432	0.3103	1	1.029
CG6865	0.0887	1	2.897
CG4927	0.3842	1	0.7574
CG6592	0.6778	1	0.1726
CG18681	0.8622	1	0.03013
CG12133	0.9828	1	0.000465
CG16710	0.8797	1	0.02292

Statistical analysis for Figure 14 (after infection with *S. aureus*)

CG number	p-value	df	Chi square
CG15002	0.4403	1	0.5956
CG15873	0.2902	1	1.119
CG1304	0.9632	1	0.00213
CG4386	0.0558	1	3.658
CG14088	0.1608	1	1.967
CG3700	0.9632	1	0.002125
CG18420	0.6246	1	0.2395
CG7754	0.5765	1	0.3119
CG15046	0.9816	1	0.000533
CG3650	0.4013	1	0.7043
CG1773	0.1967	1	1.667
CG8213	0.7207	1	0.1278
CG4650	0.787	1	0.07304
CG7432	0.1748	1	1.841
CG6865	0.3848	1	0.7554
CG4927	0.1375	1	2.206
CG6592	0.243	1	1.363
CG18681	0.4214	1	0.6464
CG12133	0.5636	1	0.3335
CG16710	0.8843	1	0.02118

Statistical analysis for Figure 14 (after infection with *E. faecalis*)

CG number	p-value	df	Chi square
CG15002	0.1269	1	2.33
CG15873	0.7012	1	0.1472
CG1304	0.9285	1	0.008055
CG4386	0.1021	1	2.673
CG14088	0.0538	1	3.72
CG3700	0.1835	1	1.769
CG18420	0.0693	1	3.299
CG7754	0.6106	1	0.2593
CG15046	0.2652	1	1.242
CG3650	0.7971	1	0.06612
CG1773	0.3298	1	0.9498
CG8213	0.2064	1	1.597
CG4650	0.787	1	0.07299
CG7432	0.3844	1	0.7567
CG6865	0.8983	1	0.01635
CG4927	0.8742	1	0.02506
CG6592	0.0506	1	3.823
CG18681	0.2517	1	1.314
CG12133	0.2993	1	1.078
CG16710	0.7012	1	0.1472

Statistical analysis for Figure 14 (after infection with *B. bassiana*)

Figure S1: Resistance of the 24 SP-RNAi flies to *E. Coli* infection.

This graph presents the bacterial CFU count for the 24 analyzed SP-RNAi flies after 48 hours post-infection. Infected and non-infected wild-type flies (w^{1118}) are used as a controls.

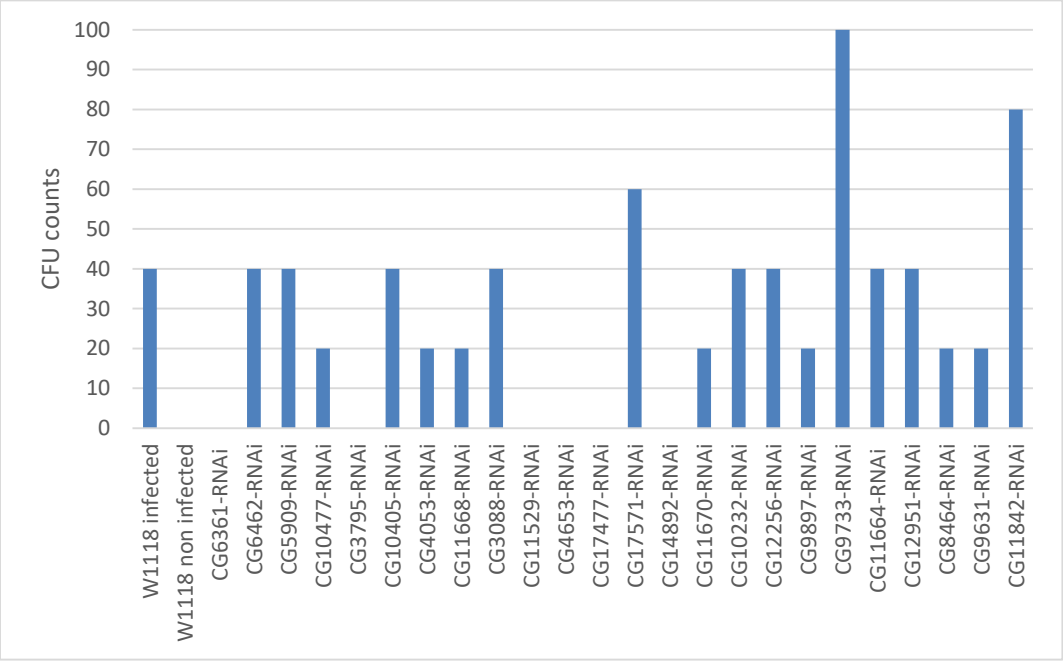
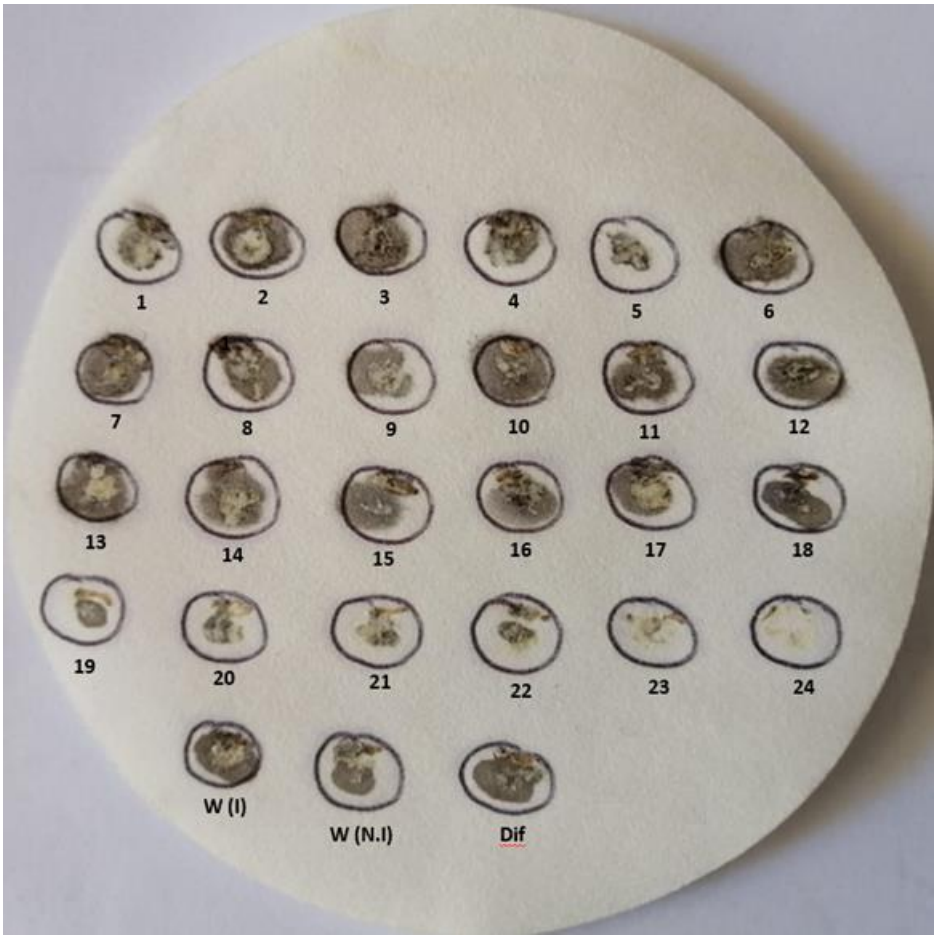


Figure S2: Melanotic spots of the 44 SP-KD flies' hemolymph.

The phenoloxidase (PO) activity was assessed semi-quantitatively. The darker and larger melanization spots as compared to the control flies (infected and non-infected wild-type flies, and *Dif-RNAi* flies) reflect the higher PO activity.

1: CG6361 (Hayan)	2: CG6462	3: CG5909	4: CG10477	5: CG3795	6: CG10405
7: CG4053	8: CG11668	9: CG3088	10: CG11529	11: CG4653	12: CG17477
13: CG17571	14: CG14892	15: CG11670	16: CG10232	17: CG12256	18: CG9897
19: CG9733	20: CG11664	21: CG12951	22: CG8464	23: CG9631	24: CG11842
25: wild-type (I; infected)		26: wild-type (N.I; non-infected)			27: Dif



B. Identification of fungi that can be used as potential insect biocontrol agents

Table S3: Number of occurrences of each isolated fungus among the collected cadavers.

In the table below, each isolated fungus is listed, its class and known mode of life are given in columns 2 and 3 respectively. A + sign indicates that the fungus was present on the cadaver of a given arthropod (arthropods ID s are given as in table 1).

Species	Class	Mode of life	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Aspergillus ustus	Eurotiomycete	Saprophyte	+																
Aspergillus candidus	Eurotiomycete	Saprophyte	+																
Aspergillus sclerotium	Eurotiomycete	Saprophyte	+		+														
Aspergillus nomius	Eurotiomycete	Saprophyte	+																
Wallemia sp.	Wallemiomycete	Saprophyte		+															
Scopulariopsis brevicaulis	Sordariomycete	Human pathogen			+														
Aspergillus fumigatus	Eurotiomycete	Opportunistic pathogen				+									+			+	
Aspergillus ruber	Eurotiomycete	Saprophyte				+													
Aspergillus Glaucus	Eurotiomycete	Saprophyte				+													
Chaetomium globosum	Sordariomycete	Saprophyte					+												
Pyrenophora dictyoides	Dothideomycete	Plant pathogen						+											
Fusarium tricinctum	Eurotiomycete	Saprophyte						+											
Botrytis cinerea	Leotiomycete	Plant pathogen							+										
Alternaria alternata	Dothideomycete	Plant pathogen							+										
Fomes fomentarius	Agaricomycete	Saprophyte							+	+	+								
Talaromyces amestolkiae	Eurotiomycete	Saprophyte								+			+					+	+
Cladosporium cladosporioides	Dothideomycete	Saprophyte						+	+	+	+	+	+	+	+	+	+	+	+
Stachybotrys chartarum	Sordariomycete	Saprophyte								+									
Ascomycota sp.	Leotiomycete	Undefined								+									
Alternaria infectoria	Dothideomycete	Plant pathogen									+					+			
Simplicillium sympodiophorum	Sordariomycete	Saprophyte									+								
Penicillium digitatum	Eurotiomycete	Saprophyte										+			+				
Periconia sp.	Sordariomycete	Saprophyte										+							
Penicillium freii	Eurotiomycete	Saprophyte											+				+		
Chaetomium nigricolor	Sordariomycete	Saprophyte												+					
Chaetomium bostrychodes	Sordariomycete	Saprophyte																	
Engyodontium album	Sordariomycete	Plant endophyte																	
Penicillium commune	Eurotiomycete	Saprophyte																	
Phoma herbarum	Dothideomycete	Plant pathogen																	
Embellisia abundans	Dothideomycete	Plant pathogen																	
Penicillium polonicum	Eurotiomycete	Saprophyte																	

Table S4: Statistical analysis for survival experiments of the different fungal species.

For statistical analysis of all survival data (Figures 18, 19, 20 and 22), F-Test for Variance were calculated and Gehan-Breslow-Wilcoxon test was applied using GraphPad Prism 5 software. Results with a p-value of less than 0.05 were considered as significant.

Species	Mean	Variance	F-value	p-value	df	Chi square
Aspergillus ustus	85	518.2	0.4	0.0553	1	3.673
Aspergillus candidus	100	0	0	0.0001	1	36.39
Aspergillus sclerotium	77.3	781.8	0.57	0.2595	1	1.271
Aspergillus candidus	72.7	278.7	0.27	0.006	1	7.543
Aspergillus nomius	52.5	1675	1.35	0.1073	1	2.594
Aspergillus sclerotium	75	990.9	0.8	0.4194	1	0.6519
Wallemia sp.	85.4	307.8	0.25	0.5679	1	0.3262
Scopulariopsis brevicaulis	93.3	24.2	0.02	0.0025	1	9.161
Aspergillus ruber	89.1	69.09	0.05	0.202	1	1.628
Aspergillus glaucus	100	0	0	0.0001	1	17.8
Chaetomium globosum	89.2	62.9	0.04	0.0046	1	8.038
Alternaria alternata	56.7	330.2	0.19	0.5283	1	0.3977
Fomes fomentarius	93.8	3.2	0.001	0.0001	1	20.02
Cladosporium cladosporioides	100	0	0	0.0001	1	28.1
Alternaria infectoria	97.6	68	0.03	0.0001	1	26.27
Penicillium digitatum	93.3	170.9	0.1	0.0001	1	28.59
Periconia sp.	91.3	59.7	0.03	0.0001	1	20.33
Penicillium freii	86	123	0.06	0.0002	1	13.45
Engyodontium album	99.3	4.4	0.002	0.0001	1	27.57
Penicillium commune	60	967.9	0.44	0.2788	1	1.173
Phoma herbarum	81.8	85.3	0.03	0.0008	1	11.23
Embellisia abundans	94.5	78.3	0.04	0.0001	1	22.69
Penecillium polonicum	80	145.1	0.06	0.0004	1	12.66
Talaromyces amestolkiae	96.1	100.7	0.04	0.0001	1	28.84

Statistical analysis for Figure 18

Species	Mean	Variance	F-value	p-value	df	Chi square
Aspergillus ustus	71.3	106.6	0.09	0.0128	1	6.196
Aspergillus sclerotium	33.8	1351.6	1.18	0.7565	1	0.0962
Aspergillus nomius	22	1253.9	1.09	0.1648	1	1.93
Aspergillus sclerotium	40.5	1855.3	1.62	0.4739	1	0.5128
Wallemia sp.	90.8	33.6	0.03	0.0001	1	18.57
Aspergillus ruber	45.6	1206.3	1.09	0.5	1	0.4549
Alternaria alternata	60	866.7	0.75	0.0231	1	5.163
Periconia sp.	90.3	26.8	0.02	0.0001	1	18.35
Penicillium commune	60.5	470.1	0.41	0.0414	1	4.161
Phoma herbarum	91.8	30.8	0.03	0.0001	1	20.49
Penecillium polonicum	93.3	140.7	0.11	0.0001	1	27.64
Talaromyces amestolkiae	76.9	137.9	0.12	0.0056	1	7.67

Statistical analysis for Figure 19

Species	Mean	Variance	F-value	p-value	df	Chi square
Aspergillus nomius	48.3	1173.8	0.75	0.4462	1	0.5803
Wallemia sp.	94.4	39.6	0.07	0.0013	1	10.34
Aspergillus ruber	79.1	666.4	0.44	0.0002	1	13.81
Alternaria alternata	80.3	345.2	0.23	0.0013	1	10.34
Penicillium digitatum	95	53.1	0.06	0.0002	1	14.35
Periconia sp.	82.8	489.9	0.35	0.0003	1	13.04
Penicillium freii	97.9	6.4	0.01	0.0001	1	18.03
Penicillium commune	97.8	10.6	0.01	0.0001	1	15.99
Talaromyces amestolkiae	80.6	662.1	0.44	0.0001	1	14.71

Statistical analysis for Figure 20

Species	Mean	Variance	F-value	p-value	df	Chi square
Aspergillus nomius	57.6	767.7	1.54	0.0507	1	3.818

Statistical analysis for Figure 22

CHAPTER VIII

REFERENCES

1. Fearon, D.T. and R.M. Locksley, *The instructive role of innate immunity in the acquired immune response.* Science, 1996. **272**(5258): p. 50-54.
2. Medzhitov, R. and C.A. Janeway Jr, *Innate immunity: impact on the adaptive immune response.* Current opinion in immunology, 1997. **9**(1): p. 4-9.
3. Nie, L., et al., *Toll-like receptors, associated biological roles, and signaling networks in non-mammals.* Frontiers in Immunology, 2018. **9**.
4. Adams, M.D., et al., *The genome sequence of Drosophila melanogaster.* Science, 2000. **287**(5461): p. 2185-2195.
5. Reiter, L.T., et al., *A systematic analysis of human disease-associated gene sequences in Drosophila melanogaster.* Genome research, 2001. **11**(6): p. 1114-1125.
6. Lloyd, T.E. and J.P. Taylor, *Flightless flies: Drosophila models of neuromuscular disease.* Annals of the New York Academy of Sciences, 2010. **1184**: p. e1.
7. Buchon, N., N. Silverman, and S. Cherry, *Immunity in Drosophila melanogaster—from microbial recognition to whole-organism physiology.* Nature reviews immunology, 2014. **14**(12): p. 796-810.
8. Vallet-Gely, I., B. Lemaitre, and F. Boccard, *Bacterial strategies to overcome insect defences.* Nat Rev Microbiol, 2008. **6**(4): p. 302-13.
9. Shklyar, B., et al., *Caspase activity is required for engulfment of apoptotic cells.* Molecular and cellular biology, 2013. **33**(16): p. 3191-3201.
10. Siva-Jothy, M.T., Y. Moret, and J. Rolff, *Insect immunity: an evolutionary ecology perspective.* Advances in insect physiology, 2005. **32**: p. 1-48.
11. Lundgren, J.G. and J.L. Jurat-Fuentes, *Physiology and ecology of host defense against microbial invaders*, in *Insect Pathology (Second Edition)*. 2012, Elsevier. p. 461-480.
12. Mace, K.A., J.C. Pearson, and W. McGinnis, *An epidermal barrier wound repair pathway in Drosophila is mediated by grainy head.* Science, 2005. **308**(5720): p. 381-385.
13. Davis, M.M. and Y. Engström, *Immune response in the barrier epithelia: lessons from the fruit fly Drosophila melanogaster.* Journal of innate immunity, 2012. **4**(3): p. 273-283.
14. Kuraishi, T., et al., *Genetic evidence for a protective role of the peritrophic matrix against intestinal bacterial infection in Drosophila melanogaster.* Proceedings of the National Academy of Sciences, 2011. **108**(38): p. 15966-15971.
15. Wagner, C., et al., *Molecular architecture of the fruit fly's airway epithelial immune system.* BMC genomics, 2008. **9**(1): p. 446.

16. Tzou, P., et al., *Tissue-specific inducible expression of antimicrobial peptide genes in Drosophila surface epithelia.* Immunity, 2000. **13**(5): p. 737-748.
17. Tingvall, T.Ö., E. Roos, and Y. Engström, *The imd gene is required for local Cecropin expression in Drosophila barrier epithelia.* EMBO reports, 2001. **2**(3): p. 239-243.
18. Bergman, P., S.S. Esfahani, and Y. Engström, *Drosophila as a model for human diseases—focus on innate immunity in barrier epithelia*, in *Current topics in developmental biology*. 2017, Elsevier. p. 29-81.
19. Theopold, U., et al., *The coagulation of insect hemolymph.* Cellular and Molecular Life Sciences CMLS, 2002. **59**(2): p. 363-372.
20. Scherfer, C., et al., *Isolation and characterization of hemolymph clotting factors in Drosophila melanogaster by a pullout method.* Current biology, 2004. **14**(7): p. 625-629.
21. Lesch, C., et al., *A role for Hemolectin in coagulation and immunity in Drosophila melanogaster.* Developmental & Comparative Immunology, 2007. **31**(12): p. 1255-1263.
22. Scherfer, C., et al., *The Toll immune-regulated Drosophila protein Fondue is involved in hemolymph clotting and puparium formation.* Developmental biology, 2006. **295**(1): p. 156-163.
23. Karlsson, C., et al., *Proteomic analysis of the Drosophila larval hemolymph clot.* Journal of Biological Chemistry, 2004. **279**(50): p. 52033-52041.
24. Wang, Z., et al., *Pathogen entrapment by transglutaminase—a conserved early innate immune mechanism.* PLoS pathogens, 2010. **6**(2): p. e1000763.
25. Lanot, R., et al., *Postembryonic hematopoiesis in Drosophila.* Developmental biology, 2001. **230**(2): p. 243-257.
26. Samakovlis, C., et al., *The immune response in Drosophila: pattern of cecropin expression and biological activity.* The EMBO journal, 1990. **9**(9): p. 2969-2976.
27. Theopold, U., R. Krautz, and M.S. Dushay, *The Drosophila clotting system and its messages for mammals.* Developmental & Comparative Immunology, 2014. **42**(1): p. 42-46.
28. Vanha-aho, L.-M., S. Valanne, and M. Rämet, *Cytokines in Drosophila immunity.* Immunology Letters, 2016. **170**: p. 42-51.
29. Martinek, N., et al., *Haemocyte-derived SPARC is required for collagen-IV-dependent stability of basal laminae in Drosophila embryos.* Journal of cell science, 2008. **121**(10): p. 1671-1680.
30. Honti, V., et al., *The cell-mediated immunity of Drosophila melanogaster: hemocyte lineages, immune compartments, microanatomy and regulation.* Developmental & Comparative Immunology, 2014. **42**(1): p. 47-56.
31. Avet-Rochex, A., et al., *An in vivo RNA interference screen identifies gene networks controlling Drosophila melanogaster blood cell homeostasis.* BMC developmental biology, 2010. **10**(1): p. 65.
32. Owusu-Ansah, E. and U. Banerjee, *Reactive oxygen species prime Drosophila haematopoietic progenitors for differentiation.* Nature, 2009. **461**(7263): p. 537.

33. Rizki, T., R.M. Rizki, and E. Grell, *A mutant affecting the crystal cells inDrosophila melanogaster.* Wilhelm Roux's archives of developmental biology, 1980. **188**(2): p. 91-99.
34. Cosson, P. and T. Soldati, *Eat, kill or die: when amoeba meets bacteria.* Current opinion in microbiology, 2008. **11**(3): p. 271-276.
35. Hillyer, J.F. and M.R. Strand, *Mosquito hemocyte-mediated immune responses.* Current opinion in insect science, 2014. **3**: p. 14-21.
36. Shklover, J., F. Levy-Adam, and E. Kurant, *Apoptotic cell clearance in development*, in *Current topics in developmental biology*. 2015, Elsevier. p. 297-334.
37. Serizier, S.B. and K. McCall, *Scrambled eggs: Apoptotic cell clearance by non-professional phagocytes in the Drosophila ovary.* Frontiers in immunology, 2017. **8**: p. 1642.
38. Segawa, K. and S. Nagata, *An apoptotic 'eat me' signal: phosphatidylserine exposure.* Trends in cell biology, 2015. **25**(11): p. 639-650.
39. Kounatidis, I. and P. Ligoxygakis, *Drosophila as a model system to unravel the layers of innate immunity to infection.* Open biology, 2012. **2**(5): p. 120075.
40. Franc, N.C., et al., *Croquemort, a novel Drosophila hemocyte/macrophage receptor that recognizes apoptotic cells.* Immunity, 1996. **4**(5): p. 431-443.
41. Kucharova, S. and R. Farkas, *Hormone nuclear receptors and their ligands: Role in prgrammed cell death.* Endocrine regulations, 2002. **36**(1): p. 37-60.
42. Devitt, A. and C.D. Gregory, *Innate immune mechanisms in the resolution of inflammation*, in *The Resolution of Inflammation*. 2008, Springer. p. 39-56.
43. Williams, M.J., *Regulation of antibacterial and antifungal innate immunity in fruitflies and humans.* Advances in immunology, 2001. **79**: p. 225-260.
44. Awasaki, T., et al., *Essential role of the apoptotic cell engulfment genes draper and ced-6 in programmed axon pruning during Drosophila metamorphosis.* Neuron, 2006. **50**(6): p. 855-867.
45. Ziegenfuss, J.S., et al., *Draper-dependent glial phagocytic activity is mediated by Src and Syk family kinase signalling.* Nature, 2008. **453**(7197): p. 935-939.
46. McPhee, C.K. and E.H. Baehrecke, *The engulfment receptor Draper is required for autophagy during cell death.* Autophagy, 2010. **6**(8): p. 1192-1193.
47. Tung, T.T., et al., *Phosphatidylserine recognition and induction of apoptotic cell clearance by Drosophila engulfment receptor Draper.* The Journal of Biochemistry, 2013. **153**(5): p. 483-491.
48. Williamson, A.P. and R.D. Vale, *Spatial control of Draper receptor signaling initiates apoptotic cell engulfment.* Journal of Cell Biology, 2018. **217**(11): p. 3977-3992.
49. Rämet, M., et al., *Drosophila scavenger receptor CI is a pattern recognition receptor for bacteria.* Immunity, 2001. **15**(6): p. 1027-1038.
50. Yamada, Y., et al., *Scavenger receptor family proteins: roles for atherosclerosis, host defence and disorders of the central nervous system.* Cellular and Molecular Life Sciences CMLS, 1998. **54**(7): p. 628-640.
51. Mukhopadhyay, S. and S. Gordon, *The role of scavenger receptors in pathogen recognition and innate immunity.* Immunobiology, 2004. **209**(1-2): p. 39-49.

52. Pearson, A., A. Lux, and M. Krieger, *Expression cloning of dSR-CI, a class C macrophage-specific scavenger receptor from Drosophila melanogaster.* Proceedings of the National Academy of Sciences, 1995. **92**(9): p. 4056-4060.
53. Philips, J.A., E.J. Rubin, and N. Perrimon, *Drosophila RNAi screen reveals CD36 family member required for mycobacterial infection.* Science, 2005. **309**(5738): p. 1251-1253.
54. Nakanishi, Y. and A. Shiratsuchi, *Mechanisms and roles of phagocytosis in Drosophila and Caenorhabditis elegans.* Invertebrate Survival Journal, 2006. **3**(2): p. 89-96.
55. Rämet, M., et al., *Functional genomic analysis of phagocytosis and identification of a Drosophila receptor for E. coli.* Nature, 2002. **416**(6881): p. 644.
56. Choe, K.-M., H. Lee, and K.V. Anderson, *Drosophila peptidoglycan recognition protein LC (PGRP-LC) acts as a signal-transducing innate immune receptor.* Proceedings of the National Academy of Sciences, 2005. **102**(4): p. 1122-1126.
57. Kaneko, T., et al., *PGRP-LC and PGRP-LE have essential yet distinct functions in the drosophila immune response to monomeric DAP-type peptidoglycan.* Nature immunology, 2006. **7**(7): p. 715-723.
58. Kocks, C., et al., *Eater, a transmembrane protein mediating phagocytosis of bacterial pathogens in Drosophila.* Cell, 2005. **123**(2): p. 335-346.
59. Chung, Y.-S.A. and C. Kocks, *Recognition of pathogenic microbes by the Drosophila phagocytic pattern recognition receptor Eater.* Journal of Biological Chemistry, 2011. **286**(30): p. 26524-26532.
60. Ertürk-Hasdemir, D. and N. Silverman, *Eater: a big bite into phagocytosis.* Cell, 2005. **123**(2): p. 190-192.
61. Bretscher, A.J., et al., *The Nimrod transmembrane receptor Eater is required for hemocyte attachment to the sessile compartment in Drosophila melanogaster.* Biology open, 2015. **4**(3): p. 355-363.
62. Kurucz, É., et al., *Nimrod, a putative phagocytosis receptor with EGF repeats in Drosophila plasmatocytes.* Current Biology, 2007. **17**(7): p. 649-654.
63. Wang, W., et al., *A novel phagocytic receptor (CgNimC) from Pacific oyster Crassostrea gigas with lipopolysaccharide and gram-negative bacteria binding activity.* Fish & shellfish immunology, 2015. **43**(1): p. 103-110.
64. Shiratsuchi, A., et al., *Independent recognition of Staphylococcus aureus by two receptors for phagocytosis in Drosophila.* Journal of Biological Chemistry, 2012. **287**(26): p. 21663-21672.
65. Somogyi, K., et al., *Evolution of genes and repeats in the Nimrod superfamily.* Molecular biology and evolution, 2008. **25**(11): p. 2337-2347.
66. Kurant, E., et al., *Six-microns-under acts upstream of Draper in the glial phagocytosis of apoptotic neurons.* Cell, 2008. **133**(3): p. 498-509.
67. Watson, F.L., et al., *Extensive diversity of Ig-superfamily proteins in the immune system of insects.* Science, 2005. **309**(5742): p. 1874-1878.
68. Lazzaro, B.P., *Natural selection on the Drosophila antimicrobial immune system.* Current opinion in microbiology, 2008. **11**(3): p. 284-289.
69. Hemani, Y. and M. Soller, *Mechanisms of Drosophila Dscam mutually exclusive splicing regulation*. 2012, Portland Press Ltd.

70. Ulvila, J., L.M. VANHA-AHO, and M. RÄMET, *Drosophila phagocytosis–still many unknowns under the surface.* Apmis, 2011. **119**(10): p. 651-662.
71. Mamali, I., et al., *A β integrin subunit regulates bacterial phagocytosis in medfly haemocytes.* Developmental & Comparative Immunology, 2009. **33**(7): p. 858-866.
72. Ulvila, J., et al., *Double-stranded RNA is internalized by scavenger receptor-mediated endocytosis in Drosophila S2 cells.* Journal of Biological Chemistry, 2006. **281**(20): p. 14370-14375.
73. Stroschein-Stevenson, S.L., et al., *Phagocytosis of Candida albicans by RNAi-treated Drosophila S2 cells*, in *Host-Pathogen Interactions*. 2009, Springer. p. 347-358.
74. Cheng, L.W. and D.A. Portnoy, *Drosophila S2 cells: an alternative infection model for Listeria monocytogenes.* Cellular microbiology, 2003. **5**(12): p. 875-885.
75. Stuart, L.M. and R.A. Ezekowitz, *Phagocytosis and comparative innate immunity: learning on the fly.* Nature Reviews Immunology, 2008. **8**(2): p. 131-141.
76. Melcarne, C., B. Lemaitre, and E. Kurant, *Phagocytosis in Drosophila: From molecules and cellular machinery to physiology.* Insect biochemistry and molecular biology, 2019. **109**: p. 1-12.
77. Pech, L. and M. Strand, *Encapsulation of foreign targets by hemocytes of the moth Pseudoplusia includens [Chrysodeixis includens] (Lepidoptera: Noctuidae) involves an RGD-dependent cell adhesion mechanism.* Journal of Insect Physiology (United Kingdom), 1995.
78. Nappi, A.J., et al., *Superoxide anion generation in Drosophila during melanotic encapsulation of parasites.* European journal of cell biology, 1995. **68**(4): p. 450-456.
79. Russo, J., et al., *Insect immunity: early events in the encapsulation process of parasitoid (Leptopilina boulardi) eggs in resistant and susceptible strains of Drosophila.* Parasitology, 1996. **112**(1): p. 135-142.
80. Strand, M.R. and L.L. Pech, *Immunological basis for compatibility in parasitoid-host relationships.* Annual review of entomology, 1995. **40**(1): p. 31-56.
81. Satyavathi, V.V., A. Minz, and J. Nagaraju, *Nodulation: an unexplored cellular defense mechanism in insects.* Cellular Signalling, 2014. **26**(8): p. 1753-1763.
82. Hillyer, J.F., *Insect immunology and hematopoiesis.* Developmental & Comparative Immunology, 2016. **58**: p. 102-118.
83. Miller, J.S., T. Nguyen, and D.W. Stanley-Samuelson, *Eicosanoids mediate insect nodulation responses to bacterial infections.* Proceedings of the National Academy of Sciences, 1994. **91**(26): p. 12418-12422.
84. Trudeau, D., J.O. Washburn, and L.E. Volkman, *Central role of hemocytes in Autographa californica M nucleopolyhedrovirus pathogenesis in Heliothis virescens and Helicoverpa zea.* Journal of Virology, 2001. **75**(2): p. 996-1003.
85. Pech, L.L. and M.R. Strand, *Granular cells are required for encapsulation of foreign targets by insect haemocytes.* Journal of cell science, 1996. **109**(8): p. 2053-2060.
86. Grizanova, E.V., et al., *Maintenance of redox balance by antioxidants in hemolymph of the greater wax moth Galleria mellonella larvae during*

encapsulation response. Archives of insect biochemistry and physiology, 2018. **98**(4): p. e21460.
87. Pereira, T.C., et al., *Recent advances in the use of Galleria mellonella model to study immune responses against human pathogens.* Journal of Fungi, 2018. **4**(4): p. 128.
88. Hoffmann, J.A. and J.-M. Reichhart, *Drosophila innate immunity: an evolutionary perspective.* Nature immunology, 2002. **3**(2): p. 121.
89. Hultmark, D., *Drosophila immunity: paths and patterns.* Current opinion in immunology, 2003. **15**(1): p. 12-19.
90. Yokoi, K., et al., *Antimicrobial peptide gene induction, involvement of Toll and IMD pathways and defense against bacteria in the red flour beetle, Tribolium castaneum.* Results in immunology, 2012. **2**: p. 72-82.
91. De Gregorio, E., et al., *The Toll and Imd pathways are the major regulators of the immune response in Drosophila.* The EMBO journal, 2002. **21**(11): p. 2568-2579.
92. Tanji, T., et al., *Toll and IMD pathways synergistically activate an innate immune response in Drosophila melanogaster.* Molecular and cellular biology, 2007. **27**(12): p. 4578-4588.
93. Mahla, R.S., et al., *Sweeten PAMPs: role of sugar complexed PAMPs in innate immunity and vaccine biology.* Frontiers in immunology, 2013. **4**: p. 248.
94. Govind, S., *Innate immunity in Drosophila: Pathogens and pathways.* Insect science, 2008. **15**(1): p. 29-43.
95. Kurata, S., S. Ariki, and S.-i. Kawabata, *Recognition of pathogens and activation of immune responses in Drosophila and horseshoe crab innate immunity.* Immunobiology, 2006. **211**(4): p. 237-249.
96. Bianchi, M.E., *DAMPs, PAMPs and alarmins: all we need to know about danger.* Journal of leukocyte biology, 2007. **81**(1): p. 1-5.
97. Zindel, J. and P. Kubes, *DAMPs, PAMPs, and LAMPs in immunity and sterile inflammation.* Annual Review of Pathology: Mechanisms of Disease, 2020. **15**: p. 493-518.
98. Royet, J., D. Gupta, and R. Dziarski, *Peptidoglycan recognition proteins: modulators of the microbiome and inflammation.* Nature Reviews Immunology, 2011. **11**(12): p. 837-851.
99. Kang, D., et al., *A peptidoglycan recognition protein in innate immunity conserved from insects to humans.* Proceedings of the National Academy of Sciences, 1998. **95**(17): p. 10078-10082.
100. Valanne, S., J.-H. Wang, and M. Rämet, *The Drosophila toll signaling pathway.* The Journal of Immunology, 2011. **186**(2): p. 649-656.
101. Gobert, V., et al., *Dual activation of the Drosophila toll pathway by two pattern recognition receptors.* Science, 2003. **302**(5653): p. 2126-2130.
102. Michel, T., et al., *Drosophila Toll is activated by Gram-positive bacteria through a circulating peptidoglycan recognition protein.* Nature, 2001. **414**(6865): p. 756-759.
103. Gottar, M., et al., *Dual detection of fungal infections in Drosophila via recognition of glucans and sensing of virulence factors.* Cell, 2006. **127**(7): p. 1425-1437.

104. Buchon, N., et al., *A single modular serine protease integrates signals from pattern-recognition receptors upstream of the Drosophila Toll pathway.* Proceedings of the National Academy of Sciences, 2009. **106**(30): p. 12442-12447.
105. El Chamy, L., et al., *Sensing of'danger signals' and pathogen-associated molecular patterns defines binary signaling pathways' upstream'of Toll.* Nature immunology, 2008. **9**(10): p. 1165.
106. Jang, I.-H., et al., *A Spätzle-processing enzyme required for toll signaling activation in Drosophila innate immunity.* Developmental cell, 2006. **10**(1): p. 45-55.
107. Weber, A.N., et al., *Binding of the Drosophila cytokine Spätzle to Toll is direct and establishes signaling.* Nature immunology, 2003. **4**(8): p. 794.
108. Tauszig-Delamasure, S., et al., *Drosophila MyD88 is required for the response to fungal and Gram-positive bacterial infections.* Nature immunology, 2002. **3**(1): p. 91.
109. Belvin, M.P. and K.V. Anderson, *A conserved signaling pathway: the Drosophila toll-dorsal pathway.* Annual review of cell and developmental biology, 1996. **12**(1): p. 393-416.
110. Horng, T. and R. Medzhitov, *Drosophila MyD88 is an adapter in the Toll signaling pathway.* Proceedings of the National Academy of Sciences, 2001. **98**(22): p. 12654-12658.
111. Issa, N., et al., *The circulating protease Persephone is an immune sensor for microbial proteolytic activities upstream of the Drosophila Toll pathway.* Molecular cell, 2018. **69**(4): p. 539-550. e6.
112. Robertson, A.S., et al., *Characterization of the necrotic protein that regulates the Toll-mediated immune response in Drosophila.* Journal of Biological Chemistry, 2003. **278**(8): p. 6175-6180.
113. Kaneko, T., et al., *Monomeric and polymeric gram-negative peptidoglycan but not purified LPS stimulate the Drosophila IMD pathway.* Immunity, 2004. **20**(5): p. 637-649.
114. Kaneko, T. and N. Silverman, *Bacterial recognition and signalling by the Drosophila IMD pathway.* Cellular microbiology, 2005. **7**(4): p. 461-469.
115. Choe, K.-M., H. Lee, and K.V. Anderson, *Drosophila peptidoglycan recognition protein LC (PGRP-LC) acts as a signal-transducing innate immune receptor.* Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(4): p. 1122-1126.
116. Leulier, F., et al., *Inducible expression of double-stranded RNA reveals a role for dFADD in the regulation of the antibacterial response in Drosophila adults.* Current Biology, 2002. **12**(12): p. 996-1000.
117. Leulier, F., et al., *The Drosophila caspase Dredd is required to resist Gram-negative bacterial infection.* EMBO reports, 2000. **1**(4): p. 353-358.
118. Myllymäki, H., S. Valanne, and M. Rämet, *The Drosophila imd signaling pathway.* The Journal of Immunology, 2014. **192**(8): p. 3455-3462.
119. Leone, P., et al., *Crystal structure of Drosophila PGRP-SD suggests binding to DAP-type but not lysine-type peptidoglycan.* Molecular immunology, 2008. **45**(9): p. 2521-2530.

120. Weber, A.N., et al., *Binding of the Drosophila cytokine Spätzle to Toll is direct and establishes signaling.* Nature immunology, 2003. **4**(8): p. 794-800.
121. Hu, X., et al., *Multimerization and interaction of Toll and Spätzle in Drosophila.* Proceedings of the National Academy of Sciences, 2004. **101**(25): p. 9369-9374.
122. Tauszig-Delamasure, S., et al., *Drosophila MyD88 is required for the response to fungal and Gram-positive bacterial infections.* Nature immunology, 2002. **3**(1): p. 91-97.
123. Nicolas, E., et al., *In Vivo Regulation of the IκB Homologuecactus during the Immune Response ofDrosophila.* Journal of Biological Chemistry, 1998. **273**(17): p. 10463-10469.
124. Ip, Y.T., et al., *Dif, a dorsal-related gene that mediates an immune response in Drosophila.* Cell, 1993. **75**(4): p. 753-763.
125. Hu, S. and X. Yang, *dFADD, a novel death domain-containing adapter protein for theDrosophila caspase DREDD.* Journal of Biological Chemistry, 2000. **275**(40): p. 30761-30764.
126. Stöven, S., et al., *Activation of the Drosophila NF-κB factor Relish by rapid endoproteolytic cleavage.* EMBO reports, 2000. **1**(4): p. 347-352.
127. Stöven, S., et al., *Caspase-mediated processing of the Drosophila NF-κB factor Relish.* Proceedings of the National Academy of Sciences, 2003. **100**(10): p. 5991-5996.
128. Silverman, N., et al., *A Drosophila IκB kinase complex required for Relish cleavage and antibacterial immunity.* Genes & development, 2000. **14**(19): p. 2461-2471.
129. Lemaitre, B. and J. Hoffmann, *The host defense of Drosophila melanogaster.* Annu. Rev. Immunol., 2007. **25**: p. 697-743.
130. Agaisse, H. and N. Perrimon, *The roles of JAK/STAT signaling in Drosophila immune responses.* Immunological reviews, 2004. **198**(1): p. 72-82.
131. Binari, R. and N. Perrimon, *Stripe-specific regulation of pair-rule genes by hopscotch, a putative Jak family tyrosine kinase in Drosophila.* Genes & development, 1994. **8**(3): p. 300-312.
132. Barillas-Mury, C., et al., *Anopheles gambiae Ag-STAT, a new insect member of the STAT family, is activated in response to bacterial infection.* The EMBO journal, 1999. **18**(4): p. 959-967.
133. Myllymäki, H. and M. Rämet, *JAK/STAT pathway in Drosophila immunity.* Scandinavian journal of immunology, 2014. **79**(6): p. 377-385.
134. Morin-Poulard, I., A. Vincent, and M. Crozatier, *The Drosophila JAK-STAT pathway in blood cell formation and immunity.* Jak-Stat, 2013. **2**(3): p. e25700.
135. Xu, J. and S. Cherry, *Viruses and antiviral immunity in Drosophila.* Developmental & Comparative Immunology, 2014. **42**(1): p. 67-84.
136. Hombría, J.C.-G., et al., *Characterisation of Upd2, a Drosophila JAK/STAT pathway ligand.* Developmental biology, 2005. **288**(2): p. 420-433.
137. Wright, V.M., et al., *Differential activities of the Drosophila JAK/STAT pathway ligands Upd, Upd2 and Upd3.* Cellular signalling, 2011. **23**(5): p. 920-927.
138. Brown, S., N. Hu, and J.C.-G. Hombría, *Identification of the first invertebrate interleukin JAK/STAT receptor, the Drosophila gene domeless.* Current Biology, 2001. **11**(21): p. 1700-1705.

139. Ghiglione, C., et al., *The Drosophila cytokine receptor Domeless controls border cell migration and epithelial polarization during oogenesis.* Development, 2002. **129**(23): p. 5437-5447.
140. Luo, H., et al., *The Hopscotch Jak kinase requires the Raf pathway to promote blood cell activation and differentiation in Drosophila.* Molecular Genetics and Genomics, 2002. **267**(1): p. 57-63.
141. Hou, X.S., M.B. Melnick, and N. Perrimon, *Marelle acts downstream of the Drosophila HOP/JAK kinase and encodes a protein similar to the mammalian STATs.* Cell, 1996. **84**(3): p. 411-419.
142. Dostert, C., et al., *The Jak-STAT signaling pathway is required but not sufficient for the antiviral response of drosophila.* Nature immunology, 2005. **6**(9): p. 946-953.
143. Lagueux, M., et al., *Constitutive expression of a complement-like protein in toll and JAK gain-of-function mutants of Drosophila.* Proceedings of the National Academy of Sciences, 2000. **97**(21): p. 11427-11432.
144. Agaisse, H., et al., *Signaling role of hemocytes in Drosophila JAK/STAT-dependent response to septic injury.* Developmental cell, 2003. **5**(3): p. 441-450.
145. Boutros, M., H. Agaisse, and N. Perrimon, *Sequential activation of signaling pathways during innate immune responses in Drosophila.* Developmental cell, 2002. **3**(5): p. 711-722.
146. Ekengren, S., et al., *A humoral stress response in Drosophila.* Current biology, 2001. **11**(9): p. 714-718.
147. Ekengren, S. and D. Hultmark, *A family of Turandot-related genes in the humoral stress response of Drosophila.* Biochemical and biophysical research communications, 2001. **284**(4): p. 998-1003.
148. Akhouayri, I., et al., *Toll-8/Tollo negatively regulates antimicrobial response in the Drosophila respiratory epithelium.* PLoS Pathog, 2011. **7**(10): p. e1002319.
149. Peng, J., P. Zipperlen, and E. Kubli, *Drosophila sex-peptide stimulates female innate immune system after mating via the Toll and Imd pathways.* Current Biology, 2005. **15**(18): p. 1690-1694.
150. Nehme, N.T., et al., *A model of bacterial intestinal infections in Drosophila melanogaster.* PLoS Pathog, 2007. **3**(11): p. e173.
151. Daffre, S., et al., *The lysozyme locus in Drosophila melanogaster: an expanded gene family adapted for expression in the digestive tract.* Molecular and General Genetics MGG, 1994. **242**(2): p. 152-162.
152. Buchon, N., N.A. Broderick, and B. Lemaitre, *Gut homeostasis in a microbial world: insights from Drosophila melanogaster.* Nature Reviews Microbiology, 2013. **11**(9): p. 615-626.
153. Ryu, J.-H., E.-M. Ha, and W.-J. Lee, *Innate immunity and gut–microbe mutualism in Drosophila.* Developmental & Comparative Immunology, 2010. **34**(4): p. 369-376.
154. Ryu, J.-H., et al., *Innate immune homeostasis by the homeobox gene caudal and commensal-gut mutualism in Drosophila.* science, 2008. **319**(5864): p. 777-782.
155. Zaidman-Rémy, A., et al., *The Drosophila amidase PGRP-LB modulates the immune response to bacterial infection.* Immunity, 2006. **24**(4): p. 463-473.

156. Ferrandon, D., et al., *A drosomycin–GFP reporter transgene reveals a local immune response in Drosophila that is not dependent on the Toll pathway.* The EMBO journal, 1998. **17**(5): p. 1217-1227.
157. Önfelt Tingvall, T., E. Roos, and Y. Engström, *The imd gene is required for local Cecropin expression in Drosophila barrier epithelia.* EMBO reports, 2001. **2**(3): p. 239-243.
158. Kylsten, P., et al., *The lysozyme locus in Drosophila melanogaster: different genes are expressed in midgut and salivary glands.* Molecular and General Genetics MGG, 1992. **232**(3): p. 335-343.
159. Huhmark, D., H. Steiner, and T. Rasmuson, *Purification and properities of three inducible bactericidal proteins from hemolymph of immunized pupae of Hyalophora cecmpia.* European Joumal of Biochemistry, 1980. **1**: p. O6.
160. Steiner, H., et al., *Sequence and specificity of two antibacterial proteins involved in insect immunity.* Nature, 1981. **292**(5820): p. 246-248.
161. QU, X.m., et al., *Insect immunity: isolation and structure of cecropins B and D from pupae of the Chinese oak silk moth, Antheraea pernyi.* European journal of biochemistry, 1982. **127**(1): p. 219-224.
162. Boman, H., *Cecropins: Antibacterial peptides from insects and pigs.* Phylogenetic perspectives in immunity: the insect host defense, 1994: p. 3-17.
163. Moore, A.J., et al., *Antimicrobial activity of cecropins.* Journal of Antimicrobial Chemotherapy, 1996. **37**(6): p. 1077-1089.
164. Zhou, X., T. Nguyen, and D.A. Kimbrell, *Identification and characterization of the Cecropin antibacterial protein gene locus in Drosophila virilis.* Journal of molecular evolution, 1997. **44**(3): p. 272-281.
165. Cavallarin, L., D. Andreu, and B. San Segundo, *Cecropin A—derived peptides are potent inhibitors of fungal plant pathogens.* Molecular plant-microbe interactions, 1998. **11**(3): p. 218-227.
166. Delucca, A.J., et al., *Fungicidal activity of cecropin A.* Antimicrobial agents and chemotherapy, 1997. **41**(2): p. 481-483.
167. Andrä, J., O. Berninghausen, and M. Leippe, *Cecropins, antibacterial peptides from insects and mammals, are potently fungicidal against Candida albicans.* Medical microbiology and immunology, 2001. **189**(3): p. 169-173.
168. Lu, D., et al., *Bombyx mori cecropin A has a high antifungal activity to entomopathogenic fungus Beauveria bassiana.* Gene, 2016. **583**(1): p. 29-35.
169. Van Hofsten, P., et al., *Molecular cloning, cDNA sequencing, and chemical synthesis of cecropin B from Hyalophora cecropia.* Proceedings of the National Academy of Sciences, 1985. **82**(8): p. 2240-2243.
170. Hultmark, D., et al., *Insect immunity: isolation and structure of cecropin D and four minor antibacterial components from Cecropia pupae.* European journal of biochemistry, 1982. **127**(1): p. 207-217.
171. Yi, H.-Y., et al., *Insect antimicrobial peptides and their applications.* Applied microbiology and biotechnology, 2014. **98**(13): p. 5807-5822.
172. Hetru, C., L. Troxler, and J.A. Hoffmann, *Drosophila melanogaster antimicrobial defense.* The Journal of infectious diseases, 2003. **187**(Supplement_2): p. S327-S334.

173. Rosetto, M., et al., *Molecular characterization of ceratotoxin C, a novel antibacterial female-specific peptide of the ceratotoxin family from the medfly Ceratitis capitata.* European journal of biochemistry, 1996. **241**(2): p. 330-337.
174. Fehlbaum, P., et al., *Insect immunity. Septic injury of Drosophila induces the synthesis of a potent antifungal peptide with sequence homology to plant antifungal peptides.* Journal of Biological Chemistry, 1994. **269**(52): p. 33159-33163.
175. Simon, A., et al., *Drosomycin-like defensin, a human homologue of Drosophila melanogaster drosomycin with antifungal activity.* Antimicrobial agents and chemotherapy, 2008. **52**(4): p. 1407-1412.
176. Zhang, Z.T. and S.Y. Zhu, *Drosomycin, an essential component of antifungal defence in Drosophila.* Insect molecular biology, 2009. **18**(5): p. 549-556.
177. Fehlbaum, P., et al., *Structure-activity analysis of thanatin, a 21-residue inducible insect defense peptide with sequence homology to frog skin antimicrobial peptides.* Proceedings of the National Academy of Sciences, 1996. **93**(3): p. 1221-1225.
178. LETELLIERII, L., C. HETRU, and J.A. HOFFMANN, *Structure-activity analysis of thanatin, 21-residue inducible insect defense peptide with sequence homology to frog skin antimicrobial peptides.*
179. Bulet, P., et al., *A novel inducible antibacterial peptide of Drosophila carries an O-glycosylated substitution.* Journal of Biological Chemistry, 1993. **268**(20): p. 14893-14897.
180. Cociancich, S., et al., *Novel inducible antibacterial peptides from a hemipteran insect, the sap-sucking bug Pyrrhocoris apterus.* Biochemical Journal, 1994. **300**(2): p. 567-575.
181. Hetru, C., *Antibacterial peptides/polypeptides in the insect host defense: a comparison with vetebrate antibacterial peptides/polypeptides.* Phylogenetic Perspectives in Immunity: The Insect Host Defense, 1994.
182. DIMARCQ, J.L., et al., *Insect immunity: purification and characterization of a family of novel inducible antibacterial proteins from immunized larvae of the dipteran Phormia terranovae and complete amino-acid sequence of the predominant member, diptericin A.* European Journal of Biochemistry, 1988. **171**(1-2): p. 17-22.
183. Hultmark, D., et al., *Insect immunity. Attacins, a family of antibacterial proteins from Hyalophora cecropia.* The EMBO journal, 1983. **2**(4): p. 571-576.
184. Meister, M., B. Lemaitre, and J.A. Hoffmann, *Antimicrobial peptide defense in Drosophila.* Bioessays, 1997. **19**(11): p. 1019-1026.
185. Kylsten, P., C. Samakovlis, and D. Hultmark, *The cecropin locus in Drosophila; a compact gene cluster involved in the response to infection.* The EMBO journal, 1990. **9**(1): p. 217-224.
186. TRYSELIUS, Y., et al., *CecC, a cecropin gene expressed during metamorphosis in Drosophila pupae.* European journal of biochemistry, 1992. **204**(1): p. 395-399.
187. DIMARCQ, J.L., et al., *Characterization and transcriptional profiles of a Drosophila gene encoding an insect defensin: a study in insect immunity.* European Journal of Biochemistry, 1994. **221**(1): p. 201-209.

188. Charlet, M., et al., *Cloning of the gene encoding the antibacterial peptide drosocin involved in Drosophila immunity: expression studies during the immune response.* European journal of biochemistry, 1996. **241**(3): p. 699-706.
189. Levashina, E.A., et al., *Metchnikowin, a novel immune-inducible proline-rich peptide from Drosophila with antibacterial and antifungal properties.* European Journal of Biochemistry, 1995. **233**(2): p. 694-700.
190. Åsling, B., M.S. Dushay, and D. Hultmark, *Identification of early genes in the Drosophila immune response by PCR-based differential display: the Attacin A gene and the evolution of attacin-like proteins.* Insect biochemistry and molecular biology, 1995. **25**(4): p. 511-518.
191. Reichhart, J.-M., et al., *Insect immunity: developmental and inducible activity of the Drosophila diptericin promoter.* The EMBO journal, 1992. **11**(4): p. 1469-1477.
192. Hultmark, D., et al., *Insect immunity. Purification and properties of three inducible bactericidal proteins from hemolymph of immunized pupae of Hyalophora cecropia.* European Journal of Biochemistry, 1980. **106**(1): p. 7-16.
193. Ekengren, S. and D. Hultmark, *Drosophila cecropin as an antifungal agent.* Insect biochemistry and molecular biology, 1999. **29**(11): p. 965-972.
194. Cociancich, S., et al., *Insect defensin, an inducible antibacterial peptide, forms voltage-dependent channels in Micrococcus luteus.* Journal of Biological Chemistry, 1993. **268**(26): p. 19239-19245.
195. Tzou, P., J.-M. Reichhart, and B. Lemaitre, *Constitutive expression of a single antimicrobial peptide can restore wild-type resistance to infection in immunodeficient Drosophila mutants.* Proceedings of the National Academy of Sciences, 2002. **99**(4): p. 2152-2157.
196. Kragol, G., et al., *The antibacterial peptide pyrrhocoricin inhibits the ATPase actions of DnaK and prevents chaperone-assisted protein folding.* Biochemistry, 2001. **40**(10): p. 3016-3026.
197. Cudic, M., et al., *Chemical synthesis, antibacterial activity and conformation of diptericin, an 82-mer peptide originally isolated from insects.* European journal of biochemistry, 1999. **266**(2): p. 549-558.
198. Hedengren, M., K. Borge, and D. Hultmark, *Expression and evolution of the Drosophila attacin/diptericin gene family.* Biochemical and biophysical research communications, 2000. **279**(2): p. 574-81.
199. Uttenweiler-Joseph, S., et al., *Differential display of peptides induced during the immune response of Drosophila: a matrix-assisted laser desorption ionization time-of-flight mass spectrometry study.* Proceedings of the National Academy of Sciences, 1998. **95**(19): p. 11342-11347.
200. Clemmons, A.W., S.A. Lindsay, and S.A. Wasserman, *An effector peptide family required for Drosophila Toll-mediated immunity.* PLoS Pathog, 2015. **11**(4): p. e1004876.
201. Lindsay, S.A., S.J. Lin, and S.A. Wasserman, *Short-Form bomanins mediate humoral immunity in Drosophila.* Journal of innate immunity, 2018. **10**(4): p. 306-314.

202. Imler, J.-L. and P. Bulet, *Antimicrobial peptides in Drosophila: structures, activities and gene regulation*, in *Mechanisms of epithelial defense*. 2005, Karger Publishers. p. 1-21.
203. Hanson, M.A. and B. Lemaitre, *New insights on Drosophila antimicrobial peptide function in host defense and beyond.* Current Opinion in Immunology, 2020. **62**: p. 22-30.
204. Tang, H., et al., *Two proteases defining a melanization cascade in the immune system of Drosophila.* Journal of Biological Chemistry, 2006. **281**(38): p. 28097-28104.
205. Nakhleh, J., L. El Moussawi, and M.A. Osta, *The melanization response in insect immunity*, in *Advances in Insect Physiology*. 2017, Elsevier. p. 83-109.
206. Christensen, B.M., et al., *Melanization immune responses in mosquito vectors.* Trends in parasitology, 2005. **21**(4): p. 192-199.
207. Tang, H., *Regulation and function of the melanization reaction in Drosophila.* Fly, 2009. **3**(1): p. 105-111.
208. Söderhäll, K. and L. Cerenius, *Role of the prophenoloxidase-activating system in invertebrate immunity.* Current opinion in immunology, 1998. **10**(1): p. 23-28.
209. Cerenius, L. and K. Söderhäll, *The prophenoloxidase-activating system in invertebrates.* Immunological reviews, 2004. **198**(1): p. 116-126.
210. Vavricka, C.J., B.M. Christensen, and J. Li, *Melanization in living organisms: a perspective of species evolution.* Protein & cell, 2010. **1**(9): p. 830-841.
211. Sanzhaeva, U., et al., *Dual effect of nitric oxide on phenoloxidase-mediated melanization.* Journal of enzyme inhibition and medicinal chemistry, 2016. **31**(6): p. 1063-1068.
212. Binggeli, O., et al., *Prophenoloxidase activation is required for survival to microbial infections in Drosophila.* PLoS pathogens, 2014. **10**(5).
213. Tang, H., *Regulation and function of the melanization reaction in*. 2009, Drosophila.
214. Binggeli, O., et al., *Prophenoloxidase activation is required for survival to microbial infections in Drosophila.* PLoS pathogens, 2014. **10**(5): p. e1004067.
215. Dudzic, J.P., et al., *Drosophila innate immunity: regional and functional specialization of prophenoloxidases.* BMC Biol, 2015. **13**: p. 81.
216. Nam, H.J., et al., *Genetic evidence of a redox-dependent systemic wound response via Hayan protease-phenoloxidase system in Drosophila.* The EMBO Journal, 2012. **31**(5): p. 1253-1265.
217. De Gregorio, E., et al., *An immune-responsive Serpin regulates the melanization cascade in Drosophila.* Developmental cell, 2002. **3**(4): p. 581-592.
218. Scherfer, C., et al., *Drosophila Serpin-28D regulates hemolymph phenoloxidase activity and adult pigmentation.* Developmental biology, 2008. **323**(2): p. 189-196.
219. Tang, H., et al., *A serpin that regulates immune melanization in the respiratory system of Drosophila.* Developmental cell, 2008. **15**(4): p. 617-626.
220. Kan, H., et al., *Molecular control of phenoloxidase-induced melanin synthesis in an insect.* J Biol Chem, 2008. **283**(37): p. 25316-23.
221. Tang, H., et al., *Two proteases defining a melanization cascade in the immune system of Drosophila.* J Biol Chem, 2006. **281**(38): p. 28097-104.

222. Ahmad, S.T., et al., *Genetic screen identifies serpin5 as a regulator of the toll pathway and CHMP2B toxicity associated with frontotemporal dementia.* Proc Natl Acad Sci U S A, 2009. **106**(29): p. 12168-73.
223. Ligoxygakis, P., et al., *A serpin mutant links Toll activation to melanization in the host defence of Drosophila.* The EMBO journal, 2002. **21**(23): p. 6330-6337.
224. Dudzic, J.P., et al., *More than black and white: complex relationships involving serine proteases regulate the Toll pathway and the melanization response in Drosophila.* bioRxiv, 2018: p. 383257.
225. Yamamoto-Hino, M. and S. Goto, *Spätzle-processing enzyme-independent activation of the Toll pathway in Drosophila innate immunity.* Cell structure and function, 2016: p. 16002.
226. Dudzic, J.P., et al., *More Than Black or White: Melanization and Toll share regulatory serine proteases in Drosophila.* Cell reports, 2019. **27**(4): p. 1050-1061. e3.
227. Di Cera, E., *Serine proteases.* IUBMB life, 2009. **61**(5): p. 510-515.
228. Kanost, M.R. and H. Jiang, *Clip-domain serine proteases as immune factors in insect hemolymph.* Current opinion in insect science, 2015. **11**: p. 47-55.
229. Stroud, R.M., *A family of protein-cutting proteins.* Scientific American, 1974. **231**(1): p. 74-89.
230. Neitzel, J.J., *Enzyme catalysis: the serine proteases.* Nature Education, 2010. **3**(9): p. 21.
231. Page, M.J. and E. Di Cera, *Serine peptidases: classification, structure and function.* Cellular and Molecular Life Sciences, 2008. **65**(7-8): p. 1220-1236.
232. Rawlings, N.D., A.J. Barrett, and A. Bateman, *MEROPS: the peptidase database.* Nucleic acids research, 2009. **38**(suppl_1): p. D227-D233.
233. Gasteiger, E., et al., *ExPASy: the proteomics server for in-depth protein knowledge and analysis.* Nucleic acids research, 2003. **31**(13): p. 3784-3788.
234. Ross, J., et al., *Serine proteases and their homologs in the Drosophila melanogaster genome: an initial analysis of sequence conservation and phylogenetic relationships.* Gene, 2003. **304**: p. 117-131.
235. Ligoxygakis, P., et al., *Activation of Drosophila Toll during fungal infection by a blood serine protease.* Science, 2002. **297**(5578): p. 114-116.
236. Kellenberger, C., et al., *Structure-function analysis of grass clip serine protease involved in Drosophila Toll pathway activation.* Journal of Biological Chemistry, 2011. **286**(14): p. 12300-12307.
237. Kambris, Z., et al., *Drosophila immunity: a large-scale in vivo RNAi screen identifies five serine proteases required for Toll activation.* Current Biology, 2006. **16**(8): p. 808-813.
238. Jang, I.-H., H.-J. Nam, and W.-J. Lee, *CLIP-domain serine proteases in Drosophila innate immunity.* BMB reports, 2008. **41**(2): p. 102-107.
239. Dudzic, J.P., et al., *Drosophila innate immunity: regional and functional specialization of prophenoloxidases.* BMC biology, 2015. **13**(1): p. 81.
240. Castillejo-Lopez, C. and U. Hacker, *The serine protease Sp7 is expressed in blood cells and regulates the melanization reaction in Drosophila.* Biochem Biophys Res Commun, 2005. **338**(2): p. 1075-82.

241. Veillard, F., L. Troxler, and J.-M. Reichhart, *Drosophila melanogaster clip-domain serine proteases: Structure, function and regulation.* Biochimie, 2016. **122**: p. 255-269.
242. Meekins, D.A., M.R. Kanost, and K. Michel. *Serpins in arthropod biology*. in *Seminars in cell & developmental biology*. 2017. Elsevier.
243. Reichhart, J.M., D. Gubb, and V. Leclerc, *The Drosophila serpins: multiple functions in immunity and morphogenesis*, in *Methods in enzymology*. 2011, Elsevier. p. 205-225.
244. Ahmad, S.T., et al., *Genetic screen identifies serpin5 as a regulator of the toll pathway and CHMP2B toxicity associated with frontotemporal dementia.* Proceedings of the National Academy of Sciences, 2009. **106**(29): p. 12168-12173.
245. Levashina, E.A., et al., *Constitutive activation of toll-mediated antifungal defense in serpin-deficient Drosophila.* Science, 1999. **285**(5435): p. 1917-1919.
246. Soukup, S.F., J. Culi, and D. Gubb, *Uptake of the necrotic serpin in Drosophila melanogaster via the lipophorin receptor-1.* PLoS genetics, 2009. **5**(6): p. e1000532.
247. Ameres, S.L. and P.D. Zamore, *Diversifying microRNA sequence and function.* Nature reviews Molecular cell biology, 2013. **14**(8): p. 475-488.
248. Fire, A., et al., *Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans.* nature, 1998. **391**(6669): p. 806.
249. Kennerdell, J.R. and R.W. Carthew, *Use of dsRNA-mediated genetic interference to demonstrate that frizzled and frizzled 2 act in the wingless pathway.* Cell, 1998. **95**(7): p. 1017-1026.
250. Kim, Y.-O., et al., *A functional genomic screen for cardiogenic genes using RNA interference in developing Drosophila embryos.* Proceedings of the National Academy of Sciences, 2004. **101**(1): p. 159-164.
251. Jankovics, F., et al., *Functional analysis of the Drosophila embryonic germ cell transcriptome by RNA interference.* PloS one, 2014. **9**(6): p. e98579.
252. Heigwer, F., F. Port, and M. Boutros, *RNA Interference (RNAi) Screening in Drosophila.* Genetics, 2018. **208**(3): p. 853-874.
253. Mohr, S.E., *RNAi screening in Drosophila cells and in vivo.* Methods, 2014. **68**(1): p. 82-88.
254. Duffy, J.B., *GAL4 system in Drosophila: a fly geneticist's Swiss army knife.* genesis, 2002. **34**(1-2): p. 1-15.
255. Brand, A.H. and N. Perrimon, *Targeted gene expression as a means of altering cell fates and generating dominant phenotypes.* development, 1993. **118**(2): p. 401-415.
256. Nishihara, S., et al., *Approach for functional analysis of glycan using RNA interference.* Glycoconjugate journal, 2004. **21**(1-2): p. 63.
257. May, R.M., *The dimensions of life on earth.* Nature and human society, 2000: p. 30-45.
258. Waterhouse, R.M., et al., *Evolutionary dynamics of immune-related genes and pathways in disease-vector mosquitoes.* science, 2007. **316**(5832): p. 1738-1743.
259. Lounibos, L.P., *Invasions by insect vectors of human disease.* Annual review of entomology, 2002. **47**(1): p. 233-266.

260. Knio, K., et al., *A two-year survey on mosquitoes of Lebanon.* Parasite, 2005. **12**(3): p. 229-235.
261. Zakhia, R., et al., *Experimental transmission of West Nile Virus and Rift Valley Fever Virus by Culex pipiens from Lebanon.* PLOS Neglected Tropical Diseases, 2018. **12**(1): p. e0005983.
262. Haddad, N., et al., *Presence of Aedes albopictus in Lebanon and Syria.* Journal of the American Mosquito Control Association, 2007. **23**(2): p. 226-228.
263. Haddad, N., et al., *Aedes albopictus in Lebanon, a potential risk of arboviruses outbreak.* BMC infectious diseases, 2012. **12**(1): p. 300.
264. Hemingway, J. and I. Bates, *Malaria: past problems and future prospects: After more than a decade of neglect, malaria is finally back on the agenda for both biomedical research and public health politics.* EMBO reports, 2003. **4**(6S): p. S29-S31.
265. Mabaso, M.L., B. Sharp, and C. Lengeler, *Historical review of malarial control in southern African with emphasis on the use of indoor residual house-spraying.* Tropical Medicine & International Health, 2004. **9**(8): p. 846-856.
266. Hemingway, J. and H. Ranson, *Insecticide resistance in insect vectors of human disease.* Annual review of entomology, 2000. **45**(1): p. 371-391.
267. Nauen, R., *Insecticide resistance in disease vectors of public health importance.* Pest Management Science: formerly Pesticide Science, 2007. **63**(7): p. 628-633.
268. Weill, M., et al., *Insecticide resistance in mosquito vectors.* Nature, 2003. **423**(6936): p. 136-137.
269. Herath, P.R., et al., *The detection and characterization of malathion resistance in field populations of Anopheles culicifacies B in Sri Lanka.* Pesticide Biochemistry and Physiology, 1987. **29**(2): p. 157-162.
270. Hemingway, J. and S. Karunaratne, *2008901. Mosquito carboxylesterases: a review of the molecular biology and biochemistry of a major insecticide resistance mechanism.* Medical and veterinary entomology, 1998. **12**(1): p. 39-45.
271. Brogdon, W.G. and A.M. Barber, *Fenitrothion-deltamethrin cross-resistance conferred by esterases in Guatemalan Anopheles albimanus.* Pesticide Biochemistry and Physiology, 1990. **37**(2): p. 130-139.
272. Vatandoost, H., A. McCaffery, and H. Townson, *An electrophysiological investigation of target site insensitivity mechanisms in permethrin-resistant and susceptible strains of Anopheles stephensi.* Transactions of the Royal Society of Tropical Medicine and Hygiene, 1996. **90**: p. 216.
273. Chandre, F., et al., *Status of pyrethroid resistance in Anopheles gambiae sensu lato.* Bulletin of the World Health Organization, 1999. **77**(3): p. 230.
274. Hemingway, J., et al., *Mechanisms of insecticide resistance in Aedes aegypti (L.)(Diptera: Culicidae) from Puerto Rico.* Bulletin of Entomological Research, 1989. **79**(1): p. 123-130.
275. MOURYA, D.T., J. HEMINGWAY, and C.J. LEAKE, *Changes in enzyme titres with age in four geographical strains of Aedes aegypti and their association with insecticide resistance.* Medical and veterinary entomology, 1993. **7**(1): p. 11-16.

276. Karunamoorthi, K. and S. Sabesan, *Insecticide resistance in insect vectors of disease with special reference to mosquitoes: a potential threat to global public health.* 2013.
277. Rose, R.I., *Pesticides and public health: integrated methods of mosquito management.* Emerging infectious diseases, 2001. **7**(1): p. 17.
278. Peterson, R.K., P.A. Macedo, and R.S. Davis, *A human-health risk assessment for West Nile virus and insecticides used in mosquito management.* Environmental Health Perspectives, 2006. **114**(3): p. 366-372.
279. Ansari, M.S., M.A. Moraiet, and S. Ahmad, *Insecticides: impact on the environment and human health*, in *Environmental deterioration and human health*. 2014, Springer. p. 99-123.
280. Nkya, T.E., et al., *Impact of environment on mosquito response to pyrethroid insecticides: facts, evidences and prospects.* Insect biochemistry and molecular biology, 2013. **43**(4): p. 407-416.
281. Iturbe-Ormaetxe, I., T. Walker, and S.L. O'Neill, *Wolbachia and the biological control of mosquito-borne disease.* EMBO reports, 2011. **12**(6): p. 508-518.
282. Walker, T., et al., *The w Mel Wolbachia strain blocks dengue and invades caged Aedes aegypti populations.* Nature, 2011. **476**(7361): p. 450-453.
283. Hoffmann, A.A., et al., *Successful establishment of Wolbachia in Aedes populations to suppress dengue transmission.* Nature, 2011. **476**(7361): p. 454-457.
284. Moreira, L.A., et al., *A Wolbachia symbiont in Aedes aegypti limits infection with dengue, Chikungunya, and Plasmodium.* Cell, 2009. **139**(7): p. 1268-1278.
285. Van den Hurk, A.F., et al., *Impact of Wolbachia on infection with chikungunya and yellow fever viruses in the mosquito vector Aedes aegypti.* PLoS neglected tropical diseases, 2012. **6**(11).
286. Lacey, L., et al., *Insect pathogens as biological control agents: back to the future.* Journal of invertebrate pathology, 2015. **132**: p. 1-41.
287. Khachatourians, G.G., *Production and use of biological pest control agents.* Trends in Biotechnology, 1986. **4**(5): p. 120-124.
288. Ignoffo, C., *Development of a viral insecticide: concept to commercialization.* Experimental Parasitology, 1973. **33**(2): p. 380-406.
289. Hernández, C.S., et al., *Isolation and toxicity of Bacillus thuringiensis from potato-growing areas in Bolivia.* Journal of Invertebrate Pathology, 2005. **88**(1): p. 8-16.
290. Palma, L., et al., *Bacillus thuringiensis toxins: an overview of their biocidal activity.* Toxins, 2014. **6**(12): p. 3296-3325.
291. Jisha, V.N., R.B. Smitha, and S. Benjamin, *An overview on the crystal toxins from Bacillus thuringiensis.* Advances in Microbiology, 2013. **3**(05): p. 462.
292. Promdonkoy, B. and D.J. Ellar, *Investigation of the pore-forming mechanism of a cytolytic δ-endotoxin from Bacillus thuringiensis.* Biochemical Journal, 2003. **374**(1): p. 255-259.
293. Höfte, H. and H. Whiteley, *Insecticidal crystal proteins of Bacillus thuringiensis.* Microbiological reviews, 1989. **53**(2): p. 242-255.
294. Höfte, H. and H. Whiteley, *Insecticidal crystal proteins of Bacillus thuringiensis.* Microbiology and Molecular Biology Reviews, 1989. **53**(2): p. 242-255.

295. Van Frankenhuyzen, K., *Insecticidal activity of Bacillus thuringiensis crystal proteins.* Journal of invertebrate pathology, 2009. **101**(1): p. 1-16.
296. Rubio-Infante, N. and L. Moreno-Fierros, *An overview of the safety and biological effects of Bacillus thuringiensis Cry toxins in mammals.* Journal of Applied Toxicology, 2016. **36**(5): p. 630-648.
297. Ramírez-Lepe, M. and M. Ramírez-Suero, *Biological control of mosquito larvae by Bacillus thuringiensis subsp. israelensis.* Insecticides—Pest Engineering, 2012.
298. Bravo, A., S.S. Gill, and M. Soberón, *Mode of action of Bacillus thuringiensis Cry and Cyt toxins and their potential for insect control.* Toxicon, 2007. **49**(4): p. 423-435.
299. Ambethgar, V., *Potential of entomopathogenic fungi in insecticide resistance management (IRM): A review.* Journal of Biopesticides, 2009. **2**(2): p. 177-193.
300. Lomer, C., et al., *Biological control of locusts and grasshoppers.* Annual review of entomology, 2001. **46**(1): p. 667-702.
301. Meyling, N.V. and J. Eilenberg, *Ecology of the entomopathogenic fungi Beauveria bassiana and Metarhizium anisopliae in temperate agroecosystems: potential for conservation biological control.* Biological control, 2007. **43**(2): p. 145-155.
302. Chase, A., L. Osborne, and V. Ferguson, *Selective isolation of the entomopathogenic fungi Beauveria bassiana and Metarhizium anisopliae from an artificial potting medium.* Florida Entomologist, 1986: p. 285-292.
303. Quesada-Moraga, E., A. Ruiz-García, and C. Santiago-Alvarez, *Laboratory evaluation of entomopathogenic fungi Beauveria bassiana and Metarhizium anisopliae against puparia and adults of Ceratitis capitata (Diptera: Tephritidae).* Journal of Economic Entomology, 2006. **99**(6): p. 1955-1966.
304. Bukhari, T., W. Takken, and C.J. Koenraadt, *Development of Metarhizium anisopliae and Beauveria bassiana formulations for control of malaria mosquito larvae.* Parasites & vectors, 2011. **4**(1): p. 23.
305. Howard, A.F., et al., *Pyrethroid resistance in Anopheles gambiae leads to increased susceptibility to the entomopathogenic fungi Metarhizium anisopliae and Beauveria bassiana.* Malaria Journal, 2010. **9**(1): p. 168.
306. de Paula, A.R., et al., *Susceptibility of adult Aedes aegypti (Diptera: Culicidae) to infection by Metarhizium anisopliae and Beauveria bassiana: prospects for Dengue vector control.* Biocontrol Science and Technology, 2008. **18**(10): p. 1017-1025.
307. Kirkland, B.H., G.S. Westwood, and N.O. Keyhani, *Pathogenicity of entomopathogenic fungi Beauveria bassiana and Metarhizium anisopliae to Ixodidae tick species Dermacentor variabilis, Rhipicephalus sanguineus, and Ixodes scapularis.* Journal of medical entomology, 2004. **41**(4): p. 705-711.
308. Shah, P. and J. Pell, *Entomopathogenic fungi as biological control agents.* Applied microbiology and biotechnology, 2003. **61**(5-6): p. 413-423.
309. Thomas, M.B. and A.F. Read, *Fungal bioinsecticide with a sting.* Nature biotechnology, 2007. **25**(12): p. 1367-1368.
310. McNeil, J., *Fungi for the biological control of insect pests*. 2011.
311. Butt, T.M. and L.G. Copping, *Fungal biological control agents.* Pesticide Outlook, 2000. **11**(5): p. 186-191.

312. Ortiz-Urquiza, A., Z. Luo, and N.O. Keyhani, *Improving mycoinsecticides for insect biological control.* Applied microbiology and biotechnology, 2015. **99**(3): p. 1057-1068.
313. Leger, R.J.S. and C. Wang, *Genetic engineering of fungal biocontrol agents to achieve greater efficacy against insect pests.* Applied Microbiology and Biotechnology, 2010. **85**(4): p. 901-907.
314. Piao, S., et al., *Crystal structure of a clip-domain serine protease and functional roles of the clip domains.* The EMBO Journal, 2005. **24**(24): p. 4404-4414.
315. Irving, P., et al., *A genome-wide analysis of immune responses in Drosophila.* Proceedings of the National Academy of Sciences, 2001. **98**(26): p. 15119-15124.
316. De Gregorio, E., et al., *Genome-wide analysis of the Drosophila immune response by using oligonucleotide microarrays.* Proceedings of the National Academy of Sciences, 2001. **98**(22): p. 12590-12595.
317. El Chamy, L., et al., *Sensing of'danger signals' and pathogen-associated molecular patterns defines binary signaling pathways' upstream'of Toll.* Nature immunology, 2008. **9**(10): p. 1165-1170.
318. Kachlishvili, E., et al., *Trinitrotoluene and mandarin peels selectively affect lignin-modifying enzyme production in white-rot basidiomycetes.* Springerplus, 2016. **5**(1): p. 1-9.
319. ITO, H., M. SUGIURA, and T. MIYAZAKI, *Antitumor polysaccharide fraction from the culture filtrate of Fomes fomentarius.* Chemical and Pharmaceutical Bulletin, 1976. **24**(10): p. 2575-2575.
320. Park, Y.-M., et al., *Anti-inflammatory and anti-nociceptive effects of the methanol extract of Fomes fomentarius.* Biological and Pharmaceutical Bulletin, 2004. **27**(10): p. 1588-1593.
321. Roussel, B., et al., *History of the therapeutic uses of the tinder polypore, Fomes fomentarius (L.: Fr.* Revue d'Histoire de la Pharmacie, 2002. **50**(336): p. 599-614.
322. Zalar, P., et al., *Taxonomy and phylogeny of the xerophilic genus Wallemia (Wallemiomycetes and Wallemiales, cl. et ord. nov.).* Antonie van Leeuwenhoek, 2005. **87**(4): p. 311-328.
323. Pitt, J.I. and A.D. Hocking, *Penicillium and related genera*, in *Fungi and food spoilage*. 1997, Springer. p. 203-338.
324. Tekaia, F. and J.-P. Latgé, *Aspergillus fumigatus: saprophyte or pathogen?* Current opinion in microbiology, 2005. **8**(4): p. 385-392.
325. Foley, K., et al., *The distribution of Aspergillus spp. opportunistic parasites in hives and their pathogenicity to honey bees.* Veterinary Microbiology, 2014. **169**(3-4): p. 203-210.
326. Paterson, R.R.M., *Fungi and fungal toxins as weapons.* Mycological research, 2006. **110**(9): p. 1003-1010.
327. Matskevich, A.A., J. Quintin, and D. Ferrandon, *The Drosophila PRR GNBP3 assembles effector complexes involved in antifungal defenses independently of its Toll-pathway activation function.* European journal of immunology, 2010. **40**(5): p. 1244-1254.
328. Lu, H.-L. and R.S. Leger, *Insect immunity to entomopathogenic fungi*, in *Advances in genetics*. 2016, Elsevier. p. 251-285.

329. Seto, Y. and K. Tamura, *Extensive differences in antifungal immune response in two Drosophila species revealed by comparative transcriptome analysis.* International journal of genomics, 2013. **2013**.
330. George, J., et al., *Malaria mosquitoes attracted by fatal fungus.* PLoS One, 2013. **8**(5): p. e62632.
331. García-Munguía, A.M., et al., *Transmission of Beauveria bassiana from male to female Aedes aegypti mosquitoes.* Parasites & vectors, 2011. **4**(1): p. 1-6.
332. Lecellier, G. and P. Silar, *Rapid methods for nucleic acids extraction from Petri dish-grown mycelia.* Current genetics, 1994. **25**(2): p. 122-123.
333. White, T.J., et al., *Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics.* PCR protocols: a guide to methods and applications, 1990. **18**(1): p. 315-322.
334. NCBI. *Genbank*. 2016; Available from: (<http://www.ncbi.nlm.nih.gov/genbank/>.)