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MicroRNAs with a key role in the *Drosophila melanogaster* immune response to bacteria

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“I decided a while ago… You know what? I am going for art.
I may miss the mark by a lot, but I am going for something special.”

Conan O’Brien, Gettin' Better Podcast

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Resumo em Português

O sistema imunitário é responsável por uma eficiente neutralização de agentes infeciosos e toxinas, que põem em risco o estado de saúde de um indivíduo. Frequentemente, a resposta imune é descrita como tendo duas componentes: imunidade inata e adaptativa. Insetos, tal como *Drosophila melanogaster*, dependem apenas de estratégias de defesa inatas para eliminar patogéneos e possíveis danos que resultam de uma infeção. Mecanismos inatos são ativados após o reconhecimento de padrões moleculares conservados em micróbios, levando a uma resposta coordenada e eficiente. Esta resposta imune apresenta semelhanças com organismos de maior complexidade, sendo *D. melanogaster* o foco de estudo de trabalhos de investigação sobre imunidade inata.

Na natureza, *D. melanogaster* está exposta a um conjunto diverso de patogéneos cuja principal via de infeção é por ingestão oral. Como tal, a primeira linha de defesa no hospedeiro é ao nível dos tecidos epiteliais, que em si são uma barreira física a invasões microbianas. As células do epitélio gastrointestinal têm defesas locais adicionais em resposta a infeções, tais como a produção local de péptidos antimicrobianos (AMPs, do inglês *antimicrobial peptides*) e espécies reativas de oxigénio (ROS, do inglês *reactive oxygen species*). Quando os agentes infeciosos são capazes de subsistir à ação destes mecanismos e infiltrar a cavidade corporal do inseto, induzem a produção sistémica de AMPs no corpo adiposo pelas vias de sinalização Toll e Imd (do inglês *immunodeficency*). Enquanto a via Toll é ativada por reconhecimento imune de fungos e bactérias Gram-positivas, a via Imd é induzida na presença de bactérias Gram-negativas. A coordenação entre a resposta imune local e a sistémica é estabelecida por células imunes especializadas denominadas de hemócitos. Estas células migratórias são importantes vigilantes imunes, contribuindo também para a remoção de micróbios por fagocitose e mecanismos de reparação de tecido.

A sobrevivência do hospedeiro num contexto de infeção é dependente do dano infligido pelo patogéneo nos seus tecidos e função metabólica. Além disso, mecanismos de defesa, como a produção ROS, também têm um impacto prejudicial nos tecidos do hospedeiro. Assim sendo, existem duas estratégias de sobrevivência para os quais mecanismos imunes contribuem. A primeira estratégia consiste na eliminação ou expulsão do agente infecioso, e inclui os chamados mecanismos de resistência. A segunda está relacionada com mecanismos de tolerância que previnem ou reparam o dano tecidual causado pela infeção, sem necessariamente atacar o patogéneo. De modo a manter uma resposta imune adequada, os organismos multicelulares evoluíram no sentido de possuírem reguladores eficientes do balanço entre mecanismos de resistência e tolerância.

Ao nível da expressão génica, a resposta imune pode ser regulada pós-transcricionalmente por pequenas moléculas de RNA não codificantes tais como microRNAs (miRNAs). Estas sequências ligam-se a zonas complementares na região 3’ não traduzida (UTR, do inglês *untraslated region*) de transcritos alvo, inibindo a sua tradução ou promovendo o seu decaimento. Os miRNAs já foram envolvidos em vários processos biológicos de humanos e insetos, incluindo processos de desenvolvimento e metabolismo. Recentemente, miRNAs específicos foram implicados na expressão de péptidos antimicrobianos, mas estudos bioinformáticos sugerem que o potencial de regulação destas sequências abrange outros mecanismos da resposta imune.

Este projeto tem como objetivo principal a identificação e caracterização de miRNAs com um papel importante na resposta imune de *D. melanogaster*. Para tal, investigou-se o impacto de uma infeção sistémica com *Pseudomonas entomophila*, uma bactéria entomopatogénica, em trinta e três linhas deletadas em miRNAs. Resultados prévios sugerem que este é um conjunto de miRNAs que têm um impacto significativo na sobrevivência de *D. melanogaster* após este tipo de infeção.

A primeira abordagem consistiu em validar resultados anteriores, ao replicar um *screen* de sobrevivência dos mutantes de miRNA após infeção com *P. entomophila*. Paralelamente, mediu-se a carga bacteriana no momento da morte (BLUD, do inglês *bacterial load upon death*) para cada linha mutante. O estudo destes dois fenótipos permite identificar miRNAs que têm um impacto no desfecho final da infeção, e, portanto, na resposta imune do hospedeiro. Os nossos resultados mostram que catorze mutantes têm uma sobrevivência alterada após a infeção. Seis linhas mutantes – miR-11, -957, -955, -92a, -278 e -285 – têm um risco de mortalidade mais elevado que linhas controlo, revelando que estes miRNAs são importantes para uma defesa imune eficaz. A acção de oito miRNAs – miR-986, -959/960/961/962, -965, -1000, -966, -137, -2a-21/2a-1/2b-2, e -100/let-7/125 – é prejudicial na infeção com *P. entomophila*, uma vez que os respetivos mutantes apresentam uma maior probabilidade de sobrevivência na sua ausência. Os resultados de BLUD mostram que alguns mutantes que têm a sobrevivência alterada também apresentam diferenças significativas na carga bacteriana quando sucumbem à infeção. Isto indica que mecanismos de resistência ou tolerância estão possivelmente afetados nas linhas mutantes.

A segunda abordagem foi caracterizar os mecanismos pelos quais os miRNAs poderão influenciar a resposta imune. Neste sentido, estudou-se as interações patogéneo-hospedeiro com base na proliferação bacteriana ao longo da infeção. Isto foi feito em cinco linhas mutantes, cada uma com um fenótipo conjunto diferente de sobrevivência e BLUD. Esta análise permite compreender se a sobrevivência está de algum modo relacionada com alterações na resistência ao patogéneo, que é medida indiretamente como o inverso da carga bacteriana. A razão pela qual alguns mutantes não apresentam diferenças na proliferação de *P. entomophila*, sugere que mecanismos de resistência não contribuem para o fenótipo de sobrevivência que observamos em cada mutante. Nestes casos, interpretamos as alterações nos níveis de saúde do hospedeiro como resultado de mecanismos de tolerância, que contribuem para a sobrevivência do organismo sem necessariamente afetar a proliferação bacteriana. A ausência de miR-965 e miR-966 aumenta a probabilidade de sobrevivência do hospedeiro, sem, no entanto, influenciar o crescimento bacteriano. Estes dados sugerem que tanto o miR-965 como o miR-966 são inibidores da tolerância, visto que os mutantes respetivos apesar de terem a mesma quantidade de patogéneo, lidam melhor com o dano que advém da infeção. De um modo semelhante, a regulação do *cluster* miR-100/let-7/125 aparenta ser prejudicial para a tolerância de *D. melanogaster* ao patogéneo. Em fases finais da infeção, os mutantes do miR-11 e miR-955 apresentam uma capacidade diminuída de controlar a proliferação bacteriana e uma mortalidade mais elevada. Isto sugere que ambos os miRNAs são importantes para manter a resistência a *P. entomophila*.

Em suma, este trabalho mostra que a regulação da expressão génica por miRNAs tem um papel impactante nas interações patogéneo-hospedeiro, refletindo-se em fenótipos distintos de sobrevivência. No futuro, será importante continuar a investigar a ação destes miRNAs, especificamente quais são os genes alvo desta regulação, e de que maneira a sua expressão afeta a imunidade de *D. melanogaster*.

Palavras-chave

Drosophila melanogaster; Imunidade: MicroRNAs; Tolerância; Resistência.

Summary

The immunity of *Drosophila melanogaster* is an efficient and multifaceted system, comprised of innate mechanisms that protect the host from pathogens it is naturally exposed to. In order to survive the action of infectious agents, *D. melanogaster* relies on two distinct strategies referred to as host resistance and disease tolerance. Resistance mechanisms consist of killing or expelling an immune elicitor, while tolerance mechanisms deal with the inevitable damage of infection. The adequate balance between the two is achieved by the action of effective regulators of the immune response, such as microRNAs (miRNAs). These small non-coding RNA molecules control gene expression at a post-transcriptional level, inhibiting the expression of target messenger RNAs (mRNAs). Although miRNA regulation is well characterized in biological processes such as development, we are just now starting to unravel their potential impact in *D. melanogaster* immunity. With this project we aimed to identify and characterize miRNAs with a key role in *D. melanogaster* immune response to bacteria. Our approach was to investigate the impact of systemic infection with *Pseudomonas entomophila* in a set of thirty-three miRNA knockout lines. Focusing on post-infection survival, we identified fourteen mutants that had significant differences from control lines. Six knockout lines – miR-11, -957, -955, -92a, -278 and -285 – had a higher mortality rate than control flies, suggesting that these miRNAs are important for the host immune response to *P. entomophila*. In contrast, eight miRNA candidates – miR-986, -959/960/961/962, -965, -1000, -966, -137, -2a-2/2a-1/2b-2, and -100/let-7/125 – appeared to be detrimental for host defence against *P. entomophila*, since the respective miRNA knockout line had a significantly better chance of survival after infection. We decided to further characterize the role of five miRNA candidates, by studying host-pathogen interactions in the respective knockout lines. The absence of miR-965 and miR-966 increased the chances of host survival to infection, without impacting within-host bacterial proliferation. These data suggested that miR-965 and miR-966 are potential inhibitors of host tolerance against *P. entomophila*. In a similar way, the miR-100/let-7/125 cluster may be a potential inhibitor of host disease tolerance. Finally, in later stages of infection, miR-11 and miR-955 mutants showed an impaired ability to control bacterial proliferation, which suggests that both miRNAs are important for immune function maintenance during infection with *P. entomophila*. Our findings show that gene expression regulation by miRNAs can impact host-pathogen interactions, leading to distinct survival phenotypes in *D. melanogaster*.

Key words

Drosophila melanogaster; Immunity; MicroRNAs; Disease Tolerance; Resistance.

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List of abbreviations, acronyms and symbols

% – percentage
°C – Celsius degree
Ago1 – Argonaute 1
AMP – antimicrobial peptide
BLUD – bacterial load upon death
CFU – colony forming unit
CO_2 – dioxide carbon
DAMP – danger associated molecular pattern
DAP – diaminopimelic acid
Dcr-1 – Dicer-1
dDuox – dual oxidase
Dif – Dorsal-related immunity factor
Dredd – death related ced-3/Nedd2-like caspase
E2F1 – E2F transcription factor 1
Ebs – enteroblasts
Exp-5 – Exportin-5
Fadd – Fas-associated death domain
Freq(0) – Frequency of null observations
GNBP – gram-negative binding protein
h – hour
H_2O_2 – hydrogen peroxide
HClO – hypochlorous acid
IKK – I-kappaB kinase
Imd – immune deficiency
IRC – immune responsive catalase
ISC – intestinal stem cell
JNK - JUN N-terminal kinase
K – carrying capacity
KO – knockout
LB – Lysogeny broth
let-7 – lethal-7
ln – natural logarithm
Log_2 – base two logarithm
miRNA – microRNA
ml – milliliter
modSP – modular serine protease
mRNA – messenger RNA
n.s.d. – no significant differences
N_0 – initial inoculum
NADPH – nicotinamide adenine dinucleotide phosphate
NF-κB – factor nuclear kappa B
OD – optical density
PAMP – pathogen-associated molecular pattern
PBS – phosphate-buffered saline
PDL – pathogen load dynamics
PGN – peptidoglycan
PGRP – peptidoglycan receptor protein
PHD – peroxidase-like domain
PO – phenoloxidase
PPO – prophenoloxidase
pre-miRNA – precursor-miRNA
pri-miRNA – primary-miRNA
r – bacterial growth rate
RanGTP – RAs-related nuclear guanosine-5'-triphosphate
Rel – Relish
RISC – RNA-induced silencing complex
RNA – ribonucleic acid
RNA pol II – RNA polymerase II
RNA-seq – RNA-sequencing
ROS – reactive oxygen species
SD – standard deviation
SPE – Spätzle-processing enzyme
Tab2 – TAK1-associated binding protein 2
Tak1 – TGF-β activated kinase 1
TEP – thioester-containing protein
UAS – upstream activation sequence
UTR – untranslated region
wt – wild type
µl – microliter

1. Introduction

In multicellular organisms, the immune system is responsible for the adequate biological response against infectious microbes, toxins and any other entity that may prompt the individual to a state of disease[1]. The immune response is delivered by cells and molecules that act in a collective and coordinated way, allowing for the recognition and elimination or containment of pathological agents whilst essential physiological functions are maintained[2]. The first line of defence against such agents is referred to as innate immunity, specified to confine infection in the early hours after exposure[3]. This response evolved to discriminate infectious microbes from self by utilizing invariant receptors that bind to conserved molecular patterns, common to most pathogens[3]. The ability to recognize all pathogens specifically and to provide enhanced protection against reinfection are unique features of adaptive immunity, which is based on clonal selection of lymphocytes bearing antigen-specific receptors[4]. Contrary to the innate immune response, thought to be common to all metazoans, adaptative immunity is a vertebrate exclusive subsystem. Nevertheless, invertebrates, such as insects, are able to develop an intricate and multifaceted immune response to invading organisms[5].

In later years, *Drosophila melanogaster* has been established as a relevant model organism for the study of innate immunity, given that many of the key signalling pathways and transcriptional regulators are conserved between insects and mammals[6,7].

1.1 The *Drosophila* Immune Response: An Overview

In nature, *D. melanogaster* is exposed to a wide variety of pathogens, including the ones that colonize decaying fruit where it lays its eggs, develops as a larva and forages on as an adult. Therefore, its main infection gateway is thought to be through oral ingestion of microorganisms, although wild flies can also be infected due to an accidental breaching of the cuticle or assisted entrance via an entomophagous nematode[8]. Once inside the host, pathogens will encounter physical barriers at the level of the epithelia that prevent their entrance into the body cavity, local and systemic production of antimicrobial substances and specialized immune cells with phagocytic proprieties[6].

The hallmark of *D. melanogaster* systemic immune response is the synthesis and secretion of antimicrobial peptides (AMPs) that are released into the haemolymph upon infection[6]. This type of antimicrobial activity is mainly attributed to the fat-body, a large size organ that is located inside the insect open circulatory system where it can readily produce and secrete AMPs so that they reach their effective concentrations[6]. This immune response is induced by the recognition of microbial cell wall components by host's PGRPs (peptidoglycan receptor proteins) and GNBPs (Gram-negative binding proteins), which triggers one of two principal signalling cascades: The Toll pathway or the Imd (immune deficiency) pathway[9]. Each pathway is triggered by a specific group of pathogens, ultimately leading to the nuclear translocation of NF-κB-like transcription factors that stimulate the expression of a distinct set of AMPs (Figure 1.1).

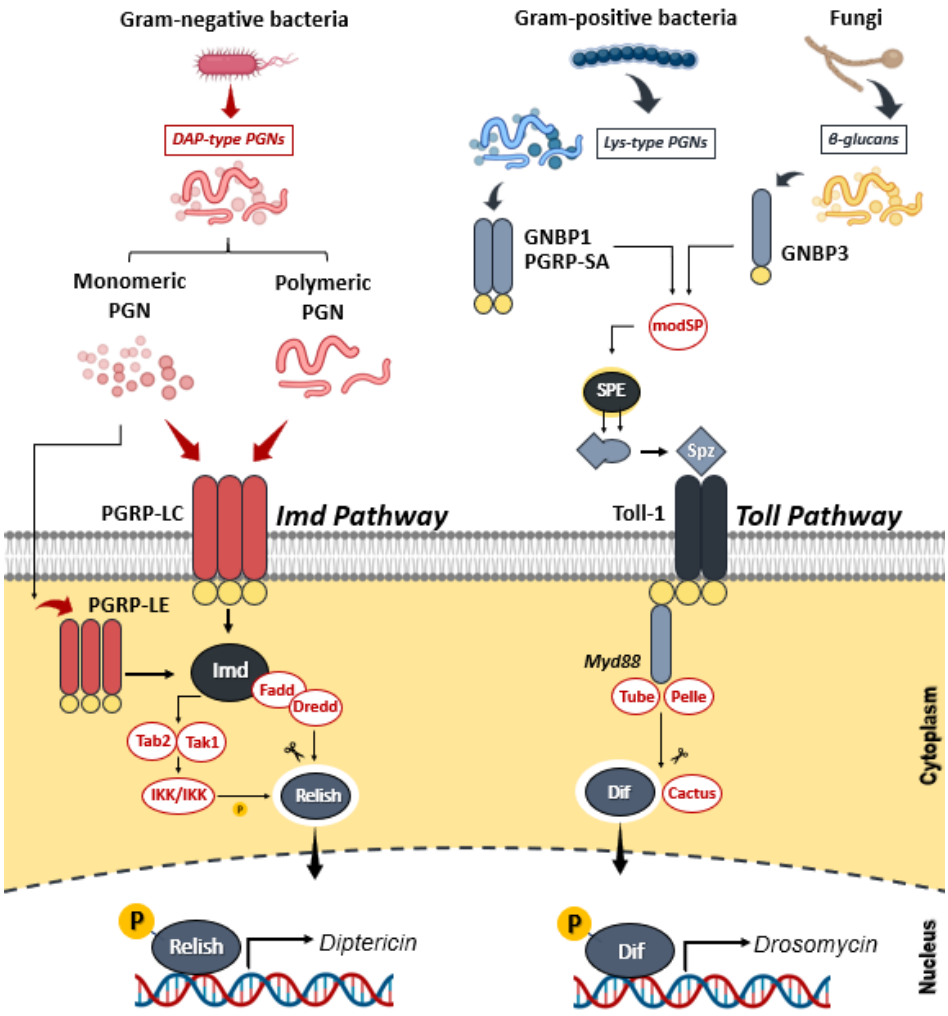

Figure 1.1 **AMP production by activation of the Toll and Imd Pathways.** The Imd pathway is activated upon immune recognition of DAP-type PGNs from Gram-negative and *Bacillus* bacteria proteins by PGRP-LC and -LE, which initiates a signal transduction involving Imd, Fadd and Dredd. Once activated, Dredd cleaves Imd, leading to the recruitment and stimulation of the Tab2/Tak1 complex that phosphorylates the *Drosophila* IKK complex. This complex activates Relish by phosphorylation, leading to cleavage of Relish and subsequent translocation to the nucleus, where it promotes the transcription of AMP genes such as *diptericin*. The Toll pathway is activated by extracellular recognition of Lys-type PGNs of Gram-positive bacteria by the PGRP-SA and GNBP1 complex, and of β-glucans of fungi by GNBP3. Both trigger protease cascades that culminate in the activation of the modular serine protease (modSP), leading to the activation of SPE that cleaves the cytokine Spätzle. Spätzle binds to the Toll transmembrane protein, resulting in the assembly of an intracellular complex, consisting of MyD88, Tube and Pelle. This leads to the phosphorylation and degradation of Cactus, which releases Dif to translocate to the nucleus and activate transcription of AMP genes such as *drosomycin*. The Toll pathway is only inducible in the fat-body, while the Imd pathway is active both in the fat body and in barrier epithelial surfaces. (Adapted from: Buchon et al. (2013)[10])

The Toll pathway is activated upon cleavage of the Spätzle cytokine that in this form binds to the surface membrane receptor Toll-1[9]. This happens in the presence of fungi and Gram-positive bacteria, which are primarily recognized by the β-glucan circulating receptor GNBP3 and the lysine-type peptidoglycan GNBP1 and PGRP-SA receptor complex, respectively. Both ligand–receptor complexes triggers a proteolytic cascade with a function core consisting of several serine proteases (SPs), that culminates in Spätzle cleavage by the activated Spätzle-processing enzyme (SPE)[11]. Upon recognition, Toll-1 will alter its conformation generating an intracellular proteolytic cascade that ultimately leads to the nuclear translocation of transcription factor Dif (Dorsal-related immunity factor), to induce the expression of AMP genes such as *drosomycin*[9,12]. Notably, the Toll pathway is also activated by microbial proteases and endogenous signals released by necrotic cells, which are sensed by the host protease Persephone that stimulates SPE activity[6]. These molecular elicitors are

often referred to as DAMPs (danger associated molecular patterns), and accumulate during cell injury and death, leading to the inflammatory response consecutive to tissue injury and innate immune response[13].

The Imd pathway is triggered by the binding of surface-bound PGRP-LC or cytosolic PGRP-LE to diaminopimelic acid (DAP)-type peptidoglycans, which is a common surface feature of Gram-negative bacteria and *Bacillus* species of Gram-positive bacteria[9]. The intracellular IMD adaptor indirectly interacts with the cytoplasmatic domains of both PGRP-LC and PGRP-LE, functioning as a signalling platform that enables Relish (Rel) activation. This transcription factor migrates to the nucleus, where it promotes the expression of AMPs such as Diptericin, by binding to κB binding sites of target genes[9,12]. Microbial recognition by PGRP-LC also activates the JUN N-terminal kinase (JNK) signalling cascade, which branches from the Imd pathway downstream of TAK1[14]. The JNK pathway is known to regulate the expression of certain immune related genes including that of AMPs and cell remodelling cytoskeletal factors necessary for phagocytosis[14]. Lastly, cytosolic PGRP-LE signalling enables an additional antibacterial autophagic response that is essential for the control of intracellular pathogens such as *Listeria* species[15].

Once secreted from the fat-body into the haemolymph, AMPs kill pathogens via a variety of mechanisms including membrane disruption, interference with bacterial metabolism, and targeting of microbial cytoplasmic components[16]. This mode of action is directly related to the degree of interaction between these effectors and the surface membrane of microbes. In some instances, specific AMPs are exquisitely efficient in the interaction with a pathogen and are the primary contributors to a successful defence. This is the case for Diptericin against *Providencia rettgeri* and of Drosocin against *Enterobacter cloacae*[17]. However, in most cases antimicrobial effectors show a broad-spectrum importance against many pathogens, and the combined action of AMPs is what ultimately allows for an efficient immune response[12].

Another key feature of *D. melanogaster* immunity is the existence of an efficient blood system with specialized cells, haemocytes, that in case of infection enable a tissue coordinated immune response[18–20]. This is possible given that haemocytes are found both in circulating haemolymph and in close proximity to the fat body and many surface epithelia, such as the respiratory and gastrointestinal tracts[21]. In adults, the predominant haemocytes are plasmatocytes that function in the phagocytic removal of microbial pathogens, having also an important role in tissue repair and immune surveillance[18,21]. During infection, their function is mediated by cell surface receptors that recognize pathogen-associated molecular patterns (PAMPs). This is the case of the Nimrod transmembrane receptors, NimC1 and Eater, that recognize both gram-positive and gram-negative bacteria, leading to their rapid engulfment and degradation[22]. Additionally, plasmatocytes express opsonins that bind to microbes and favour their phagocytosis, namely thioester-containing proteins (TEPs) which are transcriptionally induced in case of infection[23,24]. AMP production is also increased in circulating plasmatocytes in response to invading pathogens by activation of the Imd pathway, even though its contribution to haemolymph antimicrobial activity is minimal. Instead, plasmatocytes mainly contribute to the processing of microbial ligands so that they are easily accessible by the major AMP-producing tissues[6,18]. For example, defensin expression in the fat body depends on pathogen degradation in plasmatocytes, which is mediated by the lysosomal protein Psidin[25]. Following injury or infection-related tissue damage, plasmatocytes further enable wound healing mechanisms such as clotting, being the main producers of an important clot component, hemolectin[6]. Clot formation is often followed by melanization, which is the rapid synthesis and deposition of melanin as well as some microbicidal oxidative by-products[6,26]. This reaction is triggered upon cleavage of prophenoloxidase (PPO) to active phenoloxidase (PO). This enzyme

catalyses the oxidation of monophenols and diphenols to orthoquinones, which polymerize into melanin[6]. The formation of the initial clot and its further darkening by melanin deposition are important immune defences, consisting of a secondary barrier to infection that limits haemolymph loss and immobilizes bacteria[6].

The barrier epithelia, including the digestive, respiratory and reproductive tract possess a set of efficient physiological conditions that allow for the control of microbial growth. For example, the *Drosophila* gut and trachea are lined with a chitinous layer that separates epithelial cells from luminal contents. Additionally, lysozymes are constitutively expressed in the gut lumen, where pH levels are low, making it a hostile medium for microbial colonization[10,27]. Local production of reactive oxygen species (ROS) and AMPs are also two defence mechanisms against infection at the barrier epithelia. In the gut, both are basally activated by microbiota and ingested microorganisms, and strongly induced by microbial infection[10]. Synthesis of ROS in the gut is catalysed by the dual oxidase, dDuox, that acts downstream of the Hedgehog signalling pathway. The NADPH domain of dDuox catalyses hydrogen peroxide (H_2O_2) production, that can be further transformed to hypochlorous acid (HClO), an oxidant of higher antimicrobial activity, by the peroxidase-like domain (PHD)[28]. However, excessive ROS production is deleterious for the host, eliciting delamination of enterocytes from the epithelium. This is compensated by the ROS-removing action of the immune responsive catalase (IRC), and also by the induction of epithelial renewal. The latter is a two-step process that starts with the differentiation of quiescent enteroblasts (Ebs), to immediately repair damage, followed by the division of intestinal stem cells (ISCs), to fully replace damaged cells and return to homeostatic conditions[10,29]. Local AMPs are expressed in a diverse set of organs, including the digestive, respiratory and reproductive tracts, as well as Labellar glands and Malpighian tubules. Each one has specific group of AMPs whose synthesis may be induced upon by certain microorganisms[30]. For instance, in the *D. melanogaster* gut, production of Diptericin and Attacin is induced by activation of the Imd pathway upon recognition of DAP-type peptidoglycans of Gram-negative bacteria[10].

An important aspect to consider is that alternate infection routes trigger different types of immune mechanisms leading to distinct physiological responses, which also can be specific to the pathogen[31,32]. Often experimental protocols are based in one of two ways of infecting *D. melanogaster* – oral administration by feeding the host, or septic injury, directly inserting the pathogen into the insect's body cavity. The impact that the route of infection has on host's immune response is well described for a particular entomopathogenic Gram-negative bacterium, *Pseudomonas entomophila*[10,31,32]. Oral and septic injury administration of *P. entomophila* in wild-type *D. melanogaster* display different expression patterns of genes related to the host immune response[32]. Additionally, AMP and phagocytosis genes that are common to both routes of infection present a more rapid induction kinetics after systemic than after oral infection. This happens even if their maximal expression is similar, reflecting a delay in the activation of immune signalling cascades when bacteria are orally transmitted[32]. These differences are due to the fact that microbes inserted into the body cavity bypass the local gut response to pathogens[10,31]. Oral infection is shown to induce a strong epithelial response namely by the modulation of physical barriers and gut epithelium renewal, limiting crossing of the bacteria to the body cavity[10]. On the other hand, administrating *P. entomophila* systemically leads to the rapid canonical action of AMPs and plasmatocytes[31].

A way to study immunity in *D. melanogaster* is to evaluate the progress of infection itself inside the host, specifically how it progresses in terms of host-pathogen interactions. Assessing the dynamic interplay between host and pathogen during infection and how it affects each one highlights the mechanisms that are important for host survival.

1.1.1 Resistance and Tolerance to Microbial Infection

The pathological outcome of infection is set by the degree of homeostatic disruption inflicted upon the host's tissues and metabolic function[2]. Even though infectious agents may be the cause for this dysfunction, host's immune defence mechanisms may also contribute to tissue damage and inflammation that ultimately increase the chances of succumbing to infection[2,10,33]. For instance, local ROS production in the gut, which is critical for the elimination of certain infectious bacteria, can be harmful to the host and to the delamination of enterocytes from the epithelium[10]. *D. melanogaster* possesses a wide range of innate defence strategies that are activated upon infection. The best described mechanisms work in the sense of promoting the elimination of pathogens, by killing or expelling them[33]. This includes the immune killing mechanisms and the signalling pathways that activate them, such as AMP and ROS production and the phagocytic processes that are activated in haemocytes upon microbial recognition. However, some mechanisms focus on dealing with the inevitable damage of infection, either by preventing it or inducing a tissue repair response[33]. One example of this is that of haemocytes in the gut that promote tissue repair by inducing intestinal stem cells proliferation upon infection, which in part offsets the negative effects of ROS in host health[21].

These two ways for surviving infection are referred to as resistance and disease tolerance[33,34]. The impact of these type of mechanisms can be investigated by studying how host-pathogen interactions fluctuate throughout. Resistance mechanisms increase the host's fitness by reducing the number of immune elicitors attacking it[33,34]. In this context, resistance can be measured as the inverse of the within-host microbial concentration – an individual is more resistant than another if it has a lower parasite load[33,34]. Tolerance mechanisms improve the host's fitness by limiting the damage impact of infection, without necessarily affecting parasite numbers[2,33]. Therefore, tolerance can be viewed as the fitness response of a host to an immune elicitor, meaning that when two hosts have the same within-organism microbial load, the most tolerant is the one that has the highest fitness[33]. In this context, fitness can be measured as the host health status, which is reflected on phenotypic readouts such as survival after infection, longevity, fertility and fecundity [33,35,36].

One point of interest is that resistance mechanisms are tightly regulated, which allows the host to mount as great an immune response as needed[33]. For example, activation of the Toll pathway in response to an immune challenge prompts a negative feedback loop that limits the nuclear translocation of Dorsal[37]. The main synthesis of Drosomycin is in this way interrupted, which minimizes the potential health hazard of AMP overproduction and ensures the energetic efficiency of this resistance response[33,37]. Avoiding an overly exuberant resistance to a microbe is a prime example of how tolerance can also be manifested in regulation. Not only because tissue damage is prevented, reducing the pathological impact of infection, but also because energetic costs associated to immune responses require trade-offs with other physiological processes that are energy demanding[33].

In order to maintain the adequate balance between resistance and disease tolerance, *D. melanogaster* has evolved effective regulators of immune processes[38,39]. One example of these are small non-coding RNA molecules that modulate the expression of immune activator genes at a post-transcriptional level[38,40]. These small molecules are potentially very useful for immune regulation. Firstly, there is a known wide variety of regulatory RNAs, some which are highly specific for their respective target genes, making the process less prone to error[38,41]. Secondly, because their action can be reversible in some cases, enabling a quick reactivation of repressed targets[38,41]. In this project, we aim to study how a particular class of small RNA regulators, microRNAs, is able to affect host disease tolerance and resistance to bacterial infection.

1.2 MicroRNAs Biogenesis and Function

MicroRNAs (miRNAs) are small non-coding RNA molecules that silence gene expression at a post-transcriptional level by targeting messenger RNAs (mRNAs) that are complementary to them[42]. The first time a small RNA was described as a key regulator of mRNA translation was in 1993, when the work of two groups brought to light the role of lin-4 in lin-14 expression in *Caenorhabditis elegans*[43,44]. The 22 nucleotide lin-4 was complementary to 3' untranslated region (UTR) in the lin-14 mRNA and, in a manner related to this, was able to inhibit the protein translation. This finding prompted the identification of several other miRNAs not only in invertebrates but also in vertebrates, plants, fungi and viruses[45]. Nowadays, we have a better understanding of how miRNAs can impact diverse cellular processes in a wide variety of organisms by knowing the principles of miRNA biogenesis (Figure 1.2).

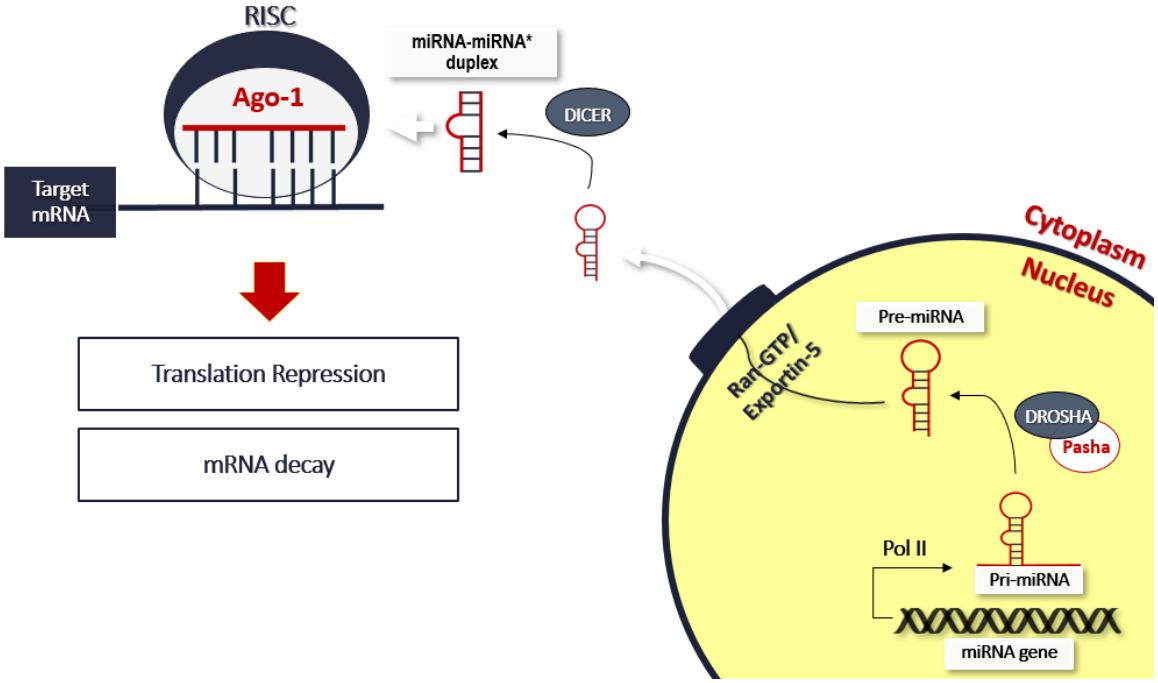

Figure 1.2 **MicroRNA biogenesis in *Drosophila melanogaster***. MicroRNA loci are typically transcribed by RNA polymerase II (Pol II). The transcripts fold into the pri-miRNA hair-loop structure, which is processed in the nucleus by the Drosha/Pasha complex forming the pre-miRNA. When exported to the cytoplasm by the RanGTP/Exp-5 mechanism, pre-miRNA undergoes further processing by Dicer-1 (Dcr-1) to form the miRNA-miRNA* duplex. After sorting, a miRNA guide strand is loaded into the RISC complex in association with Argonaute 1 (Ago1). The miRNA seed sequence binds complementary seed match sites within the 3'UTR of mRNAs, resulting in either translational inhibition or mRNA degradation. (Adapted from: Lucas et al. (2013))[45]

In *Drosophila melanogaster*, mature miRNAs can arise from intergenic or non-intergenic regions of the genome. In the first case, miRNA *loci* contain their own promoters and are expressed as independent mono or polycistronic units, while non-intergenic miRNA are located in intronic sites of a host-gene with which they are typically co-expressed[46]. Polycistronic miRNA transcriptional units, often referred to as miRNA clusters, are comprised of 2-7 gene sequences that have evolved to co-ordinately regulate functionally related genes[47].

Regardless of their genomic location, most miRNA genes are transcribed by the RNA polymerase II originating an approximately 30 base pairs stem structure with a terminal loop and flanking segments, known as primary miRNA (pri-miRNA). Pri-miRNA transcripts are processed by the RNase III enzyme Drosha and its co-factor, Pasha, to yield the approximately 70 nucleotide-long pre-miRNA precursor (pre-miRNA). Non-intergenic miRNAs, such as miRtrons, bypass this process

and are instead originated by host-mRNA splicing[48]. After Drosha-Pasha processing, the pre-miRNA product will be exported through a nuclear pore into the cytoplasm by a well characterized Exp-5/Ran-GTP mediated mechanism[42]. Once in the cytoplasm, the pre-miRNA is further processed by Dicer-1, an RNase III enzyme that excises the stem-loop yielding a mature miRNA-miRNA* duplex. This structure is then unwound, and a single miRNA guide strand is loaded into the RNA-induced silencing complex (RISC) in association with an Argonaute protein, typically Ago1[49]. It is important to mention that although most miRNA are ready for mRNA targeting after they are bound to Ago1, some require additional processing by the 3'-5' exoribonuclease Nibbler[50]. The complete RISC complex is guided by the bound miRNA to inhibit the translation of a target mRNA. Target specificity is determined by complementarity between the miRNA 5' end *seed* sequence (2-8 nucleotides) and mRNA functional binding sites, which are usually located in the 3'UTR, although there are also findings for 5'UTR and ORF[45,51–53]. Perfect or nearly perfect base-pairing between the two sequences will promote mRNA decay, while an imperfect mRNA-miRNA binding will only repress mRNA translation[42].

1.2.1 MicroRNAs in *D. melanogaster* Immune Response to Microbial Infection

In *D. melanogaster*, miRNAs regulate the expression of genes involved in a wide range of physiological and pathological processes including development, cell proliferation and differentiation, apoptosis and immunity[45,46]. Following infection, the differential abundance of host miRNAs is commonly observed in many host-pathogen systems[54]. The relative expression pattern of miRNAs is also thought to vary according to the stage of infection and type of pathogen involved[54]. These perceptions about miRNA regulation during infection are attributed to deep sequencing analysis techniques, such as microarrays and RNA-sequencing (RNA-seq) that enabled a helpful understanding of miRNA expression profiles[54]. However, assigning a particular role to a miRNA in this context becomes difficult not only because different transcripts may be regulated by the same miRNA, but also because different miRNAs may regulate the same mRNA[46]. Nevertheless, there has been new insightful bioinformatic work that complements deep sequencing findings by focusing on the prediction of conserved putative target sites in immune related genes[46,55,56]. For instance, Fullaondo and colleagues (2011) identified seven miRNAs that may be involved in regulating immune related processes based on their putative mRNA targets, including miR-1003, -1016, -12, -283, -304, -31b and -33[46]. Among the targets were three pattern recognition receptors, PGRP-LC, PGRP-SD and PGRP-LE. In addition to those seven miRNAs, over seventy others were predicted to potentially target transcripts of genes involved in major immune pathways in insects, including Toll, Imd and melanization[46].

In order to establish the function of miRNAs in immune defence pathways it is necessary to have an experimental validation of the expression profile and target predictions. This was the approach taken by Li and co-workers (2017), that initially measured miRNA expression levels following an infection with *Escherichia coli* by RNA-seq[57]. Of the several candidates obtained, miR-9a and -981 stood out, since they have binding sites on the 3´UTR of *diptericin* mRNA. The potential repressive action of miR-9a and -981 was validated *in vivo*, by demonstrating that their overexpression led to decreased expression levels of Imd-induced Diptericin[57]. In a recent publication, the authors used a similar approach to validate that miR-317 represses the expression of its predicted target, Drosomycin. In this case, they measured survival output in miR-317 overexpressing mutants and inferred that their increased mortality rates after infection with *Micrococcus luteus* were partially due to the lack of Drosomycin antimicrobial activity[56]. In a work done by Atilano and colleagues (2017), putative target prediction was used to elaborate on experimental data that suggested that 6 miRNA knockout mutants had differential survival after systemic infection with *Candida albicans*[55]. For

example, mutants for miR-193, predicted to bind to the *drosomycin* mRNA, have a higher post-infection survival chance. Given this, the authors speculated that miR-193 mutants might have an enhanced antimicrobial activity[55].

In addition to the mentioned miRNAs, other important publications have highlighted the central role of another three miRNAs in AMP production. Mir-8 was shown to negatively regulate the expression of AMPs such as Drosomycin and Diptericin, in order to maintain their low basal expression in non-infectious conditions[58]. It was also proposed that *let-7*, a ubiquitous conserved miRNA involved in insect development, may target the 3' UTR of *diptericin* mRNA and repress its translation. The authors suggest that this regulation may contribute to set a threshold for antimicrobial activity following immune induction, and thereby avoid overstimulation of the innate immune response[59]. The miRNA cluster 959–964 has been associated with immune response regulation in *D. melanogaster*, since miRNA 959–964 cluster knockout mutants have altered levels of mRNAs involved in immune responses, including *drosomycin* mRNA[60].

Together these works show that some miRNAs have an established role in the *D. melanogaster* immune response. However, it remains widely unknown what are the mechanisms by which these regulatory RNAs modulate host-pathogen interactions. With this project we aim at identifying miRNAs with a role in *D. melanogaster* immunity by studying their impact on host-pathogen interactions.

2. Aims

Gene expression regulation by miRNAs is known to be a fine-tuning process that critically underwrites aspects of development and physiology in a wide range of organisms. In *Drosophila melanogaster*, we are now starting to unravel the activity and function of some miRNAs in specific biological processes, however this has yet to be determined for most of them.

Previous results show that deletions of thirty-three miRNA genes have an impact on *D. melanogaster* survival after infection with *Pseudomonas entomophila*, a Gram-negative entomopathogenic bacterium[61]. We hypothesised that some of these non-coding RNAs might be associated with the vinegar fly's innate immunity. This project was developed with the main objective of further identifying and characterizing miRNAs with an important role in *D. melanogaster* immune response. Our approach was to devise a series of studies in microRNA knockout flies infected with *P. entomophila*, having these specific aims as guidelines:

i. Identify miRNAs with an impact on *D. melanogaster* immunity by analysing the post-infection survival and bacterial load upon death phenotypes in miRNA mutants.
ii. Investigate the mechanisms regulated by each miRNA candidate that underlie the immune response to *P. entomophila*.
    a. Characterize the miRNA role in the resistance and disease tolerance mechanisms of the host by studying pathogen proliferation in miRNA knockout mutants.
    b. Determine if the impact of the miRNA is related to an immune response of a specific tissue.

Although we have developed a strategy that allowed us to investigate if miRNA function is related to a certain tissue (specific aim 2.2), during the time frame of this thesis results could not be generated. Nevertheless, the experimental design of a tissue-specific assay for our miRNA candidates will be elaborated upon in the Conclusion and Future Perspectives section of this dissertation.

3. Materials and Methods

3.1 MiRNA Knockout Fly Lines

In order to investigate the role of miRNAs in *D. melanogaste*r immunity, we performed a series of studies in a group of thirty-three miRNA knockout mutants (Table S1), that have previously shown significant differences in post-infection survival[61]. This set is comprised of lines that either lack expression of a single miRNA, or of a cluster of miRNAs. Each mutant was generated by ends-out homologous recombination resulting in the deletion of the loci region encoding the miRNA hairpin or the eight base pair *seed* sequence. For loci encoding miRNA clusters, all precursors were excised in a single deletion.

The original miRNA mutant library was created by Chen *et al.* (2014) and provided to us by Claudio Alonso Lab (University of Sussex, UK)[62]. In our lab, control (y^1,w^* and w^{1118}) and miRNA deficient lines were crossed with one of these three balancer chromosomes, depending on the deletion site: y^1, w^*, N^1*/FM7c, P{w[+mC]=GAL4-twi.G}108.4, P{UAS-2xEGFP}AX* (X chromosome mutations), w^{1118}*; In(2LR)Gla,* $wg^{Gla\text{-}1}$*/ CyO, P{w[+mC]=GAL4-twi.G}2.2, P{UAS-2xEGFP}AH2.2* (second chromosome mutations) and w^**; ;*$ry^{506}Dr^1$*/ TM3, P{Dfd-GMR-nvYFP}3,* Sb^1 (third chromosome mutations). The control for a certain miRNA knockout line is a wild type line that has been crossed with the same balancer chromosome. Our experiments were performed on homozygous knockout mutants, except for the ones that have a reduced (≤ 24%) or null KO/KO viability. The former, indicated in Table S1, will be maintained heterozygous for this deletion with a balancer chromosome. Lines were compared to equally balanced or non-balanced controls.

3.2 Fly stocks maintenance

Fly stocks were kept under constant temperature (18°C) and fed with a standard cornmeal–agar medium. Crosses and experiments were performed at 25°C, 60-70% humidity and 12:12hr light-dark cycles.

3.3 Bacterial Infection and Survival

In order to validate previous results, we performed a systemic survival screen of miRNA deficient flies with *P. entomophila* that was a gift by Bruno Lemaitre (École Polytechnique Fédérale de Lausanne, SWI). Bacteria stock was kept in glycerol at -80°C. For use, a portion of the stock was streaked in a Petri dish and incubated until visible colonies grew. A single colony was picked and left to grow over night in standard LB medium at 29°C. The concentration of the bacterial suspension was then adjusted to OD_{600}=0,01 (approximately $5x10^6$ cells/ml) with PBS 1x. For **survival assay**, 70 adult males (4-6 days old) from mutant lines and their respective background control were randomly selected, anesthetized with CO_2 and then pricked in the thoracic region with a tungsten needle previously dipped in the *P. entomophila* suspension. As a technical control, a set of 30 males from each line was pricked instead with a needle soaked in PBS 1x. Flies were kept in narrow vials with *ad libitum* access to food in groups of ten. For each line, survival was followed for 50 infected and 20 not-infected individuals for 72 hours after infection. The remaining individuals were used for initial inoculum estimation. The same method was followed for the **within-host pathogen proliferation** assay in live flies. In this case, 250 flies from each line and control were infected in each experiment.

3.4 Bacterial Load Estimations

For within-host bacterial load estimation, collected flies were dipped in a solution of bleach (50%), ethanol (70%) and sterilized miliQ water to limit external contamination. Depending on the assay (see below), we loaded either single or three flies onto a plaque well with 100µl of PBS 1x and a single glass bead and mashed at a frequency of 23/s for 1 minute and 30 seconds. After the homogenate was spun down and properly diluted, samples were plated in a *Petri* dish with standard Agar + LB medium and incubated for 16 hours at 29°C. As a technical control, the homogenate of flies pricked with a PBS solution was plated at the same time. Finally, the number of colony forming units (CFUs) was counted.

For **initial inoculum assessment**, infected flies were collected shortly after recovering from CO_2 anaesthesia and loaded onto a mashing well-plate in pools of three. This was necessary to achieve detection limit. No homogenate dilution was done before plating. To estimate the **bacterial load upon death** (BLUD), infected flies were checked every 30 minutes and newly dead flies (flies on their side or back that did not move) were collected, as described by Duneau *et al.* (2017). This was done in the time period when we observed a large mortality rate across all lines (19 to 24 hours post-infection). Single fly homogenate was diluted up to a factor of 1:1000, and a 5µl droplet of each replicate was plated. The **within-host pathogen proliferation** assay involved the random selection of live flies every two hours after infection with *P. entomophila*, for a period of 24 hours. Selected live flies varied in infection symptoms, going from apparently asymptomatic to moribund individuals (reduced mobility). From 0 up to 10 hours post-infection three flies were homogenised in PBS, and from 12 hours onwards we only homogenised single flies.

3.5 Statistical Analysis

All statistical analyses were done in R studio (version 1.2.5001), and data plotting on GraphPad Prism 6. For **survival analysis** the percent of live flies in each time-point was estimated using the Kaplan-Meyer method. Using a Cox's mixed effects model, we calculated the hazard ratio regression coefficient for each mutant line. In this case, the hazard ratio is an estimate that gives us the risk of death of a given miRNA line in relation to the respective background control. This method assumes that differences between the control and mutant line are constant over time, and only vary according to a fixed factor[63]. We set as a fixed factor the miRNA knockout line and as random factors the replicate vials and date of the experiment. Multiple comparisons between the tested lines and their control were corrected using the Bonferroni or the Holm-Bonferroni tests. For **bacterial load** analysis, we calculated the binary logarithm of samples and tested them for normality using a Shapiro-Wilk test. Comparison of each line with its control was done by using a t-test when normally distributed or a Wilcoxon-Mann-Whitney test when the distribution departed from normal. Multiple comparisons from mutant and control data sets were corrected using the Bonferroni or the Holm-Bonferroni tests. We did this for both initial inoculum and bacterial load upon death estimations. **Within-host pathogen proliferation** analysis involved the fitting of a logistic growth curve to our data set using the *Growthcurver* R package. This software finds the best values for the curve's parameters (K, r and N_0) using the implementation of the nonlinear least-squares Levenberg-Marquardt algorithm[64]. From our experimental measurements only not-null data points (presented viable colony forming units) were considered for curve fitting. Goodness of fit was estimated with an F-test. Curve comparisons are done based on the 95% confidence intervals of the curve parameters, that we calculated from the standard error output provided by the model.

4. Results and Discussion

4.1 MicroRNAs impact the outcome of infection of *Drosophila melanogaster* with *Pseudomonas entomophila*

In order to select miRNA candidates that are interesting to study in the context of *Drosophila melanogaster* immune response, we were determined to investigate how the absence of a miRNA influences the host's survival in a post-infection context. In our lab, a primary screen showed that thirty-three out of eighty-three miRNA knockout lines had significant differences in mortality when infected with the gram-negative bacterium *Pseudomonas entomophila*[61]. This specific bacterium is a known natural killer of *D. melanogaster*, able to trigger systemic humoral defences such as the production of antimicrobial peptides by Imd pathway activation in the fat body[32]. Our first approach was to replicate the primary post-infection survival screen for the thirty-three hits, in order to validate previous results and understand which candidates would be interesting to pursue. Adult miRNA mutant males were infected systemically with *P. entomophila*, as well as adult males from a genetic background control specific for each mutant. The infection method consisted in inserting the inoculum directly into the insect body cavity, thereby bypassing the initial steps of a natural host-pathogen interaction that are present in oral infection. Survival was followed for three days for mutant and control lines, at time-points 0-, 5-, 19-, 24-, 48- and 72-hours post-infection. Hazard ratio of death in infected flies was then calculated for each line in comparison to their respective background control (Table S2; Figure 4.1A). In Figure 4.1A, positive deviations from the control base line (x=0) refer to miRNA mutants that survive less than control, and negative deviations are in reference to mutants that survive more than control.

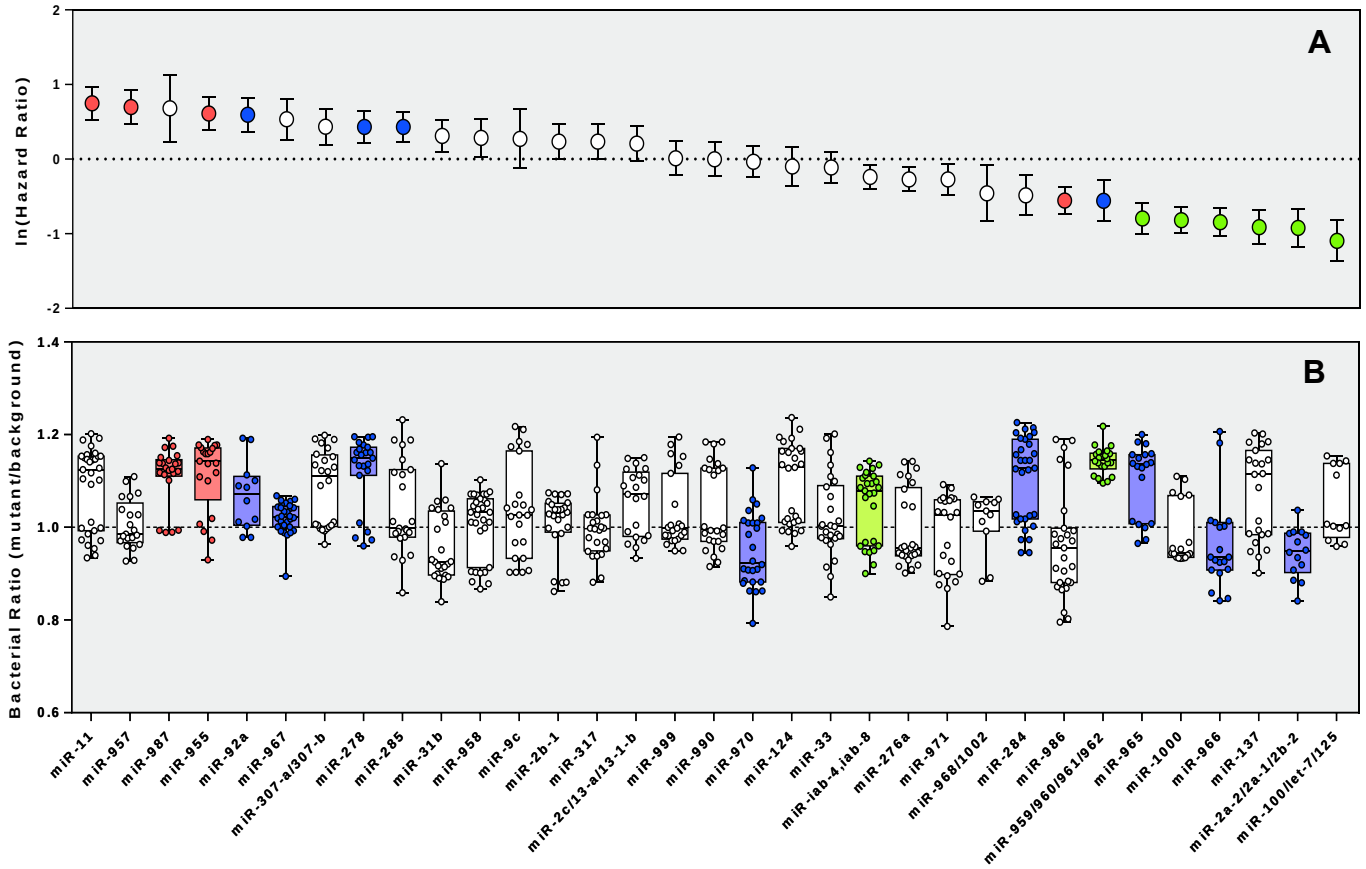

Figure 4.1 **Outcome of *Pseudomonas entomophila* infection in microRNA knockout lines**. **(A)** Survival of infected adult males (4-6 days old) monitored for three days post-infection. For each miRNA mutant, survival is presented as the natural logarithm of hazard ratio in relation to the survival of the respective control line (x=0). Error bars show standard deviation (SD) of the estimated natural logarithm of hazard ratio. **(B)** Bacterial load upon death in infected adult males (4-6 days old) that died between 19- and 24-hours post-infection. Each replicate value, presented as a dot, is normalized for the average BLUD of the background control. Sample size for each candidate is presented in Table S3. All data shown is representative of 2 independent experiments. ● p-value<0,05; ● p-value <0,01; ● p-value <0,001.

Results show that six miRNA knockout lines have a higher mortality compared to control lines, and therefore the respective miRNAs seem to be important for *D. melanogaster* survival to infection with *P. entomophila*. In contrast, the absence of eight miRNAs confers a better chance of survival to *D. melanogaster* in this type of infection. To make sure that mortality differences were not related to the pricking process, a set of flies from each line was pricked with PBS 1x. Survival estimation did not show any significant differences to control (Figure S1). Additionally, we did not detect differences in the initial inoculum between mutant and control lines that could have an effect on the survival phenotypes (Figure S2).

In addition to survival, there was a need for an extra criterion that could give us an idea about the outcome of infection. In this context, we decided to estimate the bacterial load upon death (BLUD) in newly dead flies collected between 19- and 24-hours post-infection. The BLUD corresponds to the maximal bacterial load that can be sustained before an individual dies from the infection, and therefore is a measure of host's susceptibility to the pathogen[65]. In Figure 4.1B, BLUD for each miRNA candidate is presented in relation to the respective background control. Taking into consideration the post-infection survival phenotype with BLUD estimation for each candidate, we were able to analyse the impact of the miRNA candidates in the outcome of infection with *P. entomophila*. There are seven possible outcome of infection phenotype categories in which our miRNA deletion lines can be organized (Table 4.1).

Table 4.1 **Summary of outcome of infection phenotypes for microRNA knockout lines**

		Bacterial Load Upon Death		
		↑	n.s.d	↓
Post-Infection Survival	↑	**Category 1** miR-965 miR-959/960/961/962	**Category 2** miR-986 miR-100/let-7/125 miR-137	**Category 3** miR-966 miR-2a-2/2a-1/2b-2
	n.s.d	**Category 4** miR-987 miR-967 miR-9c miR-iab-4,iab-8 miR-284	Remaining miRNA knockout lines	**Category 5** miR-970
	↓	**Category 6** miR-955 miR-92a miR-278	**Category 7** miR-11 miR-957 miR-285	

↑ - miRNA mutant has a significant higher value than control
↓ - miRNA mutant has a significant lower value than control
n.s.d – no significant differences between mutant and control

This type of categorization allows us to have an assessment of how pathogen-host interactions are mediated in specific lines, even though we do not yet know how the relationship between the survival phenotype and pathogen load varies over time. We based our interpretation of the results on the premise that lower pathogen load at the time of death can mean that flies have higher resistance that impairs bacterial proliferation and/or that flies are less tolerant to the damage implications of infection being able to only sustain low amounts of *P. entomophila* without dying. In the same way, higher BLUD can be a result of a sub-standard resistance response or a higher tolerance to the pathogen itself. Mutant lines in **category 1** survive more than control, whilst having a higher pathogen burden when they die, making it a possibility they are more tolerant to the pathogen, and only higher bacterial burdens eventually lead to death. On the other hand, there are also cases in which the survival is higher and BLUD is lower (**category 3**), the latter suggesting a higher susceptibility to the pathogen. This could be explained if the flies that are surviving infection were either the ones that were able to keep pathogen burden below a certain threshold, or that had an intrinsic higher threshold making them less prone of dying of infection. This also applies to flies that survive better to infection with no significant differences in bacterial load when they die (**category 2**). Significant differences in BLUD can happen without an implication in host's mortality in this type of infection like in mutant flies of **category 4** and **5**. However, differences in BLUD for the same surviving chance suggest that mutant flies diverge from control lines in tolerance: lines that have higher BLUD are less susceptible to bacteria, and the lower BLUD ones are more. In **category 6**, lines present a lower susceptibility to bacteria that does not translate into a higher chance of survival. We hypothesize that the impairment of resistance is substantial in these lines, leading to higher amounts of bacteria. And even though flies are less susceptible to bacteria, it is not enough to overcome this immune response deficiency. Finally, we do not have evidence that susceptibility to bacteria has an important role in the lower survival of **category 7** flies, given that BLUD is not significantly different from control.

At this stage in our work, we were determined to investigate the progression of infection itself so we could better understand the outcome results, which from a logistical standpoint could only be done for a select group of miRNA candidates. Since *D. melanogaster* immune response efficiency is ultimately defined by the ability to survive infection, we thought it would be more interesting to only choose candidates that had an impact on host's post-infection survival. We decided to continue our studies in a single candidate from each phenotypical outcome of infection category that met this criterion, and, if possible, choose the one that had the most robust differences in post-infection survival. The final group of candidates includes miR-965, miR-100/let-7/125, miR-966, miR-955 and miR-11.

4.2 Within-host pathogen proliferation in *Drosophila melanogaster* microRNA mutants

Outcome of infection phenotypes revealed that some miRNAs have a role in the dynamic interplay between host and pathogen, and therefore we wanted to understand the underlying mechanisms by which miRNAs impact the *D. melanogaster* immune response. In this regard, we decided to characterize the role of miRNAs in the two possible strategies for a host to survive infection: resistance and disease tolerance. For this reason, we investigated host-pathogen interactions in miRNA knockout lines by studying bacterial proliferation within the host. This approach allowed us to understand how efficient miRNA mutants are at eliminating bacteria, and if they have an impaired host resistance that could explained the outcome of infection phenotype. In situations in which mutant lines showed no differences in controlling bacterial growth relative to the background line, we assumed that tolerance mechanisms may be the cause for the survival phenotype we observed.

For the final five miRNA candidates, we estimated host pathogen load in both miRNA knockout and control lines infected systemically with *P. entomophila*. This was done for the first twenty-four hours of infection, so that results could be taken in consideration with the first survival slope across lines (Figure S3) and BLUD estimation. In Figure 4.2 to Figure 4.6A-B within-host bacterial proliferation is shown for each miRNA mutant and background control pair, as well as a logistic growth curve that was fitted to our experimental points, excluding null observations. The later refer to flies that did not have viable CFUs in all three replicates that were sampled. Fitting allowed for the estimation of curves parameters including the initial inoculum with which flies were infected (N_0), the within-host bacterial growth rate (r) and the carrying capacity (K). Control and mutant proliferation curves were compared based on the 95% confidence intervals of the parameters (Figure 4.2 to Figure 4.6C) (Figure S4). For lines that have a difference in bacterial proliferation compared to control, significant variations in either the bacterial growth rate or the maximum carrying capacity are expected. Additionally, for the curves to be comparable they must not differ significantly in the initial inoculum, which assures that possible variations in bacterial proliferation are only related to the host itself. Pathogen proliferation analysis would not be complete without the frequency of zeros in each time-point that is also presented in Figure 4.2 to Figure 4.6 A-B. Null observations can either mean that the host was able to clean out the bacteria entirely, or that the pricking process itself was not efficient. Currently, we do not have a method that allows us to distinguish between these two possibilities, but some alternatives will be considered in the Conclusion and Future Perspectives section of this dissertation. Lastly, bacterial proliferation curves are presented in parallel with the previously estimated survival curves (Figure 4.2 to Figure 4.6A-B) and BLUD (Figure 4.2 to Figure 4.6D) for each line.

The miR-100/let-7/125 mutant line has the best surviving chance of all knockout lines after infection with *P. entomophila* (Figure 4.1A). In the first twenty-four hours of infection, we already observe approximately 25% difference in survival compared to the background control line (Figure 4.2A-B). Analysis of within-host pathogen proliferation curves shows that there are no significant differences in initial inoculum, carrying capacity or growth rate for the first 24 hours of infection (Figure 4.2A-B; Figure S4). This suggests that miR-100/let-7/125 mutants are as efficient as their respective background at controlling bacterial growth, which is also supported by BLUD results (Figure 4.2 D). Therefore, we can hypothesize that increased tolerance has an important role in this mutant´s survival. However, frequency of null observations seems to be higher in miR-100/let-7/125 mutants than in control flies between 12- and 16-hours post-infection, and we cannot rule out the possibility that this apparent clearance can be on the basis of the differences in survival that we observe from 19 hours post-infection onwards.

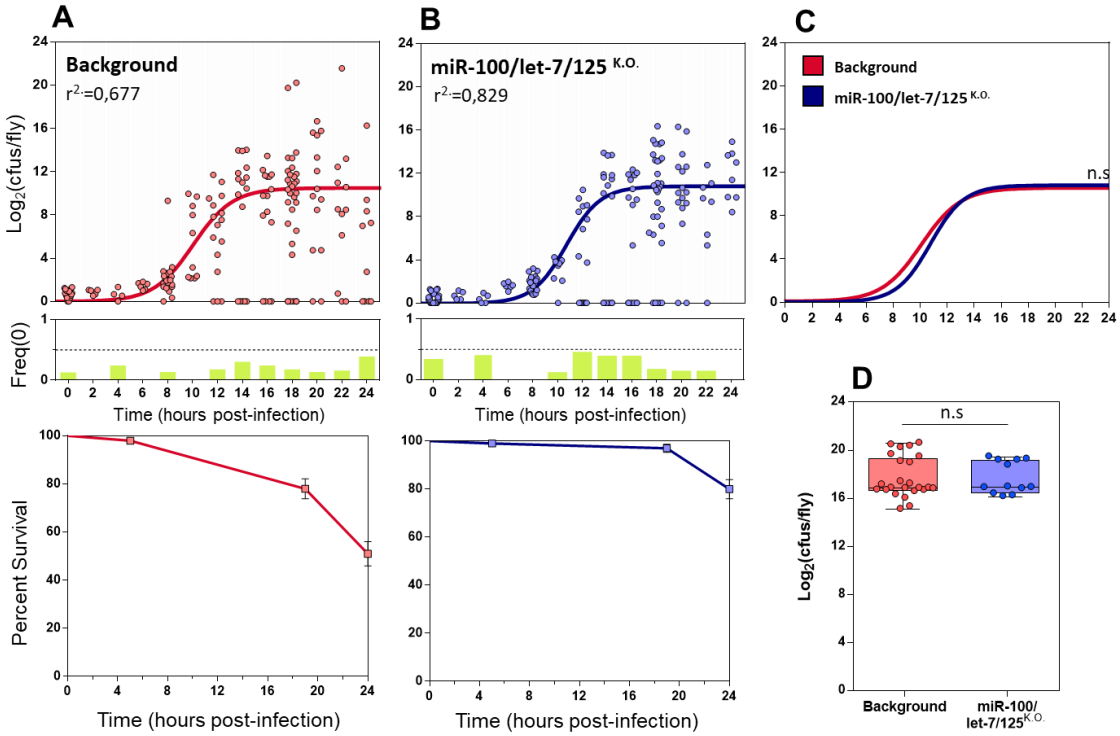

Figure 4.2 **Pathogen load dynamics for the miR-100/let-7/125 knockout line. (A) (B)** Pathogen proliferation and null observation frequency in control and miR-100/let-7/125 knockout live flies 24 hours post-infection with *P.entomophila* (OD_{600}=0,01). Goodness of fit is presented by r^2. For each line, the correspondent survival curve for the first 24 hours of infection is presented below. Error bars show standard error for the mean. **(C)** Pathogen proliferation curves comparison. Significant differences between curves were estimated based on the 95% confidence intervals of the curves' parameters (Figure S4) **(D)** BLUD of miR-100/let-7/125 knockout and control lines. All data shown is representative of 2 independent experiments n.s – not significant.

miR-965 mutants also survive more than the respective background control flies. Although survival rates are nott distinguishable at 19-hours after infection, differences between mutant and control lines become evident at 19 to 24 hours post-infection (Figure 4.3A-B; Figure S3). In Figure 4.3C, pathogen proliferation curves comparison seems to indicate that the miR-965 line tends to control better pathogen proliferation, however statistical analysis of curve parameters does not support differences in this regard (Figure S4). Likewise, we do not have a distinct difference in the frequency of null observations (Figure 4.3A-B). As the survival phenotypes do not seem to be related to how efficiently mutant flies clear out the pathogen, we hypothesize that survival in miR-965 mutants is related to tolerance mechanisms that allow the host to better cope with the damages that come with infection. This is also supported by BLUD results (Figure 4.3D), since mutants can sustain a higher bacterial load before they die.

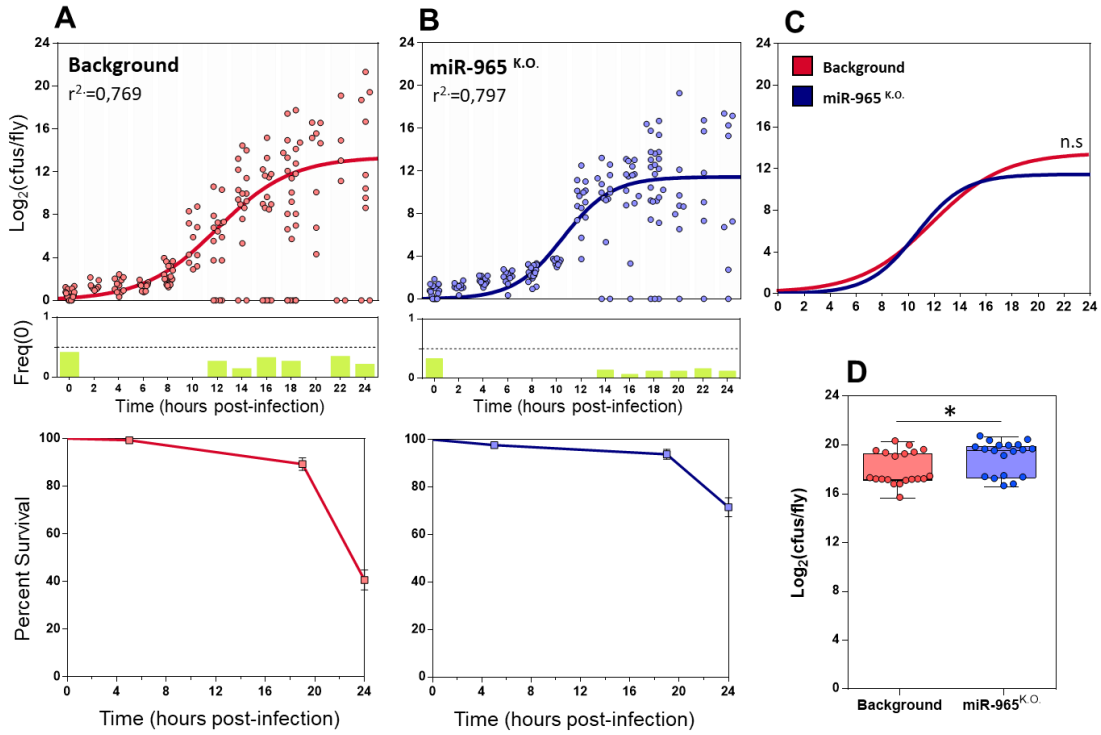

Figure 4.3 **Pathogen load dynamics for the miR-965 knockout line. (A) (B)** Pathogen proliferation and null observation frequency in control and miR-965 knockout live flies 24 hours post-infection with *P.entomophila* (OD_{600}=0,01). Goodness of fit is presented by r^2. For each line, the correspondent survival curve for the first 24 hours of infection is presented below. Error bars show standard error for the mean. **(C)** Pathogen proliferation curves comparison. Significant differences between curves were estimated based on the 95% confidence intervals of the curves' parameters (Figure S4) **(D)** BLUD of miR-965 knockout and control lines (*p-value=0,013). All data shown is representative of 2 independent experiments n.s – not significant.

Deletion of miR-966 seems to be beneficial for host survival, particularly in the time period between 5- and 19-hours post-infection, where we observe the biggest difference in the survival rate of miR-966 knockout compared to control flies (Figure S3). This phenotype does not seem to be related to differences in resistance to the pathogen, given that pathogen proliferation curves are not significantly different in either growth rate or carrying capacity (Figure 4.4A-C; Figure S4). Additionally, frequency of zeros results does not suggest that there is a difference in clearance of the pathogen between lines that could explain the survival rates in early stages of infection (Figure 4.4 A-B). Taking all these results into account, it is possible that the mechanisms that underlie host survival in early stages of infection in miR-966 mutants are related to tolerance to the pathogen instead of resistance mechanisms. However, in later stages of infection (19-24 hours) mutant flies sustain a lower bacterial load before dying (Figure 4.4 D), which suggests that they are more susceptible to the pathogen in this time period.

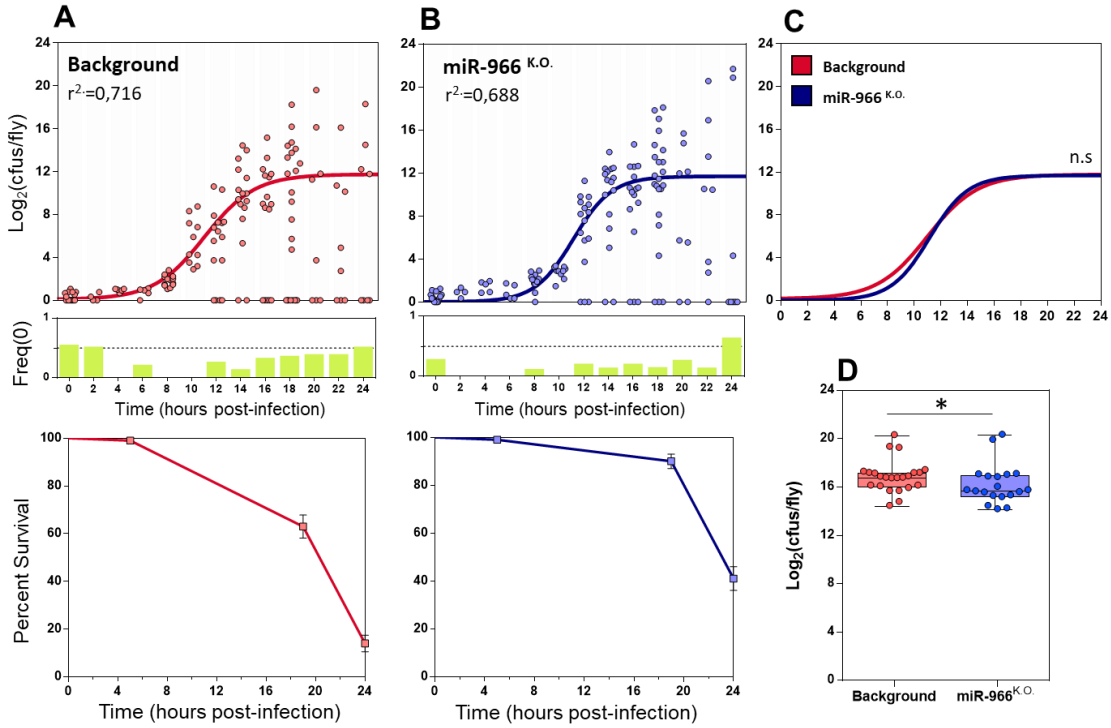

Figure 4.4 **Pathogen load dynamics for the miR-966 knockout line. (A) (B)** Pathogen proliferation and null observation frequency in control and miR-966 knockout live flies 24 hours post-infection with *P.entomophila* (OD_{600}=0,01). Goodness of fit is presented by r^2. For each line, the correspondent survival curve for the first 24 hours of infection is presented below. Error bars show standard error for the mean. **(C)** Pathogen proliferation curves comparison. Significant differences between curves were estimated based on the 95% confidence intervals of the curves' parameters (Figure S4) **(D)** BLUD of miR-966 knockout and control lines (*p-value=0,043). All data shown is representative of 2 independent experiments n.s – not significant.

Both miR-11 and miR-955 mutants survive less than the control line, having less than 10% of the population alive after 24 hours of infection (Figure 4.5 and Figure 4.6A-B). They differ in BLUD tendencies, with miR-955 mutants sustaining higher bacterial loads before dying in comparison with control (Figure 4.5 and Figure 4.6D). Analysis of within-host pathogen proliferation curves indicates that both these mutant lines present a significantly higher carrying capacity than each respective control, although there are no significant differences in growth rate (Figure 4.5 and Figure 4.6A-C; Figure S4). In both cases, there is also a shifting point in which the mutant line changes from having lower levels of pathogen load to having higher levels than control (Figure 4.5 and Figure 4.6C). The shift occurs earlier for the miR-11 knockout line, that also reaches higher levels of carrying capacity. This data suggests than miR-11 and miR-955 mutants have a higher resistance than control in earlier stages of infection, that is compromised later on during the course of infection. However, it remains to be determined if resistance mechanisms do, in some way, contribute to the high mortality rates we observe for both knockout lines.

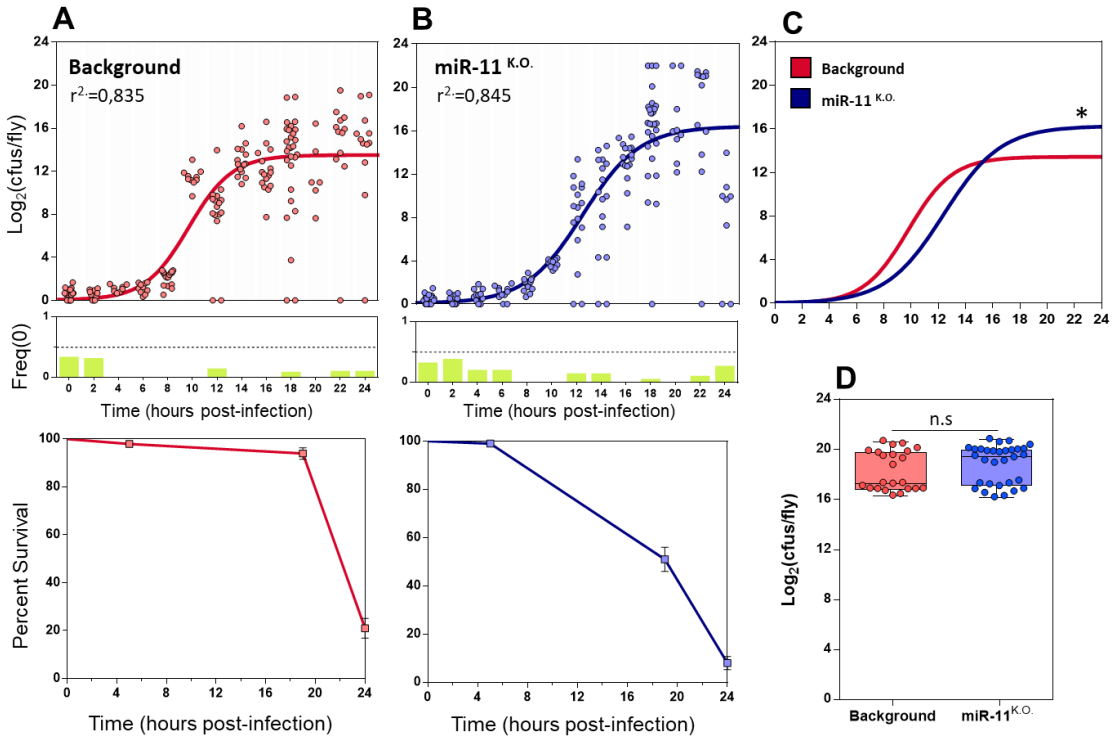

Figure 4.5 **Pathogen load dynamics for the miR-11 knockout line. (A) (B)** Pathogen proliferation and null observation frequency in control and miR-11 knockout live flies 24 hours post-infection with *P.entomophila* (OD_{600}=0,01). Goodness of fit is presented by r^2. For each line, the correspondent survival curve for the first 24 hours of infection is presented below. Error bars show standard error for the mean. **(C)** Pathogen proliferation curves comparison. Significant differences between curves were estimated based on the 95% confidence intervals of the curves' parameters (Figure S4) **(D)** BLUD of miR-11 knockout and control lines. All data shown is representative of 2 independent experiments n.s – not significant.

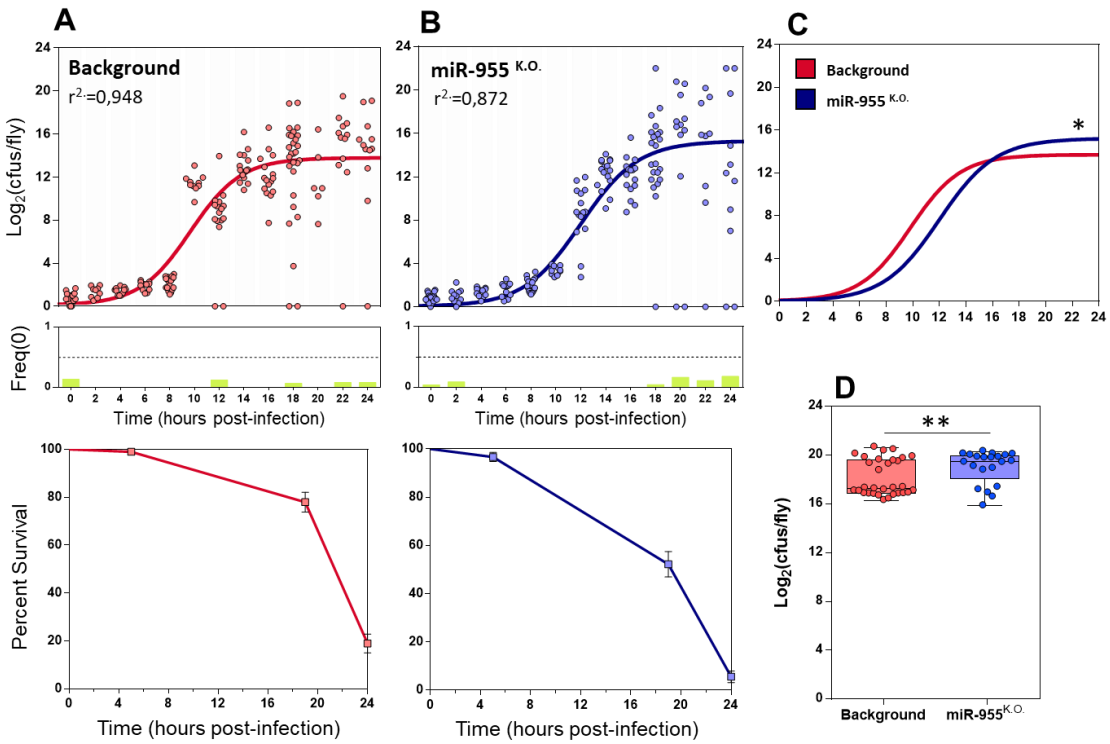

Figure 4.6 **Pathogen load dynamics for the miR-955 knockout line. (A) (B)** Pathogen proliferation and null observation frequency in control and miR-955 knockout live flies 24 hours post-infection with *P. entomophila* (OD_{600}=0,01). Goodness of fit is presented by r^2. For each line, the correspondent survival curve for the first 24 hours of infection is presented below. Error bars show standard error for the mean. **(C)** Pathogen proliferation curves comparison. Significant differences between curves were estimated based on the 95% confidence intervals of the curves' parameters (Figure S4) **(D)** BLUD of miR-955 knockout and control lines (**p-value=0,002). All data shown is representative of 2 independent experiments n.s – not significant.

5. Conclusion and Future Perspectives

The immune system is common to all metazoans, enabling an adequate biological response against any entity that may prompt the individual to a state of disease[1]. *D. melanogaster* possesses an efficient and multifaceted set of innate immune mechanisms to defend itself from pathogens that it is exposed to. These mechanisms contribute either for host resistance, allowing for the elimination of infectious agents, or host disease tolerance, by preventing or repairing infection-related damage[2,33]. As in most biological processes, gene expression regulation critically underwrites the immune response to infectious bacteria, contributing for an adequate balance between host resistance and tolerance. One of the established fine-tuning regulators of gene expression are miRNAs, that silence gene expression at a post-transcriptional level[45]. With the support of sequencing and bioinformatic analyses, it has been proposed that many miRNAs are involved in the expression of genes associated with *D. melanogaster* immunity[54]. In recent years, insightful *in vivo* and *in vitro* studies have demonstrated the mechanisms by which some miRNAs regulate the immune response, specifically how some impact the induction of AMP production[58–60]. However, the impact of miRNAs in host-pathogen interactions seems to go beyond the AMP expression, potentially affecting other immune defence mechanisms as well as physiological processes that contribute to host tolerance.

We developed this project with the objective of identifying and characterizing miRNAs with a role in *D. melanogaster* immune response to bacteria. The experimental approach was to study the impact of the absence of specific miRNAs and miRNA clusters in the immune response of *D. melanogaster*. Accordingly, our studies were conducted in a set of thirty-three miRNA knockout lines, that were subject to an immune challenge, systemic infection with *P. entomophila*. These mutants were the focus of our work since previous work by Carvalho et. al (2016) suggested that they present a distinct survival from their background control after being infected with *P. entomophila*[61]. This indicated a potential role for these miRNAs in immune mechanisms, given that they impacted the ultimate outcome of infection. Furthermore, *P. entomophila* is an interesting bacterium to study in this context since it is a natural pathogen of *D. melanogaster*, able to trigger Imd activation after systemic infection. This infection route is associated with high virulence and mortality levels at early stages of infection, even if *P. entomophila* is administered at low doses[26,32]. Therefore, we chose to study the host's immune response to this specific bacterium only for 72 hours after infection, with an increased detail for the first 24 hours.

Our first approach was to analyse the post-infection survival and BLUD phenotypes in miRNA mutants, therefore validating previous survival results and assessing host susceptibility to the pathogen[65]. Our results show that of the thirty-three miRNA knockout lines, six mutants – miR-11, -957, -955, -92a, -278 and -285 – have a higher mortality rate compared to control lines, suggesting that these miRNAs are important for the host immune response to *P. entomophila*. In contrast, eight miRNA candidates – miR-986, -959/960/961/962, -965, -1000, -966, -137, -2a-2/2a-1/2b-2, and -100/let-7/125 – appear to be detrimental for host defence against *P. entomophila*, since the respective miRNA knockout lines present a significantly better chance of survival after infection than control. Notably, nineteen mutants that in previous work showed significant differences in post-infection survival no longer do. However, it's important to have in consideration that the previous survival screen was conducted for 6 days after infection, while ours was only for 3 days. Knowing that hazard ratios are calculated based on a regression coefficient of a given survival curve, this direct comparison cannot be done unless survival estimation was done in the same time interval for both screens. BLUD analysis revealed twelve miRNAs – miR-987, -955, - 92a, -967, -278, -970, -iab-4,iab-8, -284, -959/960/961/962, -965, -966 and 2a-2/2a-1/2b-2 – that affected the pathogen burden of flies at the time of death. Considering the post-infection survival phenotype and BLUD estimation

for each candidate, there is no direct correlation that implicates one as a by-product of the other. Even though a higher pathogen burden can be associated with an enhanced virulence that ultimately impacts host survival, what ultimately determines if a host survives or succumbs to infection is the degree of damage inflicted on its parenchymal tissues[2]. Other aspects can also contribute for variations in host survival, including host tolerance to *P. entomophila*, that does not necessarily manifest in bacterial burden changes[33]. Nevertheless, these primary results highlighted the existence of seven phenotypic categories in which our mutants could be subdivided in. We chose to investigate the role of five miRNA candidates – miR-965, -100/let-7/125, -966, -955 and -11 – each one with a different outcome of infection phenotype.

In order to understand the mechanisms by which the five miRNA candidates could be impacting immune response to *P. entomophila*, we wanted to characterize their role in host resistance and tolerance. Therefore, we decided to estimate the within-host pathogen proliferation for the five different miRNA knockout lines in the first 24 hours of infection. In *D. melanogaster*, measuring tolerance in terms of host-pathogen dynamics can be difficult since the bacterial load cannot be measured without killing the fly. Therefore, there is not a direct way to correlate the bacterial load in a given fly with its mortality, since both assays are destructive. In the context of our work, we defined a miRNA knockout line as tolerant if it could survive a given level of microbes better than its control. Even though host resistance can be directly estimated as the inverse of the within-host microbial concentration, we had no method to distinguish between null observations in pathogen load that derived from a less efficient pricking process and the ones that were the result of the host clearing out the pathogen. A way to overcome this ambiguity is by administering the bacteria to the flies by microinjection, which in principle is a more efficient method than pricking.

The miR-100/let-7/125 knockout line presented the most striking post-infection survival phenotype when compared to its background. Within-host bacterial load results suggest that what mainly contributes for this distinct survival are tolerance mechanisms, since mutant and control lines seem to be equally efficient at eliminating the pathogen. However, frequency of null observations seems to be higher in miR-100/let-7/125 mutants than in control flies between 12- and 16-hours post-infection, and we cannot rule out the possibility that this apparent clearance can explain differences in survival that we observe from 19 hours post-infection onwards. Because we do not measure the pathogen proliferation continuously for a certain fly, it is possible that flies that survive infection are able to clear out the pathogen in early stages of infection. The ones that cannot do so, die with the same amount of bacterial burden as control lines. Therefore, resistance can be playing a part in survival, and this should be further investigated in the future by measuring AMP expression levels during infection. Interestingly, let-7 has already been implicated in the translation repression of *diptericin* mRNA[59]. In a simple sense, we could have expected that under expression of let-7 implicates an enhanced Diptericin expression. However, pathogen proliferation curves do not suggest that there is an enhanced antimicrobial activity in these mutants, which in part may be related to fact that results are related to cluster of genes and not only let-7.

The miR-965 and miR-966 knockout lines also had an enhanced survival chance when compared to control. However, survival rates for the miR-966 mutants mainly diverge from the background between 5- and 19-hours post-infection, while in the miR-965 knockout line differences in survival only become apparent at 19 hours post-infection. In both cases, mutants do not appear to be more efficient at eliminating the pathogen than control. Taken together, our data suggests that the enhanced survival in these lines is related to tolerance mechanisms that allow the host to cope better with the harmful implications that come with *P. entomophila* systemic infection. In miR-965 mutants this is also supported by BLUD results, since they can sustain a higher bacterial load than control before

they die. The absence of miR-966 enhanced host tolerance particularly in early stages of infection (5-19 hours), which no longer was observed at later stages (19-24 hours). In fact, BLUD results suggest that miR-966 mutants die with a lower pathogen burden at 19 to 24 post-infection, indicating that they could be more susceptible and less tolerant to the pathogen than control at this stage. To establish miR-965 and miR-966 role as inhibitors of host tolerance, further investigation needs to be done in order to identify tolerance genes that could possibly be targets of these miRNAs, such as tissue repair genes.

MiR-11 and miR-955 seem to be necessary for host post-infection survival to *P. entomophila* since both mutants have a higher mortality than control. Notably, comparison of within-host pathogen proliferation curves suggested that both mutants tend to contain better bacterial growth than background lines in early stages of infection. However, this tendency shifts during infection, with both lines having a within-host carrying capacity that is significantly higher than control. In the miR-955 knockout line, flies appear to die with a higher pathogen burden than control, which is in agreement with an impaired ability to control pathogen proliferation. Therefore, miR-11 and miR-955 mutants have a higher resistance than control in earlier stages of infection, that later becomes compromised. However, it remains to be determined if resistance impairment contributes to the lower post-infection survival observed for both knockout lines, or if it is a consequence of infection related damage that compromised the host's immune function. Additionally, we think that for both lines it would be important to do an extra experiment that confirmed the change in pathogen load that occurs in the control line at 10 hours of infection. We suspect that this tendency might be an artefact, and if exposed could imply that the curves between control and mutant lines overlap in the first hours of infection. Although neither miR-11 nor miR-955 have been directly implicated in *D. melanogaster* immune mechanisms, it is well characterized that miR-11 limits the proapoptotic activity of its host gene, *dE2f1*[66]. Apoptosis can improve the efficiency of an immune response by contributing to the turn-over of damaged cells and the killing of microbial agents. However, the trade-off of this mechanism can be particularly high, depending on the relative capacity of different tissues to withstand cell loss without compromising tissue function and homeostasis[2,67]. Therefore, it is possible that miR-11 mutants present an enhanced apoptotic activity, that may contribute to an improved resistance in early stages of infection. However, this activity could ultimately implicate a high trade-off with other physiological competences, leading to the high mortality rate of this population.

Initially, we planned to elaborate our understanding of the role of these five miRNA candidates in immunity, by investigating if miRNA regulation is related to their function in a specific immune tissue. In this context, we designed an experiment to determine if the survival phenotypes of each mutant could be rescued by expressing the respective miRNA in one of two immune tissues: haemocytes and fat-body. This would be accomplished by generating transgenic flies with the respective miRNA gene under regulation of a UAS/Gal 4 system, whose expression is promoted by a genetic driver that is exclusive to the selected tissues. However, given time constraints in the making of this master thesis, it was not possible to accomplish this experimental work. Nevertheless, we feel that this analysis can bring some insightful knowledge about the role of these miRNAs, particularly because it would help to narrow down the diversity of mechanisms by which they can be impacting host-pathogen interactions.

In conclusion, fourteen miRNAs appear to have an important role in the immune response of *D. melanogaster* to *P. entomophila* systemic infection in adults. Our findings show that gene expression regulation by miRNAs can shape host-pathogen dynamics, leading to profound changes on how *D. melanogaster* balances resistance and tolerance mechanisms.

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Supplementary Data

Table S1. **Summary of MicroRNA knockout lines**

miRNA K.O.	Genotype	K.O./K.O Viabilty (%)
miR-31b	w[*] TI{TI}mir-31b[KO]	110
miR-970	w[*] TI{TI}mir-970[KO]	103
miR-971	w[*] TI{w[+mW.hs]=GAL4}mir-971[KO]	98
miR-137	w[*]; TI{w[+mW.hs]=TI}mir-137[KO]	88
miR-278	w[*]; TI{w[+mW.hs]=TI}mir-278[KO]	*[1]
miR-2a-2/2a-1/2b-2	w[*]; Df(2L)mir-2a-2-2a-1-2b-2-KO	82
miR-2b-1	w[*]; TI{w[+mW.hs]=TI}mir-2b-1[KO]	120
miR-307-a/307-b	w[*]; Df(2R)mir-307a-307b-KO	109
miR-959/960/961/962	w[*]; Df(2L)mir-959-960-961-962-KO	201
miR-965	w[*];TI{TI}mir-965[KO]	104
miR-966	w[*]; TI{TI}mir-966[KO]	142
miR-967	w[*]; TI{TI}mir-967[KO]	145
miR-968/1002	w[*]; Df(2L)mir-968-1002-KO, TI{w[+mW.hs]=GAL4}mir-968-1002-KO	83
miR-986	w[*]; TI{TI}mir-986[KO]	175
miR-987	w[*]; TI{w[+mW.hs]=GAL4}mir-987[KO]	110
miR-990	w[*]; TI{TI}mir-990[KO]	145
miR-9c	w[*];TI{TI}mir-9c[KO]	113
miR-100/let-7/125	w[*]; Df(2L)let-7-C[KO1], TI{w[+m*]=TI}CG10283[K01]/CyO, P{w[+mC]=GAL4-twi.G}2.2, P{UAS-2xEGFP}AH2.2	0
miR-124	w[*]; TI{w[+mW.hs]=TI}mir-124[Delta177.w+]/CyO, P{w[+mC]=GAL4-twi.G}2.2, P{UAS-2xEGFP}AH2.2	0
miR-11	w[*]; TI{TI}-mir11[KO.w-]	87
miR-284	w[*]; TI{w[+mW.hs]=TI}mir-284[KO]	54
miR-285	w[*]; TI{w[+mW.hs]=TI}mir-285[KO]	83
miR-2c/13-a/13-1-b	w[*]; Df(3R)mir-2c-13a-13b-1-KO, TI{w[+mW.hs]=TI}mir-2c-13a-13b-1-KO	77
miR-317	w[*]; TI{w[+mW.hs]=TI}mir-317[KO]	100
miR-33	w[*]; TI{TI}mir-33[KO]	81
miR-92a	w[*]; TI{TI}mir-92a[KO]	81
miR-955	w[*]; TI{w[+mW.hs]=GAL4}mir-955[KO]	105
miR-957	w[*]; TI{w[+mW.hs]=TI}mir-957[KO]	90
miR-958	w[*]; TI{w[+mW.hs]=TI}mir-958[KO]	99
miR-999	w[*];TI{TI}mir-999[KO]	82
miR-1000	w[*]; TI{TI}mir-1000[KO]/TM3, P{Dfd-GMR-nvYFP}3, Sb[1]	1
miR-276a	w[*]; TI{w[+mW.hs]=TI}mir-276a[KO]/TM3, P{Dfd-GMR-nvYFP}3, Sb[1]	0
miR-iab-4,iab-8	w;;Δmir-iab-4,iab-8/TM3, P{Dfd-GMR-nvYFP}3, Sb[1]	24

***[1]**We have no information of this value, however for our experiments with this candidate we used a miR-278 knockout homozygous mutant.

Table S2. **Relative survival hazard ratios of microRNA knockout lines**.

miRNA K.O.	Hazard Ratio	Statistical Significance (p-value)*[1]
miR-31b	1.366	0.594
miR-970	0.965	0.863
miR-971	0.762	0.594
miR-137	0.402	> 0.001
miR-278	1.542	0.048
miR-2a-2/2a-1/2b-2	0.397	> 0.001
miR-2b-1	1.267	0.304
miR-307-a/307-b	1.547	0.211
miR-959/960/961/962	0.573	0.045
miR-965	0.451	> 0.001
miR-966	0.430	> 0.001
miR-967	1.708	0.053
miR-968/1002	0.632	0.225
miR-986	0.575	0.004
miR-987	1.974	0.132
miR-990	0.999	0.996
miR-9c	1.313	0.492
miR-100/let-7/125	0.335	> 0.001
miR-124	0.907	0.708
miR-11	2.116	0.002
miR-284	0.615	0.069
miR-285	1.539	0.038
miR-2c/13-a/13-1-b	1.233	0.373
miR-317	1.265	0.323
miR-33	0.896	0.601
miR-92[a]	1.812	0.010
miR-955	1.844	0.006
miR-957	2.012	0.003
miR-958	1.331	0.254
miR-999	1.012	0.960
miR-1000	0.442	> 0.001
miR-276[a]	0.762	0.686
miR-iab-4,iab-8	0.788	0.650

*[1] The statistical significance of the hazard ratios between mutant and control lines was estimated after both survival data sets were corrected for multiple comparison using a Bonferroni or Holm-Bonferroni tests.

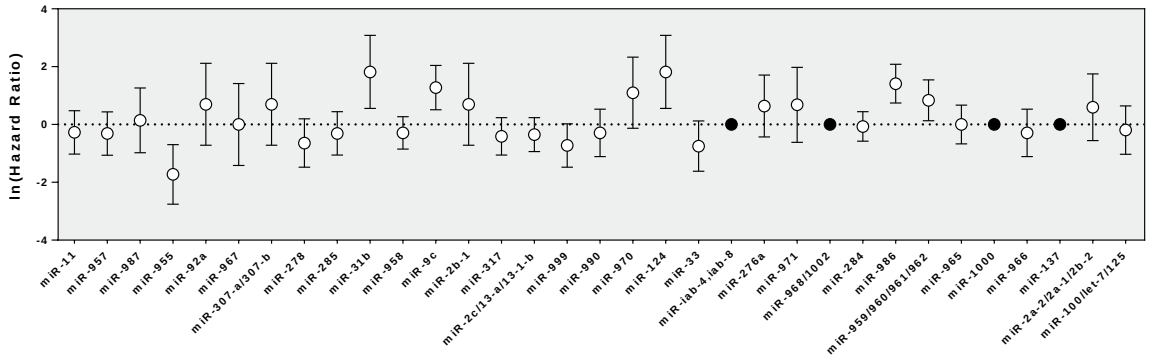

Figure S1. **Relative survival of microRNA knockout lines pricked with a PBS 1x solution**. Survival of infected adult males (4-6 days old) monitored for three days post-infection. For each miRNA mutant, survival is presented as the natural logarithm of hazard ratio in relation to the survival of the respective control line (x=0). Error bars show standard deviation (SD) of the estimated hazard ratio. Lines represented by a black dot had either a value or an SD that did not fit in the scale. For all lines there are no significant differences in survival. Data shown is representative of two independent experiments.

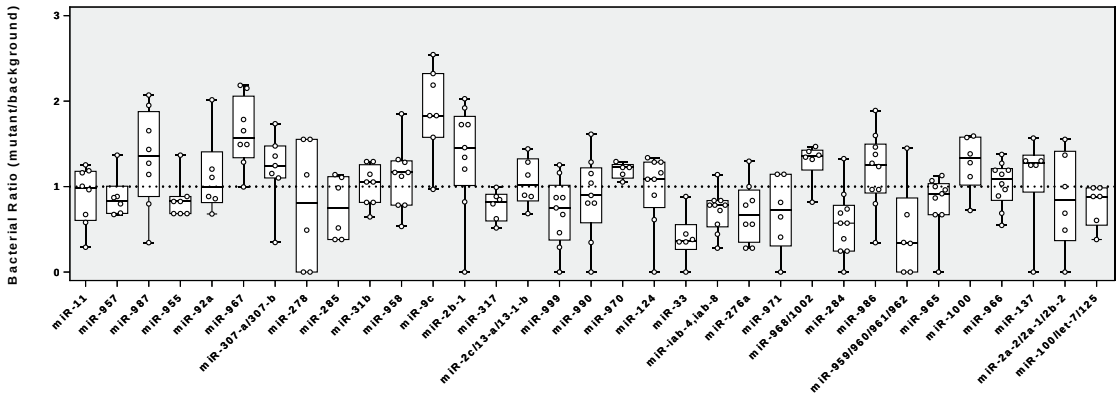

Figure S2. **Initial inoculum estimation for *D. melanogaster* infected with *Pseudomonas entomophila***. Initial inoculum for infected adult males (4-6 days old) that collected after recovering from CO_2 anaesthesia. Each replicate value, presented as a dot, is normalized for the average initial inoculum of the background control. For all lines there are no significant differences in the initial inoculum. All data shown is representative of 2 independent experiments.

Table S3. **Bacterial load upon death (BLUD) estimation for *D. melanogaster* infected with *Pseudomonas entomophila*.**

miRNA K.O.	Control			miRNA K.O.			p-value	
	N	Mean	Median	N	Mean	Median	Wilcoxon-Mann-Whitney	T-test
miR-31b	20	18.025	18.171	24	17.418	16.818	0.120	-
miR-970	20	18.025	18.171	26	17.098	16.644	-	0.046
miR-971	20	18.025	18.171	22	17.835	18.660	0.580	-
miR-137	17	17.249	16.966	23	18.195	18.902	0.262	-
miR-278	11	18.043	17.242	23	19.316	19.811	0.014	-
miR-2a-2/ 2a-1/2b-2	8	15.961	16.071	14	15.072	15.135	-	0.042
miR-2b-1	9	18.469	19.478	24	19.647	20.075	0.063	-
miR-307-a/ 307-b	16	18.280	17.390	24	18.881	19.312	0.096	-
miR-959/960/961/962	18	18.064	17.283	22	19.766	19.795	> 0.001	-
miR-965	18	18.064	17.283	19	19.039	19.628	0.013	-
miR-966	18	16.985	16.883	19	16.261	15.799	0.043	-
miR-967	14	19.022	19.516	30	19.911	19.953	0.018	-
miR-968/1002	17	18.519	19.095	11	19.310	19.763	0.145	-
miR-986	18	16.985	16.883	27	16.273	16.136	0.051	-
miR-987	13	17.414	17.214	22	19.135	19.396	0.002	-
miR-990	16	18.280	17.390	24	18.112	17.318	0.163	-
miR-9c	11	16.081	16.175	23	16.794	16.610	0.286	-
miR-100/ let-7/125	24	17.746	16.932	11	17.763	17.015	0.947	-
miR-124	24	17.746	16.932	27	18.544	19.121	0.073	-
miR-11	23	18.349	17.375	31	18.862	19.536	0.278	-
miR-284	29	17.438	16.734	31	18.457	18.873	0.040	-
miR-285	23	16.640	16.580	23	17.347	16.610	-	0.128
miR-2c/ 13-a/13-1-b	17	17.592	17.375	21	18.342	18.610	0.100	-
miR-317	23	16.640	16.580	24	16.594	16.595	-	0.902
miR-33	23	16.640	16.580	26	16.981	16.673	-	0.413
miR-92a	16	16.655	17.160	12	17.787	17.853	-	0.049
miR-955	16	17.513	17.126	21	19.059	19.573	0.002	-
miR-957	23	16.640	16.580	26	16.732	16.392	0.796	-
miR-958	28	18.620	18.988	31	19.094	19.610	0.151	-
miR-999	23	18.349	17.375	23	17.909	17.341	0.668	-
miR-1000	26	18.284	17.921	17	17.649	16.920	0.815	-
miR-276a	26	18.284	17.921	27	17.830	17.121	0.453	-
miR-iab-4,iab-8	26	18.284	17.921	27	18.780	19.332	> 0.001	-

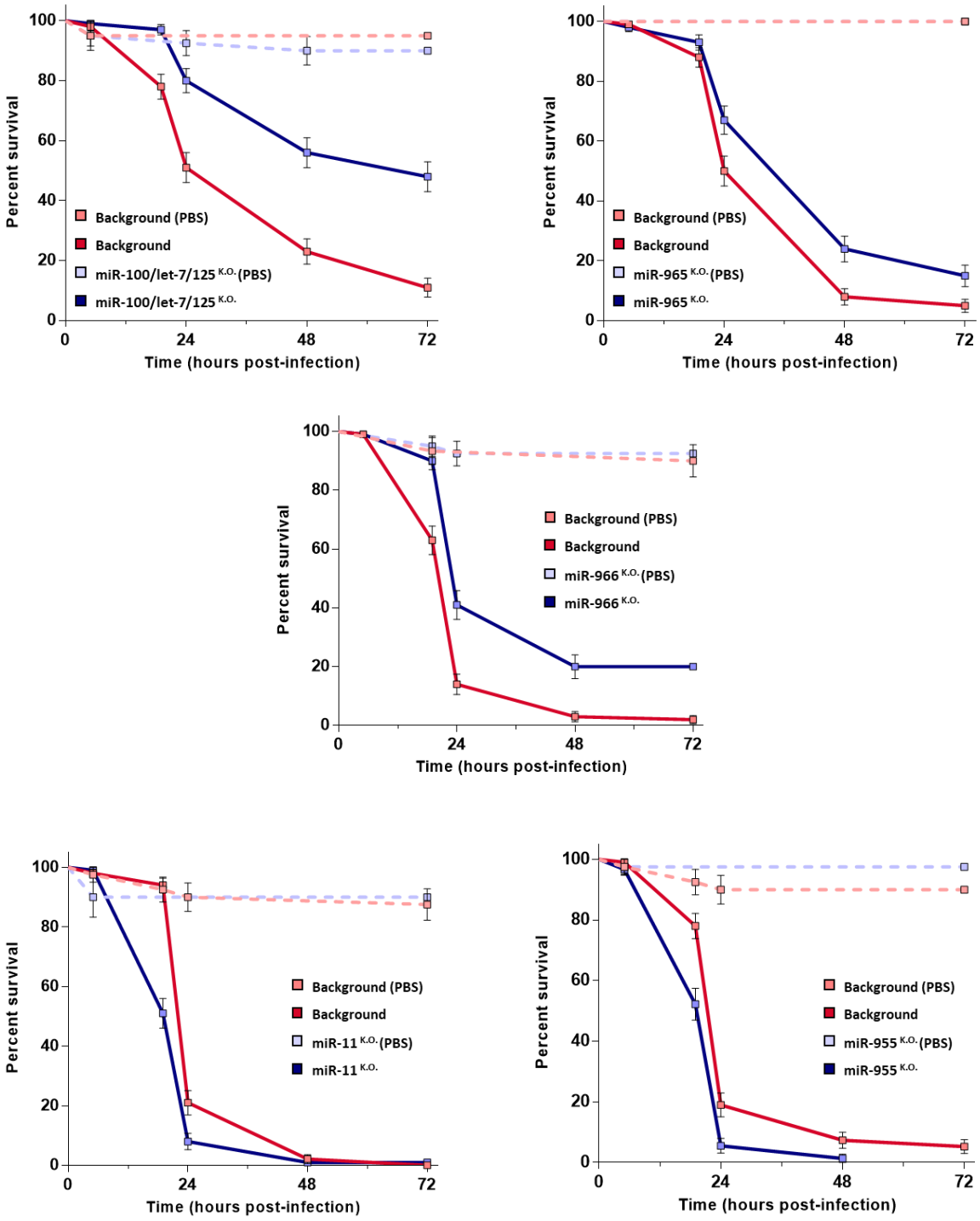

Figure S3. **Survival curves of miRNA mutants for the final group of candidates (miR-965, miR-100/let-7/125, miR-966, miR-955 and miR-11)**. Survival of adult males (4-6 days old) infected with *P.entomophila* monitored for three days after infection. PBS refers to the technical control, where flies were pricked with PBS 1x instead of the bacterial suspension. Error bars show standard error for the mean.

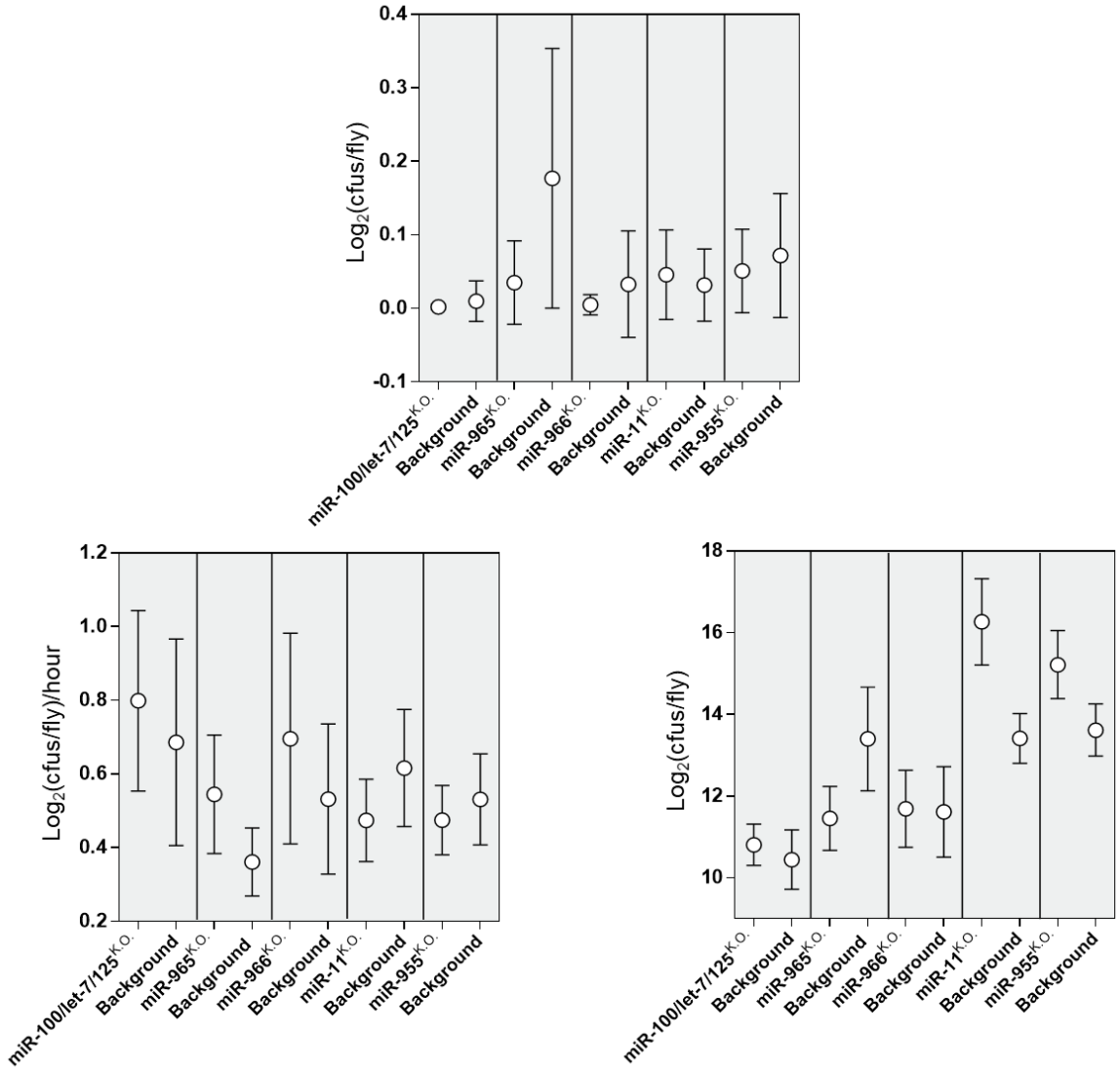

Figure S4. **Logistics in-host bacterial growth curve parameters of microRNA knockout and control lines**. Estimated value and 95% confidence intervals of **(A)** initial inoculum (N_0), **(B)** growth rate (r) and **(C)** maximum carrying capacity (k).