UNIVERSIDADE DE COIMBRA

Functional diversity of *Legionella pneumophila* Dot/Icm Effector SdhA in *Galleria mellonella* model

Dissertação de Mestrado em Biologia Celular e Molecular, orientada pela Professora Doutora Joana Costa (Universidade de Coimbra) e pelo Professor Doutor António Veríssimo (Universidade de Coimbra), apresentada ao Departamento de Ciências da Vida da Universidade de Coimbra

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Abreviations and Acronyms

^{T} – Type strain

ºC – Celsius degree

µl – microliter

µm – micrometer

ACES – N-(2-Acetamido) -2-aminoethanesulfonic acid

ATCC – American Type Culture Collection

AYE – ACES Yeast Extract

BCYE – Buffered Charcoal Yeast Extract

bp – base pair

CFU – Colony-forming unit

DNA – Deoxyribonucleic acid

Dot/Icm – Defective Organelle Trafficking/Intracellular Multiplication

LB – Luria-Bertani

LCV – *Legionella*-containing vacuole

LD – Legionnaires' disease

Lp1 – *Legionella pneumophila* serogroup 1

M – Molar

MIF – Mature Infectious Form

ml – milliliter

mM – milimolar

PBS – Phosphate-Buffered Saline

PCR – Polymerase Chain Reaction

PF – Pontiac fever

pM- picomolar

RF – Replicative form

T4BSS – Type IV Secretion System

TAE – Tris-acetate-EDTA

TF – transmissive form

Abstract

Legionella pneumophila is the major agent of Legionnaire´s Disease (LD) a severe and occasionally fatal pneumonia. This gram negative bacteria that is ubiquitous in freshwater environments and in many man-made water systems, replicates within aquatic protozoa (mainly amoeba), but also within human alveolar macrophages.

L. pneumophila virulence depends on the ability to use the phagocytic vacuole, namely *Legionella*-containing vacuole (LCV), to create a replicative niche preventing phagosome-lysosome fusion and evade the host immune system. To accomplish these tasks *L. pneumophila* translocate numerous bacterial effectors into the host cell though Dot/Icm Type IV Secretion System (TB4SS). This secretion system is responsible for the translocation of vast number of effectors that modulate diverse host cell functions. Despite this large number of recognized effectors only a few are considered to be critical for intracellular growth and disease, such as SdhA protein. This protein is crucial for the maintenance of LCV integrity in macrophages, since in the absence of this effector the LCV disruption occurs resulting in the death of both host cell and bacterium.

An increase in the use of *G. mellonella* as an infection model for human pathogens occurred in the past years due the existence of a large correlation between virulence of bacterial pathogens in the insect and in mammalian models. In this insect, mortality induced by *L. pneumophila* is both dose and functional Dot/Icm T4BSS-dependent. Moreover, the suitability of *G. mellonella* to determine the role of Dot/Icm T4BSS effectors, such SdhA, in the virulence of this bacterium was already demonstrated.

The main objective of this study was to determine if the role of the crucial virulence-related Dot/Icm T4BSS effector SdhA induced different levels of virulence

among unrelated *L. pneumophila* strains, isolated from different environments and with distinct genetic backgrounds, using *G. mellonella* larvae as an infection model.

In this study the majority of *L. pneumophila* strains induced a *sdhA*-dependent larval mortality. In addition, relevant differences on the role of *sdhA* were observed among the studied strains. In sum, SdhA induced different levels of virulence among unrelated *L. pneumophila* strains in *Galleria mellonella* infection. Importantly, some degree of functional redundancy for SdhA was detected for the first time in a *L. pneumophila* strain.

Resumo

Legionella pneumophila é o principal agente da Doença dos Legionários, uma pneumonia severa e ocasionalmente mortal. Esta bactéria gram negativa, é ubíqua em ambientes aquáticos não salinos e em sistemas artificiais de água, replicando-se dentro de protozários aquáticos (principalmente amebas), mas também dentro de macrófagos alveolares humanos.

A virulência de *L. pneumophila* depende da sua capacidade de remodelar o vacuolo fagocítico, denominado "*Legionella*-containing vacuole" (LCV), para criar um nicho replicativo prevenindo a fusão do fagossoma com o lisossoma, evitando assim o sistema imunitário do hospedeiro. Para realizar estas tarefas, *L. pneumophila* efectua a translocação de numerosos efectores bacterianos para a célula hospedeira através do sistema de secreção Dot/Icm Tipo IV (T4BSS). Este sistema de secreção é responsável pela translocação um vasto número de efectores que modulam diversas atividades da célula hospedeira. Apesar do elevado número de efetores identificados, apenas alguns são considerados críticos para o crescimento intracelular da bactéria, como a proteína SdhA. Esta proteína é importante para a manutenção da integridade do LCV em macrófagos, uma vez que na sua ausência o LCV é fragmentado resultando na morte da célula hospedeira e da bactéria.

Nos últimos anos o uso de *Galleria mellonella* como um modelo de infeção para o estudo de agentes patogénicos tem aumentado devido a existência de uma correlação entre a virulência de bactérias patogénicas em insetos e nos modelos em mamíferos. Neste inseto, a mortalidade induzida por *L. pneumophila* é dependente da dose e do sistema de secreção Dot/Icm T4BSS funcional. Para além disso, a utilização de *G. mellonella* na

determinação da função e relevância de efetores do Dot/Icm T4BSS, como o SdhA, na virulência desta bactéria já foi demonstrada.

O objetivo principal deste estudo foi determinar se o efetor SdhA translocado por Dot/Icm T4BSS e crucial para a virulência de *L. pneumophila* em *G. mellonella*, é está envolvido em diferenças de virulência em estirpes de *L. pneumophila* não relacionadas, isoladas de diferentes ambientes e com origens genéticas distintas, usando a larva *G. mellonella* como um modelo de infeção.

Neste estudo verificámos que a virulência da maioria das estirpes de *L. pneumophila* é dependente do efetor SdhA. Para além disso, a relevância deste efector na infeção por *L. pneumophila* variou entre as estirpes analisadas. Assim, concluímos que o efector SdhA é responsável pelos níveis distintos de virulência observados entre estirpes de *L. pneumophila* isoladas de ambientes distintos e com diferentes contextos genéticos. Adicionalmente, detetámos pela primeira vez a existência de redundância funcional para SdhA entre estirpes de *L. pneumophila* na infeção no modelo de *Galleria mellonella.*

Chapter I - Introduction

1.1. Historical Reference

Back to 1976, during the 58th annual convention of American Legion in Philadelphia, a strange disease affected 182 persons. From these cases, twenty-nine were fatal. Patients had an atypical form of pneumonia with symptoms like malaise, muscle aches, slight headache, dyspnea and abdominal pain. The first steps to identify the agent responsible for the infection were carry out by Fraser and McDade and their team, that were able to isolated a gram-negative bacterium in guinea pigs from lung specimens that were isolated from patients classified as having Legionnaires´ Disease (McDade *et al*., 1977). Nevertheless, the association between the bacterium and the disease was only established two years later by seroconversion. A new genus and species was described and named *Legionella pneumophila*, with *L. pneumophila* Philadelphia 1 as type strain (Brenner *et al.*, 1979). This common-source outbreak of pneumonia caused by legionellae was the first described case of Legionnaires' Disease (LD) leading to a series of investigations in *L. pneumophila* (Fraser *et al*., 1977).

With the isolation of *L. pneumophila* the origin of the unsolved outbreak at St. Elizabeth´s Hospital in Washington, D.C., in 1965, was clarified and associated to this bacterium (Thacker *et al.*, 1978). Another unsolved outbreak occurred in 1968 in the health department of Pontiac, Michigan, affecting at least 144 persons, was also related with *L. pneumophila*. This disease was characterized by acute febrile illness with symptoms as headache, myalgia, fever, and malaise but differed from LD since no

pneumonia was observed among Pontiac patients. Therefore this outbreak represented a new clinical syndrome later designated by Pontiac Fever (PF) (Glick *et al*,. 1978).

1.2. Epidemiology

1.2.1. Transmission

The genus *Legionella* is ubiquitous in freshwater environments (Fig. 1 - point 1), such as rivers, springs, lakes or streams, however in these habitats the concentration is usually low (Borella *et al*., 2005). In order to *L. pneumophila* cause disease a chain of events has to occur. Firstly, natural reservoirs that supply water distribution systems have to be colonized with *Legionella* sp. (Fig. 1 - point 2) and the water has to be distributed to man-made environments, namely cooling towers, water distribution systems, industrial equipment, domestic plumbing systems, thermal spas, among others (Castillo *et al*., 2016). In these artificial reservoirs, the environmental conditions (e.g. temperature, pH and presence of biofilms) must support *Legionella* sp. survival and promote growth (Fig. 1 - point 3) which will allow amplification of their number (Fig. 1 - point 4). The spread of this bacterium to the community requires the production of aerosols from contaminated water (Fig. 1 - point 5), and cases of disease will only occur if the bacterial strain is virulent and the populations susceptible (Fig. 1 - point 6).

The susceptibility of the human population to *Legionella* spp. differs, and has been associated with the gender, age, immunosuppression, smoking habits and underlying diseases (e.g. cancer or AIDS) (Marston *et al.*, 1994; Phin *et al*., 2014; Mercante and Winchell, 2015)

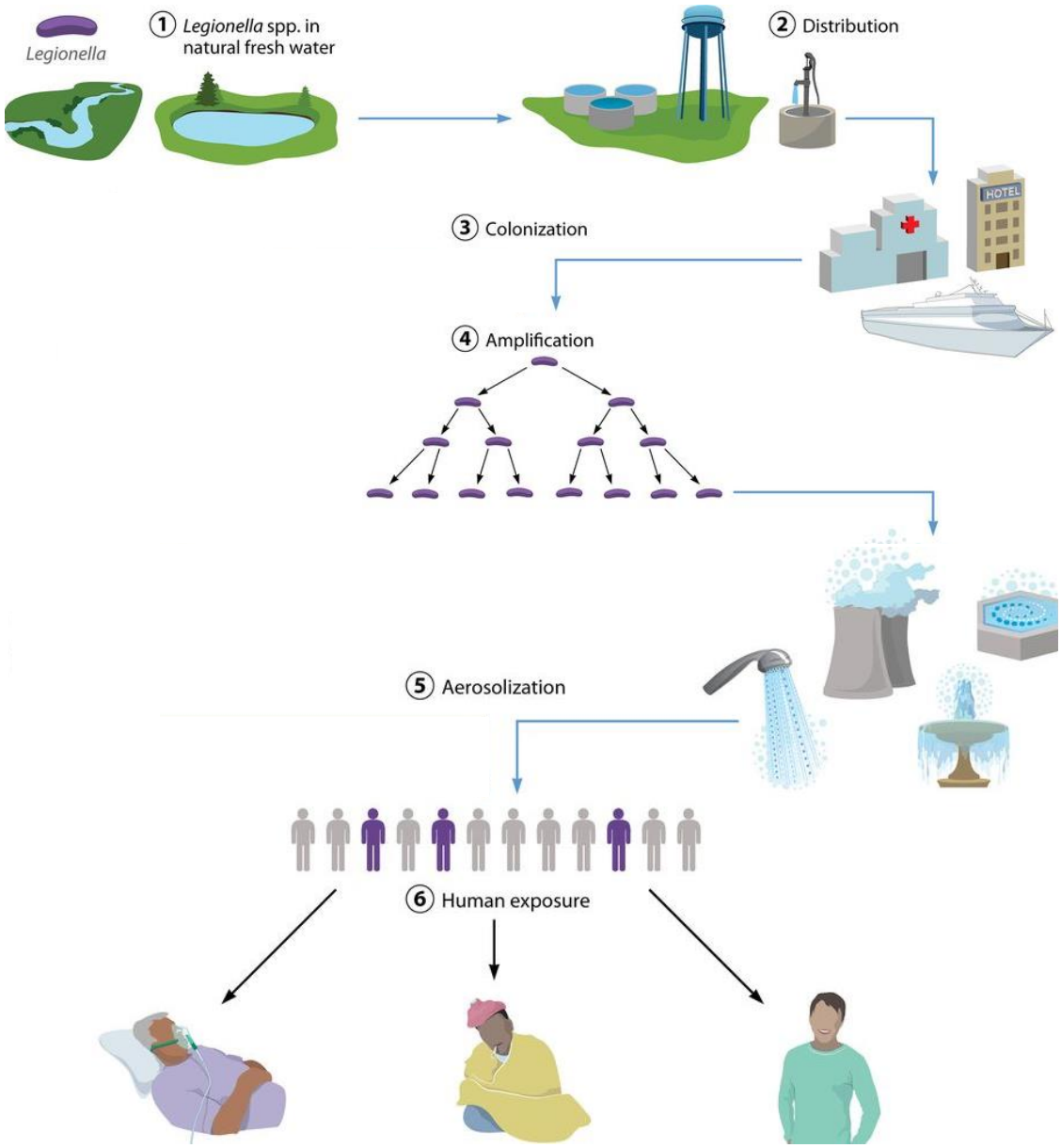

Figure 1. Chain of events leading to LD or PF cases (adapted from Mercante and Winchell, 2015).

1.2.2. Clinical Manifestations

Legionella infection in humans may present two different forms, Legionnaires' disease (LD) and Pontiac fever (PF). The first one occurs ranging from a mild cough and fever to respiratory failure but pneumonia is the main clinical manifestation (Castillo *et al.*, 2016). The incubation period of LD varies from 2 to 10 days (Castillo *et al.*, 2016), but longer and shorter periods have been observed, such in Netherlands, that 16 % (22 of

136 patient) of LD cases had, at least, 10 days of incubation periods (Den Boer *et al*., 2002). LD symptoms are non-specific and include fever, myalgia, weakness, and chills. As the disease progresses, neurologic symptoms (such headache, lethargy, confusion) and gastrointestinal symptoms (such diarrhea, nausea, vomiting) are prominent (Castillo *et al.,* 2016).

On the other hand, PF is a mild-form of *Legionella* infection, non-pneumonic, and non-fatal. Mean incubation time of PF is 36 hours, and typically is self-limited; in other words, it doesn't require any additional treatment (Castillo *et al.,* 2016; Phin *et al*., 2014).

Nowadays it is accepted that these two types of diseases may coexist within an exposed population. However it is not clear if PF is due to differences in host susceptibility to *L. pneumophila* or whether it is due to the presence of legionellae in a non-viable state. (Mercante & Winchell, 2015).

1.3. Family *Legionellaceae*

Legionellaceae comprises a single genus *Legionella* among the subdivision γ-Proteobacteria. Nowadays, the genus *Legionella* comprises over 60 species (Khodr *et al*., 2016), containing at least 70 serogroups, isolated from environmental and clinical samples (Mercante and Winchell, 2015). From these species, the best studied and well-known is *L. pneumophila*, since it is responsible for about 95 % of LD cases diagnosed

worldwide. More specific, from the 15 serogroups that compose this species, the serogroup 1 (*Lp1*) is responsible for the majority of LD cases (Khodr *et al*., 2016).

1.4. Ecology

These gram-negative bacteria are able to grow in non-saline aquatic systems with temperatures ranging between 25ºC and 42ºC, with an optimal temperature of 35ºC (Fields *et al.*, 2002). Nevertheless, they can persist at temperatures below 20ºC and above 50ºC (Cunha *et al.*, 2016; Mercante and Winchell, 2015)

L. pneumophila has been found in artificial aquatic environments, including cooling towers, water distribution systems, industrial equipment, among others (McDade, 2008). In natural habitats, *L. pneumophila* has been detected in aquatic bodies including rivers, groundwater, lakes, streams and thermal waters (Falkinham *et al*., 2015). This distribution has been associated with the ability of *L. pneumophila* to multiply in amoeba (this interaction will be better discussed in sub-chapter 1.5.1) and some ciliated protozoa which are ubiquitous in those environments (Cunha *et al.*, 2016).

The ability of *L. pneumophila* to thrive in a wide range of environments is related with biofilm formation. This strategy allows microorganisms, like *L. pneumophila*, and its hosts, to resist and thrive in adverse conditions, such high temperatures, high concentrations of chlorine, nutrients deprivation, etc. Biofilms are formed either in natural or artificial environments (McDade, 2008), and for that, public health safety control measures showed focus on the prevention of biofilm formation since once it is established, it is difficult to eliminate (Cunha *et al.*,2016).

1.5. Intracellular life cycle in amoeba - “Trojan horse”

L. pneumophila has the ability to grow outside host cells, either within microbial communities (e.g. biofilm) or *in vitro* in nutrient-rich media, and for that is considered a facultative intracellular pathogen. However, in nature, this bacteria behaves more as an obligate intracellular pathogen and less as a facultative one, and for that reason, intracellular growth is considered a fundamental process in the life cycle of *L. pneumophila* (Robertson *et al.*,2014). Therefore, understanding how the different cellular forms of this bacterium - pleomorphism, interact with the host cells and with the natural habitat is rather important.

The capacity of *L. pneumophila* to persist both intracellularly and extracellularly is mainly due to its ability to differentiate into interconvertible cell types in response to environmental and metabolic variations. During this differentiation cycle, *L. pneumophila* alternates between, at least, two distinct forms: a transmissive form (TF), which is an infectious form, metabolically active, and capable of evading lysosomal degradation; and a replicative form (RF), a noninfectious form that cannot initiate intracellular growth cycles neither synthetize cysteine, using host amino acids as primary energy source for multiply within a phagossomal compartment. This strategy of conversion between transmissive and replicative forms represents a fundamental tool in *L. pneumophila* fitness (Fonseca and Swanson, 2014; Robertson *et al.*, 2014). Briefly, when the TF of *L. pneumophila* is phagocyted by free-living amoeba (Figure 2 – point 1), which are the natural hosts of this bacterium, a specialized secretion system (Dot/Icm Type IVB Secretion System- T4BSS) is used to inject a variety of virulence effectors that arrest phagosome maturation and permit the establishment of a vacuole (Figure 2 – point 2), called Legionella containing-vacuole (LCV) (Fonseca and Swanson, 2014). Within the

LCV, *L. pneumophila* differentiates into the RF and multiplies (Figure 2 – points 2 to 3). After replication (post-exponential phase), the decrease in nutrients leads to the differentiation of *L. pneumophila* into the flagellated mature infectious form (MIF) (Figure 2 – points 3). This last form is released into the amoeba cytosol, followed by a further multiplication of *L. pneumophila*. Ultimately, the bacteria is released from the host cell by cell necrosis (Figure 2 - point 4). The released metabolic latent form of *L. pneumophila* is ready to re-infect a new host and start another intracellular cycle (Figure 2 – point 5) (Eisenreich and Heuner, 2016).

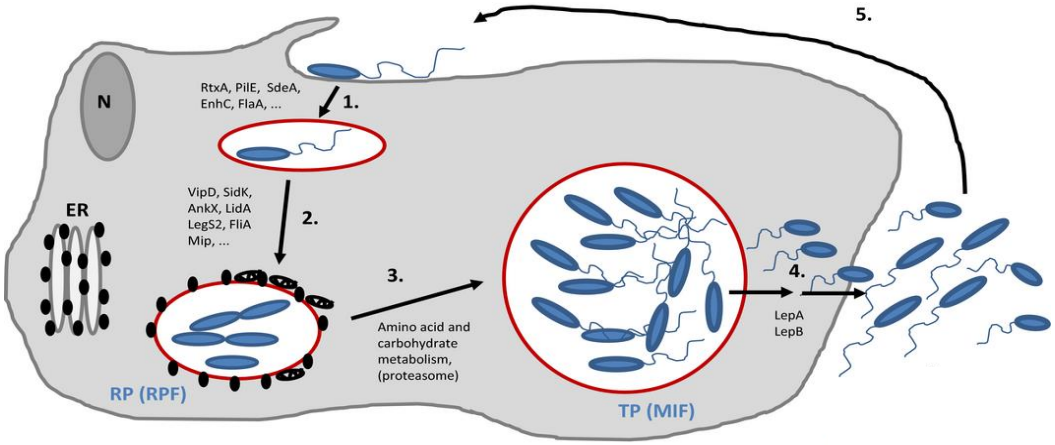

Figure 2. Biphasic life cycle of *L. pneumophila* in *Acathamoeba* sp. (adapted from Eisenreich and Heuner, 2016)

1.6. From amoeba to human macrophages

The common strategies used by this bacterium to infect and replicate in amoeba and human macrophages, associated with the extremely limited transmission between humans (Escoll *et al*., 2013), since only in one case was reported caused by a probable transmission between humans (Correia *et al*., 2016); led to the hypothesis that co-evolution with these aquatic protozoa provided the acquisition of a pool of virulence

factors which allowed *L. pneumophila* to also infect human cells (Rolando and Buchrieser, 2014). Furthermore, this co-evolution is patent in the *L. pneumophila* genome through the acquisition by horizontal gene transfer of many genes encoding eukaryotic-like proteins and protein domains that mimic host proteins allowing the subversion of the cellular mechanism and the intracellular replication of this bacteria (Nora *et al*. 2009; Rolando and Buchrieser, 2014). This major virulence strategy is frequently named molecular mimicry (Nora *et al*., 2009) and is responsible for a successfull replication within an accidental host - human macrophages (Escoll *et al*., 2013).

At cellular level, several aspects of *L. pneumophila*-infection cycle are similar between amoeba and human macrophages: *L. pneumophila* is phagocyted, LCV is formed and the LCV fusion with lysosome is impaired. Moreover, for the LCV formation in human macrophages, it is also necessary the remodelling of LCV surface by recruiting endoplasmic reticulum (ER) vesicles, ribosomes, and mitochondria (Escoll *et al*., 2013)

1.7. Pathogenicity

1.7.1. Secretion Systems

A great number of gram-negative bacteria depend on secretion systems to export virulence proteins (or in some cases, protein-protein, and DNA-protein complexes), in the case of *L. pneumophila*, directly from the cytosol into the cytoplasm of either protozoa host or human macrophage (E. Green *et al.*, 2016). Thus, this virulence-related effectors cross three phospholipid membranes (inner membrane, outer membrane, and host membrane) (E. Green *et al.,* 2016) and perform a variety of activities essential for *L. pneumophila* replication and to avoid the host immune responses (Voth *et al.,* 2012). The secretion system mostly responsible for the modulation of these activities is type IVB

secretion system known as Defective Organelle Trafficking (Dot)/Intracellular Multiplication (Icm) system (Ensminger and Isberg, 2009; Hubber and Roy, 2010)

1.7.1.1. Dot/Icm Type IVB Secretion System (T4BSS)

The T4BSS is critical for *L. pneumophila* replication and survival and, as expected, mutant's defective in Dot/Icm T4BSS compromise intracellular replication and replicative phagosome integrity (Ensminger and Isberg, 2009; Hubber and Roy, 2010).

At least, 330 *L. pneumophila* effectors are translocated to the host cell by T4BSS (Ensminger, 2016). However, most of these individual translocated effectors are dispensable for growth in macrophages (Ensminger, 2016), since the genetic deletion of this *L. pneumophila* effectors didn´t significantly impair intracellular replication (Hoffmann *et al.*, 2014). This difficulty in detecting phenotypes caused by a deletion mutations in genes that express T4BSS effectors evidenced the existence of functional redundancy since multiple effectors can carry out similar functions (Isberg *et al.*, 2009)

Nevertheless, a few virulence-related T4BSS effectors are essential for *L. pneumophila* intracellular replication, namely SdhA (involved in maintenance of LCV integrity) (Laguna *et al*., 2006), SidJ (involved in recruitment of ER to LCV) (Liu and Luo, 2007) and AnkB (involved in polyubiquitylation of LCV and proteasomal production of amino acids) (Finsel and Hilbi, 2015).

1.7.2. Dot/Icm T4BSS Effector SdhA

L. pneumophila needs the Dot/Icm T4BSS to efficiently deliver effectors that will avoid phagosome maturation (and subsequent degradation) and establish a favourable vacuole (LCV) for replication. This strategy confer to this bacterium protection against intracellular mechanisms that eukaryotic host cells use to detect and eliminate pathogens

(Creasey and Isberg, 2012; So *et al.*, 2015). SdhA protein is one of several effectors translocated by Dot/Icm T4BSS that is crucial for the intracellular growth of *L. pneumophila* in macrophages (Laguna *et al.*, 2006). Nevertheless, this effector does not represent the same level of importance among *L. pneumophila* hosts, since the absence of SdhA resulted in a mild-growth defect in amoeba and U937 human cell line (Laguna *et al*, 2006). This protein is translocated after uptake of *L. pneumophila* by host cell and is expressed throughout the intracellular growth cycle (Laguna *et al*., 2006). SdhA is critical for the maintenance of LCV integrity (Fig. 3 - A), since in the absence of this effector the LCV disruption occurs (Fig. 3 - B) (Creasey and Isberg, 2012).

When *L. pneumophila* is exposed to the macrophage cytosol, due to absence of vacuole stabilization by SdhA, occurs the activation of the host cell death. This event occurs via caspase-1 activation and via induction of type I interferon. This last pathway results in an increase in bacterial nucleic acids that trigger inflammasome - dependent cell death pathway (Fig. 3 - B). Therefore, breakdown of LCV during intracellular replication in macrophages, caused by loss of SdhA protein, results in the death of both host cell and bacterium (Creasey and Isberg, 2012; So *et al*., 2015).

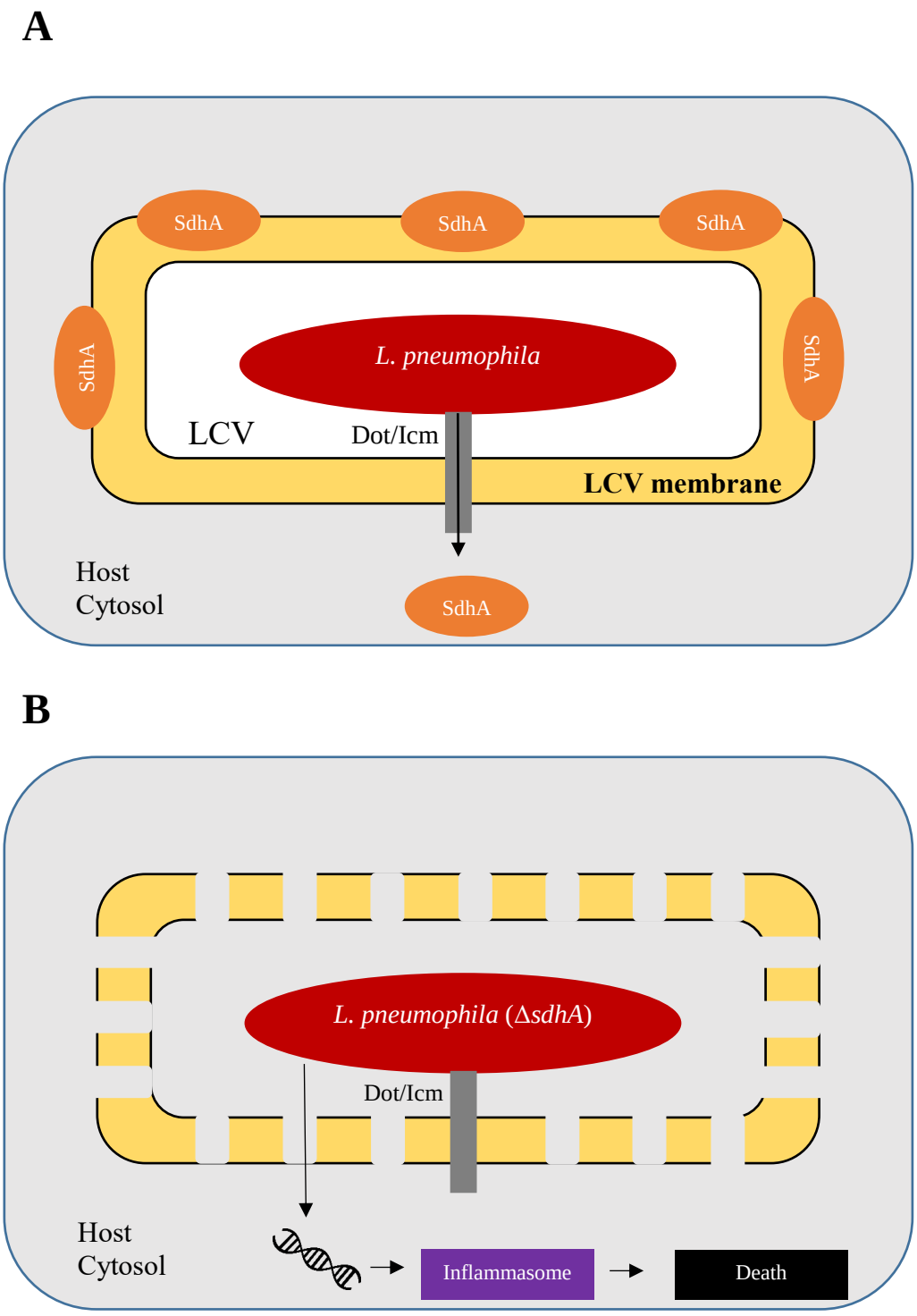

Figure 3. SdhA maintains the LCV membrane integrity. Dot/Icm-translocated SdhA protein is critical for *L. pneumophila* within macrophages (A). In its absence occurs a disruption of LCV membrane that exposes *L. pneumophila* and *Legionella*-derived molecules, such DNA, resulting in the death of both host cell and bacterium (Adapted from Harding *et al*., 2013; So *et al*, 2015)

1.8. *Galleria mellonella* as an infection model for *Legionella pneumophila*

To study molecular aspects of *L. pneumophila* pathogenesis, fresh water amoeba, such as *Acanthamoeba castellanni* or *Hartmannella vermiformis*, often serve as model hosts (Kwaik, 1996; Hilbi *et al*., 2007). However, they do not fully reflect the infection in humans because amoeba employs less complex antimicrobial mechanisms (Harding *et al.*, 2012).

Another alternative model organism used to study several bacterial pathogens, such *Legionella* spp., is the nematode *Caenorhabditis elegans* which carry an innate immune system (Brassinga *et al*., 2010). However, since *L. pneumophila* replicates in the intestinal lumen to this nematode and does not infect intestinal epithelial cells, makes this organism ineffective to study the virulence determinants needed for the intracellular replication.

Within mammalians host models, mouse strains are resistant to LD with the exception of the albino A/J mice (Brieland *et al*., 1994). On the other hand, disease progression in guinea pig is similar to human LD (Hambleton *et al.*, 1983). But due to the high cost and ethical considerations associated with the use of mammalian hosts, some alternative *L. pneumophila* insect models emerged, namely, *Drosophila melanogaster* (Scully and Bidochka, 2006) and *Galleria mellonella* (Harding *et al*., 2012).

The innate immune systems of insects displays many similarities to those used by humans, since the majority of insect species contains specialized cells, hemocytes, that phagocytes pathogens and posteriorly form aggregates which encapsulate and neutralize

these microorganisms. Activation of hemocytes leads to a series of mechanisms that ends with the production of antimicrobial compounds (Harding *et al*., 2012).

G. mellonella has become a widely used as an insect model to study a wide range of human pathogens, including *L. pneumophila* (Harding *et al*., 2012). This insect model can be incubated at human core temperature (37º C) (Tsai *et al.*, 2016) and previous studies proved that it is susceptible to *L. pneumophila* infection (Harding *et al*., 2012). In this larva model, *L. pneumophila* replicates within a LCV in hemocytes and its infection cause an antimicrobial immune response, culminating with the decrease of hemocytes and subsequent death of larva. Mortality induced by this bacterium is both dose and functional Dot/Icm T4BSS-dependent (Harding *et al*., 2012, 2013). Furthermore, previous studies demonstrated the suitability of *G. mellonella* to determine the role of Dot/Icm T4BSS effectors, namely SdhA, in disease-related *L. pneumophila* 130b strain virulence (Harding *et al.*, 2013).

1.9. Main Objective

The main objective of this study was to determine if the role of the crucial virulence-related Dot/Icm T4BSS effector SdhA induce different levels of virulence among unrelated *L. pneumophila* strains, isolated from different environments (natural environmental, man-made and disease-related) and with distinct genetic backgrounds, using *G.mellonella* larvae as an infection model.

Chapter II – Material and Methods

2.1. *Legionella pneumophila* strains

L. pneumophila strains were selected from several others assuring the maximum genetic variability inferred from the complete sequence of Type IV virulence-related effectors *dotA*, *sdhA* and *sidJ* (Costa *et al.* 2010, 2014, 2017) and type II protein secretion system related genes *pilD, lspD, lspE, proA* and *srnA* (Costa *et al*., 2012). The selected strains (Table I) comprised four isolated from natural environments, two from man-made environments and two disease-related strains. Additionally, with the exception of Micu B strain that belongs to *L. pneumophila* subsp. *pascullei* all other strains belong to *L. pneumophila* subp. *pneumophila.*

Table I. *L. pneumophila* unrelated strains used in this study.

Strain designation	**Environmental type/Origin**	**Subspecies**	**Reference**
Nmex1	Natural, New Mexico, USA	*L. pneumophila* subsp. *pneumophila*	Marrão *et al.* (1993)
Ice27	Natural, Iceland	*L. pneumophila* subsp. *pneumophila*	Costa *et al.* (2010)
HUC1	Man-made/Portugal	*L. pneumophila* subsp. *pneumophila*	Costa *et al.* (2010)
Philadelphia1 (ATCC 33152^{T})	Disease-related/USA	*L. pneumophila* subsp. *pneumophila*	(Chien *et al.* 2004)
MicuB (ATCC 33735)	Disease-related/UK	*L. pneumophila* subsp. *pascullei*	(Brenner *et al*., 1988)

^{T} – type strain

2.2. Culture Media

2.2.1. Buffered Charcoal Yeast Extract (BCYE) (Edelstein *et al*., 1981)

Table II. Buffered Charcoal Yeast Extract (BCYE) composition

Reagents	**Concentration ($g.l^{-1}$)**
Activated charcoal	2
Yeast extract	10
Agar	20
N-(2-Acetamido) -2-aminoethanesulfonic acid (ACES)	10
α-ketoglutarate	1
L-cystein	0.4
Ferric pyrophosphate	0.25

ACES and α-ketoglutarate were dissolved in 980 ml of demineralized water and pH was adjusted to 6.9 by the addition of KOH or H_2SO_4 5%, if required. The resulting solution was used to hydrate the yeast extract, the activated charcoal, and the agar. The final solution was sterilized by autoclaving at 121ºC for 25 min.

After sterilization, the temperature was stabilized in a water bath at 55ºC. After that, 10 ml of a 4% (w/v) sterile L-cysteine solution and 10 ml a 2.5% (w/v) sterile ferric pyrophosphate solution were added. The medium was homogenized and distributed into

100mm×15mm Petri dishes (about 20 ml of the medium by each petri dish), and stored at 4ºC until its use.

2.2.1.1. Ferric pyrophosphate solution

A solution of ferric pyrophosphate 2.5% (w/v) was prepared in demineralized water. The solution was sterilized by filtration through a syringe filter of 0.22 μm pore size. The final solution was distributed by sterilized 15 ml Falcon tubes and stored at -20ºC.

2.2.1.2. L-Cysteine solution

A solution of L-cysteine 4% (w/v) was prepared in demineralized water. The solution was sterilized by filtration through a syringe filter of 0.22μm pore size. The final solution was distributed by sterilized 15 ml Falcon tubes and stored at -20ºC.

2.2.2. Buffered Charcoal Yeast Extract (BCYE) with kanamycin

(Buchrieser, 2013)

This medium had a composition and preparation identical to BCYE (section 2.2.1), but with the addition of a 25 $mg.ml^{-1}$ sterile solution of kanamycin antibiotic, simultaneously added with L-Cysteine and ferric pyrophosphate.

2.2.2.1. Kanamycin solution

A 25 $mg.ml^{-1}$ solution of kanamycin was prepared in demineralized water. The solution was sterilized by filtration through of a syringe filter of 0.22μm pore size. The final solution was distributed by sterilized 1.5 ml microtubes, in aliquots of 1 ml, and stored at -20ºC.

2.2.3. ACES Yeast Extract (AYE) (Horwitz and Silverstein, 1983)

Table III. ACES Yeast Extract (AYE) composition

Reagents	**Concentration ($g.l^{-1}$)**
Yeast extract	10
Bovine Serum Albumin (BSA)	5
N-(2-Acetamido)-2-aminoethanesulfonic acid (ACES)	10
L-cysteine	0.4
Ferric pyrophosphate	0.25

BSA, yeast extract, and ACES were dissolved in 980 ml of autoclaved ultrapure water (121ºC, 25 min) and pH was adjusted to 6.9 by the addition of KOH or H_2SO_4 5% (v/v), if required. This solution was sterilized through a vacuum filtration system with a 0.22 μm filter membrane. After sterilization, the 4% (w/v) sterile L-cysteine (section 2.2.1.1) and 2.5% (w/v) sterile ferric pyrophosphate (section 2.2.1.2) solutions were added.

2.2.4. ACES Yeast Extract (AYE) with kanamycin (Buchrieser, 2013)

The medium composition and preparation was identical to AYE (section 2.2.3), with the supplementation of a 25 $mg.ml^{-1}$ sterile kanamycin solution, added along with the 4% (w/v) L-cysteine (section 2.2.1.1) and 2.5% (w/v) ferric pyrophosphate (section 2.2.1.2) sterile solutions.

2.2.5. Luria-Bertani (LB) agar

Table IV. Luria-Bertani (LB) agar composition

Reagents	**Concentration ($g.l^{-1}$)**
Tryptone	10
Sodium Chloride (NaCl)	5
Yeast Extract	10
Agar	15

Tryptone, NaCl, Yeast Extract and Agar were dissolved in 1000 ml of demineralized water. The medium was sterilized by autoclaving at 121ºC for 25 min. After sterilization, the temperature was stabilized in a water bath at 55ºC, and distributed into 100mm×15mm Petri dishes (about 25 ml of the medium by each petri dish), that were stored at 4ºC until its use.

2.2.6. Luria–Bertani (LB) agar with Ampicillin

This medium had a composition and preparation identical to LB agar (section 2.2.5), with the addition of a sterile solution of 100 $mg.ml^{-1}$ ampicillin antibiotic prior to the distribution into Petri dishes.

2.2.6.1. Ampicillin solution

A solution of ampicillin at 100 $mg.ml^{-1}$ was prepared in demineralized water. The solution was sterilized by filtration through of a syringe filter with 022μm pore size. The final solution was distributed in 1.5ml Eppendorf, in aliquots of 1ml, and stored at -20ºC.

2.2.7. Luria–Bertani (LB) Broth

Table V. Luria-Bertani (LB) broth composition

Reagents	**Concentration ($g.l^{-1}$)**
Tryptone	10
Sodium Chloride (NaCl)	5
Yeast Extract	10

Tryptone, NaCl and Yeast Extract were dissolved in 1000 ml of demineralized water. The solution was sterilized by autoclaving at 121ºC for 25 min.

2.2.8. Luria–Bertani (LB) Broth with ampicillin

This medium had a composition and preparation identical to LB broth (section 2.2.7.), but with the addition of 100 $mg.ml^{-1}$ sterile solution of ampicillin antibiotic. Ampicillin solution was prepared as previously described in section 2.2.6.1.

2.3. Construction of *L. pneumophila sdhA* deletion mutants

2.3.1. Construction of plasmid bearing internal deletion of *sdhA* gene

Briefly, *L. pneumophila* is naturally competent thus transformation and homologous recombination of a DNA construct could be used for mutant construction (Portier *et al*., 2015). Our strategy enclosed the design (SnapGene® software), synthesis and cloning of a target sequence into the pUC57 plasmid (GenScript). The designed sequence comprised conserved flanking regions of the *sdhA* target gene, a selection marker and its promoter (Fig. 4). The alignment of *sdhA* genes from the studied strains evidenced that the first and last 350 bp were conserved among all strains (Costa *et al*., personal communication). These regions were kept allowing the occurrence of homologous recombination, with the insertion of the selection marker into the transformed strain. The chosen selected marker was the gene coding for the resistance for kanamycin antibiotic (816 bp), since all tested strains were sensible to it (our results). Additionally, upstream the kanamycin resistance gene, a promoter region (120bp) was included to assure its expression in *L. pneumophila.*

Figure 4. *ΔsdhA* mutant construction cassette map. 1 to 350 bp - initial portion of *L. pneumophila* will type *sdhA* gene (blue); 351 bp to 470 bp - promoter of kanamycin resistance gene (orange); 471 bp to 1287 bp - kanamycin resistance gene (red); 1288 bp to 1637 bp – final portion of *L. pneumophila* will type *sdhA* gene (blue). The size of each fragment is represented by solid grey line.

2.3.2. *E. coli* DH5α transformation with pUC57-Δ*sdhA* - (Adapted from: Froger and Hall 2007)

pUC57-*ΔsdhA* was cloned in *E. coli* DH5α by Heat Shock Method transformation in order to replicate the plasmid. Briefly, *E. coli* DH5α competence was induced through calcium ions which neutralize negative charges of both bacteria cell wall and plasmid to dissipate electrostatic repulsion. Competent cells tranformation was performed by an abrupt increase in temperature (heat shock) allowing the formation of pores through which plasmid DNA can enter. Finally, the selection of cloned cells was confirmed by their ability to grow on agar plates supplemented with antibiotic (section 2.2.6.).

2.3.2.1. Induction of *E. coli* DH5α competence

E. coli DH5α was grown in 50 ml of LB (section 2.2.6.) overnight at 37ºC with 150 rpm. A new growth was initiated in LB with an initial OD_{610nm} of 0.1 and maintained at 37ºC with 150 rpm until reaches an OD_{610nm} between 0.2 and 0.4. Bacterial suspensions were cooled on ice and centrifuged for 30s (13200 rpm). The pellet was suspended in 2

ml of a sterilized solution of $MgCl_2$ 0.1M (section 2.3.2.1.1.) and stored on ice for 5 min. After another centrifugation (30s), cells were suspended in 1 ml of sterilized solution of $CaCl_2$ 0.1M (section 2.3.2.1.2.) and stored on ice for at least 30 min. Finally, cells were again pelleted (30s) and suspended in 300 µl of $CaCl_2$ 0.1M (section 2.3.2.1.2.).

2.3.2.1.1. $MgCl_2$ 0.1 M Solution

For a final volume of 500 ml, 4.761 g of $MgCl_2$ were diluted in 500 ml of demineralized water. The solution was sterilized by autoclaving at 121ºC for 25 min.

2.3.2.1.2. $CaCl_2$ 0.1 M Solution

For a final volume of 500 ml, 7.351 g of $CaCl_2$ were diluted in 500 ml of demineralized water. The solution was sterilized by autoclaving at 121ºC for 25 min.

2.3.2.2. Transformation

To 100 uL of bacterial suspensions previous obtained in section 2.3.2.1., 1-10 µl of plasmid DNA was added. A control was performed without the addition of exogenous DNA. Suspensions were incubated in a water bath at 42º C during 45s to induce heat shock. After that, 1 ml of LB (section 2.2.7.) was added and cells were incubated at 37 º C for 1 hour allowing the expression of the genes coding for the antibiotic resistance. Finally, bacterial suspensions were plated in LB with ampicillin (section 2.2.6.) and incubated overnight at 37ºC.

2.3.3. pUC57-Δ*sdhA* plasmid purification

Transformed *E. coli* DH5α obtained from section 2.3.2.2. were grown overnight at 37ºC with 150 rpm in 50 ml of LB supplemented with ampicillin (section 2.2.8.). The resulting bacterial suspension (4-5 ml) was centrifuged and the plasmid was purified using ZR Plasmid Miniprep™ - Classic, according to the manufacturing instructions.

Briefly, the bacterial suspension was centrifuged and the pellet was suspended in lysis buffer. This solution has a high pH and contains detergents which disrupt the bacterial membranes, thus lysing the bacteria. Next, the neutralized buffer was added to neutralize the alkaline conditions and lower the pH. By gentle mixing the genomic DNA precipitates, as well as associated proteins, while plasmid DNA remains soluble. Centrifugation of this mixture pelleted the genomic DNA/proteins precipitate. The supernatant containing plasmid DNA was placed into a column and repeated washing steps were performed with ethanol to remove endonucleases, RNA, proteins, dyes, and low-molecular weight impurities. Finally, plasmid DNA that was trapped on the filter was eluted with sterile water and stored at -20ºC.

Plasmid DNA concentration was measured with NanoDrop™ 2000 spectrophotometer in order to prepare solutions with known concentrations since an efficient *L. pneumophila* natural transformation required a final concentration of $1 \mu g.\mu l^{-1}$ plasmid. When the plasmid concentration was lower, samples were lyophilized and ultrapure water was added in order to reach the desired concentration.

2.3.3.1. Confirmation of pUC57-Δ*sdhA* construction by restriction analysis

After plasmid extraction and purification, a confirmation step was performed to guarantee the presence of the cassette insert into the plasmid. A restriction analysis was carried out with type II endonucleases, EcoRI and BamHI (Table VI), with an incubation time of at least 1 hour at 37ºC in a final volume of 30 µl. The restriction products were analyzed by electrophoresis on 1% agarose gel, stained with ethidium bromide in Tris-acetate- EDTA (TAE) (subsection 2.3.3.1.1.), for 40 min at 85V. Gels were observed using a Gel Doc XR System (BioRad, Hercules, A, EUA) system under UV light. A positive result corresponded to one band of 2710 bp (size of pUC57 cloning vector) and another band of 1637 bp (size of Δ*sdhA* mutant construction). A molecular marker (NZYDNA Ladder III®) was added for comparison purposes.

Table VI. Enzymatic restriction analysis composition

Reagents	**Volume (µl)**	**Final Concentration**
10X BamHI K Buffer	3	1X
EcoRI Enzyme (Takara ®)	1	0.5 units.µl^{-1}
BamHI Enzyme (Takara ®)	1	0.5 units.µl^{-1}
Plasmid DNA	10	0.1-02 µg.µl^{-1}
Ultrapure Water[1]	15	-

[1] Sterilized by autoclaving (121ºC, 25 min)

2.3.3.1.1. Agarose Gel 1%

For a final volume of 150 ml, 1.5g of agarose was hydrated with TAE buffer 1X. To ease the dilution, the suspension was heated in a microwave for a few minutes at 500w. After complete dilution, the remaining solution was cooled and 7 µl of a 0.5% solution of ethidium bromide were added.

2.4. Homologous recombination between pUC57-Δ*sdhA* and *L. pneumophila* strains by natural transformation

2.4.1. *L. pneumophila* natural transformation (Buchrieser and Hilbi, 2013)

The selected *L. pneumophila* strains (Table I) were cultured out of cryopreserved suspensions preserved at -80ºC. After thawing, strains were cultured in BCYE (section 2.2.1.) plates and incubated at 37ºC for 3-4 days.

Parent *L. pneumophila* strains obtained from less than one-week old cultures in BCYE plates were incubated for 18-19 hours in tubes with 5 ml of AYE (section 2.2.3.) at 37ºC with 200 rpm in a shaking incubator. To confirm sterility of the medium, a tube with AYE was used as control. After incubation, the OD_{600} was measured using a spectrophotometer (Jenway 6405 W/Vis) and new inoculums were prepared in 5 ml of AYE to a final OD_{600} of 0.1. Tubes were left overnight in a shaking incubator, as described above, until reaching an OD_{600} of 0.7 -1.2. Cells were then collected for two 1.5 ml tubes and pelleted by full speed centrifugations for 1 min. The resulting pellets were suspended in 50 µl of AYE and placed into two 25µl spots on pre-warmed BCYE

plate. Plasmid DNA was added to one of the spot at a final concentration of 1 µg.µl^{-1} and no DNA was added to the other spot (control condition). Plates were incubated at 30ºC for 2 days.

After the incubation period, the cell mass on each spot were collected separately with an inoculation loop and transferred into 300 µl of sterilized PBS (section 2.4.1.1.). The obtained cell suspension was plated on BCYE medium with kanamycin (section 2.2.2.) and incubated for 3-5 days at 37º C.

2.4.1.1. Phosphate Buffered Saline (PBS)

Table VII. Phosphate Buffered Saline (PBS) composition

Reagents	**Concentration (g.l^{-1})**
Sodium Chloride	8
Potassium Chloride	0.2
Sodium Phosphate	1.78
Potassium Phosphate	0.14

All reagents were dissolved into 500 ml of distilled water and pH was adjusted to 7.2-7.4 with NaOH or HCl. The final solution was sterilized by autoclaving at 121ºC for 25 min.

2.4.2. Confirmation of the occurrence of homologous recombination

2.4.2.1. DNA Extraction

DNA extraction was performed according to an adaption of the Wiedmann-al-Ahmad *et al*., 1994 method. Clones resulting from natural transformation (subsection 2.4.1.) were cultured in BYCE medium with kanamycin (section 2.2.2.) for 2-3 days. Cultures were observed with the help of a magnifying glass to confirm its purity. One colony from each strain was suspended in 50 µl of lysis buffer (Table VIII) and boiled at 100º C for 5 minutes to get a complete cells lysis. The remaining cell components were sediment through centrifugation for 10 minutes at 13.200 rpm. The supernatant containing DNA was transferred to a new tube and conserved at -20º C.

Table VIII. Lysis buffer composition

Reagents	**Volume (µl)**	**Final Concentration**
Tween 20 2% [1]	1500	0.01 $g.ml^{-1}$
10 x NH_4 Buffer	300	1X
Ultrapure Water [2]	200	-

[1] Previously sterilized by filtration (0.22µm filter); [2] Sterilized by autoclaving (121ºC, 25 min)

2.4.2.2. *sdhA* gene Amplification from *L. pneumophila* transformed strains by Polymerase Chain Reaction - PCR

The *sdhA* and Δ*sdhA* genes from *L. pneumophila* strains were amplified by Polymerase Chain Reaction from the genomic DNA (section 2.4.2.1.). Briefly, reaction mixtures were prepared by sequential addition of the reagents in the final concentrations described in Table IX.

Table IX. PCR amplification composition

Reagents	**Volume (µl)**	**Final Concentration**
MasterMix (NZYTech®)	25	1X
Oligonucleotide sdhA F	2	4 pM
Oligonucleotide sdhA R	2	4 pM
DNA	2	0.1-0.2 $\mu g.\mu l^{-1}$
Ultrapure Water	19	-

Amplification reactions were performed on a thermal cycle with parameters described in Table X.

Table X. Thermal cycler parameters for PCR amplification

Step	Temperature (ºC)	Time	Stage	Nº of Cycles
1	95	5 min	Initial Denaturation	1
2	95	45 s	Denaturation	29
	50	45 s	Annealing	
	72	180s	Extension	
3	72	7 min	Final Extension	1
4	4	∞	Hold	

2.4.2.2.1. Oligonucleotides

Oligonucleotides were synthetized by NZYTech®. Each oligonucleotide was suspended in ultrapure water (sterilized by filtration and autoclaving – 121º C, 25 minutes) to achieve a final concentration of $1 \mu l.\mu g^{-1}$. Work solutions were prepared with a final concentration of $0.1\ \mu l.\mu g^{-1}$.

The sequence and properties of the oligonucleotides used for *sdhA* and Δ*sdhA* amplification are described in Table XI.

Table XI. Oligonucleotides sequence and properties

Primer	Primer Sequence	Gene region (bp)	Annealing Tempeature (ºC)	Fragment Size (bp)
sdhA F	5' – ATG ACA GTG GCA CGT AAC AGA TT– 3'	1-23	50	1637
sdhA R	5' – TTA GTA AAC GCG TTC TTT CGG CTC – 3'	1613 - 1637		

2.4.2.3. Analysis of PCR amplification products by agarose gel electrophoresis

The visualization of the amplified products was performed as described in subsection 2.3.1.1**.** The *sdhA* and Δ*sdhA* genes generate a band of 1637 bp.

2.4.2.4. Purification of the amplification products

PCR products were purified using NZYGelpure® Purification Kit according to the manufacturing instructions. Briefly, the amplification products (section 2.4.2.2.) were suspended in Binding Buffer and added to a spin column. Posterior centrifugation of the spin column allowed the binding of the DNA molecules to the silica-filter while the impurities flowed through into the collection tube. In the next step the wash buffer (containing ethanol) was added to remove residual impurities. Finally, ultrapure water (previously autoclaved and filtrated) was added to the column and released DNA from the silica-filter. The purified DNA fragments were stored at -20ºC.

2.4.2.5. Restriction analysis of the amplification products

After purification of the amplification products (section 2.4.2.4.), a confirmation step was performed to guarantee the presence of Δ*sdhA* mutation. A restriction analysis was carried out with type II endonuclease, Nru I (Table XII), with an incubation time of at least 1 hour at 37ºC in a final volume of 20 µl. The resulting fragments were analyzed by agarose gel electrophoresis, performed as described in section 2.3.3.1. The presence of Δ*sdhA* was confirmed when one band of 1077 bp and another band of 560 bp were observed.

Table XII. Enzymatic restriction analysis composition

Reagents	Volume (µl)	Final Concentration
10x NruI Buffer	2	1X
0.2 % BSA	2	4×10^{-6} g.µl^{-1}
NruI Enzyme (Takara ®)	1	0.5 units.µl^{-1}
DNA	10	0.1-0.2 µg.µl^{-1}
Ultrapure Water	5	-

2.4.2.6. Δ*sdhA* mutation sequencing analysis

To confirm the Δ*sdhA* mutation sequence of *L. pneumophila* strains inferred from the restriction analysis (section 2.4.2.6.), the purified amplification products (section 2.4.2.4.) were sequenced by a commercial sequencing provider (GATC Biotech). The results were processed with Sequencing Scanner

(https://products.appliedbiosystems.com) and Bioedit software (Hall *et al*., 1999) and compared to Δ*sdhA* mutant construction (Figure 4).

2.5. Complementation of Δ*sdhA L. pneumophila* mutant strains by natural transformation

In order to guarantee that the phenotype obsorved in Δ*sdhA L. pneumophila*-infected *G. mellonella* was due to deletion of *sdhA* gene, each Δ*sdhA* strain was complemented with wild-type *sdhA* (WT-*sdhA*) by natural transformation. Briefly, the wild-type *sdhA* complementation gene was amplified and purified and used for complementation by natural transformation in *L. pneumophila* mutant strains.

2.5.1. Wild-type *sdhA* complementation gene

To generate complemented strains with the wild-type *sdhA* gene, both wild-type DNA extraction, wild type *sdhA* amplification**,** analysis by agarose gel electrophoresis and purification of amplified products were performed as previously described in subsections 2.4.2.1., 2.4.2.2, 2.4.2.3, 2.4.2.4, respectively. Moreover, wild-type *sdhA* concentration was measured with NanoDrop™ 2000 spectrophotometer in order to prepare solutions with known concentrations since an efficient *L. pneumophila* natural transformation requires a final concentration of 1µg.µl^{-1}. When the wild-type *sdhA* concentration was lower, samples were lyophilized and ultrapure water was added in order to reach the desired concentration.

2.5.2. Natural transformation – Complementation

Natural transformation of wild-type *sdhA* gene in *L. pneumophila* mutant strains was performed as described in section 2.4.1. with small adaptations. Both AYE and BCYE were supplemented with kanamycin (sections 2.2.2. and 2.2.4., respectively). Furthermore, in the last step of protocol, the remaining cell suspension was diluted by the method serial dilution until reach a final concentration of 10^7 and plated in BCYE.

2.5.3. Confirmation of the occurrence of homologous recombination – complementation

Colonies obtained by natural transformation – complementation (section 2.5.2.) were cultured in BCYE (section 2.2.1) for 2-3 days. The cultures were observed with the help of a magnifying glass to confirm its purity. One colony was cultured in BCYE supplemented with kanamycin (section 2.2.2.) for 2-3 days, and colonies that did not grew were selected as possible candidate of complementation due to its loss of resistance to kanamycin.

Following confirmation steps were performed as described in subsection 2.4.2., except that a positive result corresponded to one band of 859 bp and band of 778 bp.

2.6. *L. pneumophila* growth for injection

The selected wild-type (Table I), mutant and complemented *L. pneumophila* strains were cultured out of cryopreserved suspensions preserved at -80ºC. After thawing, strains were cultured in BCYE plates (section 2.2.1., supplemented with kanamycin for mutant strains, section 2.2.2.) and incubated at 37ºC for 3-4 days.

L. pneumophila strains obtained from BCYE plates with less one-week old cultures were incubated for 18-19 hours at 37ºC with 200 rpm in a shaking incubator, in 5 ml of AYE (section 2.2.3.) (supplemented with kanamycin for mutant strains, section 2.2.4.). To assess the sterility of the medium, a tube with only AYE was used as a control. After incubation, the OD_{600} was measured using a spectrophotometer (Jenway 6405 W/Vis) and new inoculums were prepared in 5 ml of AYE (section 2.2.3.) (supplemented with kanamycin for mutant strains, section 2.2.4.) to a final OD_{600} of 0.1. Tubes were left for 21 hours in a shaking incubator as described above, since the infection experiments in *G. mellonella*, required *L. pneumophila* strains to be in the post-exponential phase of growth (Harding et al., 2013). Sousa *et al*., 2017 calculated the relation between OD_{600} and $CFU.ml^{-1}$ for each strain (Table XIII) allowing to use a constant injection concentration for all tested strains.

Table XIII. *L. pneumophila* CFU/ml corresponding to an OD_{600} of 1 (Sousa *et al.*, 2017)

L. pneumophila strains	**$CFU.ml^{-1}$**
Philadelphia 1	1.89×10^9
Micu B	2.0×10^9
Huc1	5.82×10^9
NMex1	3.9×10^9
Ice27	3.2×10^9

2.7. *G. mellonella* infection

2.7.1. Larvae preparation

For an appropriate injection, larvae were selected with 2-3 cm of length without any signs of darkening (Harding *et al.*, 2013).

2.7.2. *G. mellonella* injection

All the following experiments related with *G. mellonella* infection with *L. pneumophila* were performed as previously described by Harding *et al*., 2013 and Sousa *et al*., 2017, with some adjustments.

After selection of 10 healthy larvae, the left pro-leg (injection site), that corresponds the most common infection route (intrahemocoelic injection) (Tsai *et al.*, 2016), was disinfected with 70% ethanol. To obtain a final concentration of 1×10^{9} $CFU.mL^{-1}$, inoculums resulting from subsection 2.7. were diluted in PBS (section 2.4.1.1.), and 10 µl of the resulting bacteria suspension were injected in each larva. As control, 10 larvae were injected with PBS, and another 10 larvae were not injected. After injection, larvae were incubated at 37ºC in the dark. Assays proceed for 72 hours since afterwards pupa formation could occasionally be seen. Larvae were obsorved at 18, 24, 48 and 72 hours post-infection. Three independent replicates (10 larvae per replicates) for each treatment (wild-type, mutant and complemented strain) were performed.

2.7.3. Larvae monitoring

For each treatment, the injected larvae were individually examined for both mortality and health status that allowed to assess both survival and the larvae health index

(Tsai *et al.*, 2016). The phenotypic evaluation parameters are described in Table XIV and represented on Figure 5.

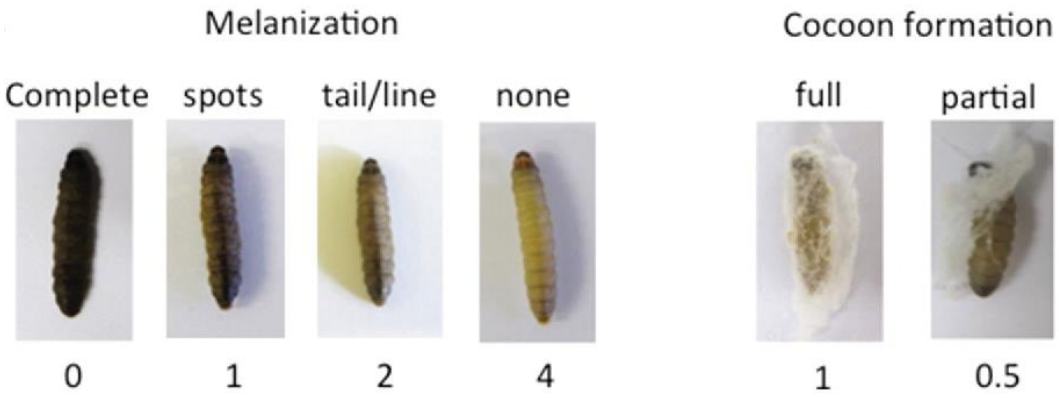

Figure 5. Health index scoring system. Melanization of larva occurs as a result of an immune response against infection and, frequently, a complete melanization correlates with death of the larva soon after. Likewise, more cocoon coincides to a heathier larva (adapted from Tsai *et al* 2016).

Table XIV. *G. mellonella* Health Index Scoring System (Adapted from: Tsai *et al*., 2016)

Category	Description	Score
	No movement	0
Activity	Minimal movement on stimulation	1
	Move when stimulated	2
	Move without stimulation	3
Cocoon Formation	No cocoon	0
	Partial cocoon	0.5
	Full cocoon	1
Melanization	Black larvae	0
	Black spots on brown larvae	1
	Spots on beige larvae	2
	No melanization	4
Survival	Dead	0
	Alive	2

2.7.4. Statistical analysis

All quantitative data were obtained from three independent assays. Standard deviation was used to calculate errors bars. The Mann-Whitney test to determine *P*-values of health index was performed using GraphPad Prism®.

The Mantel-Cox test to determine *P*-values of total survival of and survival only at 18, 24, 48 and/or 72 hours was also performed using GraphPad Prism®. Differences were considered to be statistically significant if the *P*-values were lower than 0.05.

Chapter III - Results and Discussion

3.1. *sdhA* mutant construction and complementation with wild-type *sdhA*

3.1.1. Successful introduction of Δ*sdhA* in *L. pneumophila* wild-type strains by homologous recombination

In order to induce *sdhA* mutation, the selected *L. pneumophila* strains (Table I) were submitted to a natural transformation protocol (section 2.4.1.) to induce the uptake of pUC57-Δ*sdhA* resulting on the growth of kanamycin-resistant *L. pneumophila* clones. DNA extraction (section 2.4.2.1.) from the selected strains and a subsequent *sdhA* gene amplification by PCR (section 2.4.2.2.) were performed (Fig. 6).

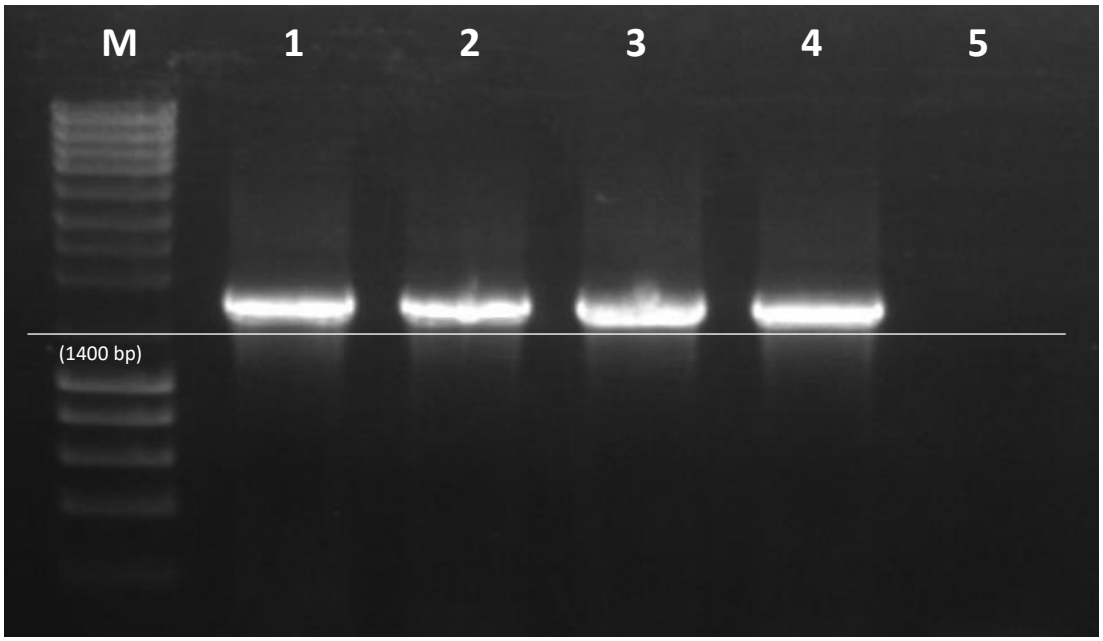

Figure 6. Analysis of *sdhA* gene PCR amplification products by agarose gel (1%) electrophoresis. Lanes: 1 to 4, amplification product**s** from putative transformed clones (1637 bp); 5, a negative control. A molecular marker (M) was added for comparison purposes.

To confirm the presence of Δ*sdhA* mutated gene, PCR amplification products were subjected to a restriction analysis and the resulting fragments were analysed by agarose gel electrophoresis. Since NruI enzyme cut at 560 bp, positive result for the presence of Δ*sdhA* was considered when two fragments of 1077 bp and 560 bp were obtained from the restriction analysis. On the contrary, a negative results was considered when two fragments of 859 bp and 778 bp were visualized (Fig. 7)

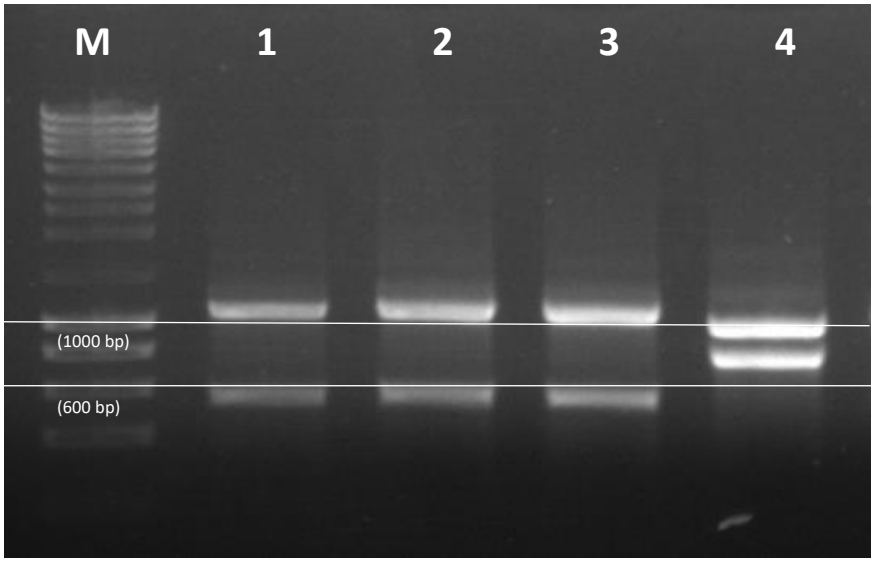

Figure 7. Analysis of the restriction profile of *sdhA* PCR amplification products by agarose gel (1%) electrophoresis. Lanes: 1 to 3, positive result for the presence of Δ*sdhA* (first fragment: 1077 bp; second fragment: 560 bp); 4, negative result for the presence of Δ*sdhA* (first fragment: 859 bp; second fragment 778 bp). A molecular marker (M) was added for comparison purposes.

Further confirmation procedures were adopted and the nucleotide sequence from each amplification product was determined by Sanger sequencing method (GATC Biotech). Comparison between the obtained sequences with Δ*sdhA* construction sequence and *sdhA* wild type sequence allowed to finally conclude which *L. pneumophila* strains carried a Δ*sdhA* gene (Fig. 8).


Start (0)
5′
3′
ATGACAGTGGCACGTAACAGATTTGATGCGGTAATTATAGGAGCTGGTGGTGCAGGAATGCGTGCTGCACTCCAGATGACAAATTCTGGTTTAAAAGTAG
TACTGTCACCGTGCATTGTCTAAACTACGCCATTAATATCCTCGACCACCACGTCCTTACGCACGACGTGAGGTCTACTGTTTAAGACCAAATTTTCATC
M T V A R N R F D A V I I G A G G A G M R A A L Q M T N S G L K V
sdhA
CGTTGTTATCAAAAGTATTTCCAACCCGCTCACATACTGTGTCGGCTCAAGGCGGTATTACAGCGGCTTTGGGCAATGCTGATGAAGACGATTGGCGTTG
GCAACAATAGTTTTCATAAAGGTTGGGCGAGTGTATGACACAGCCGAGTTCCGCCATAATGTCGCCGAAACCCGTTACGACTACTTCTGCTAACCGCAAC
A L L S K V F P T R S H T V S A Q G G I T A A L G N A D E D D W R W
sdhA
GCACATGTATGATACCGTGAAAGGCGCTGATTACATTGGTGATCAGGATTGTATAGAGTATTTGTGTAAAACTGGCCCTGAAGCAGTTTATGAACTGGAG
CGTGTACATACTATGGCACTTTCCGCGACTAATGTAACCACTAGTCCTAACATATCTCATAAACACATTTTGACCGGGACTTCGTCAAATACTTGACCTC
H M Y D T V K G A D Y I G D Q D C I E Y L C K T G P E A V Y E L E
sdhA
CATATGGGGCTACCTTTTTCACGAATGGATAATGGAAAAATTTACCAACGGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAA
GTATACCCCGATGGAAAAAGTGCTTACCTATTACCTTTTTAAATGGTTGCCTTCTAGGAAACTAGAAAAGATGCCCCAGACTGCGAGTCACCTTGCTTTT
H M G L P F S R M D N G K I Y Q R K I L S F L R G L T L S G T K
sdhA
promotor
CTCACGTTAAGGGATTTTGGTCATGAACAATAAAACTGTCTGCTTACATAAACAGTAATACAAGGGGTGTTATGAGCCATATTCAACGGGAAACGTCTTG
GAGTGCAATTCCCTAAAACCAGTACTTGTTATTTTGACAGACGAATGTATTTGTCATTATGTTCCCCACAATACTCGGTATAAGTTGCCCTTTGCAGAAC
T H V K G F W S T I K L S A Y I N S N T R G V M S H I Q R E T S C
promotor
kan
sdhA
CTCGAGGCCGCGATTAAATTCCAACATGGATGCTGATTTATATGGGTATAAATGGGCTCGCGATAATGTCGGGCAATCAGGTGCGACAATCTATCGATTG
GAGCTCCGGCGCTAATTTAAGGTTGTACCTACGACTAAATATACCCATATTTACCCGAGCGCTATTACAGCCCGTTAGTCCACGCTGTTAGATAGCTAAC
S R P R L N S N M D A D L Y G Y K W A R D N V G Q S G A T I Y R L
kan
sdhA
TATGGGAAGCCCGATGCGCCAGAGTTGTTTCTGAAACATGGCAAAGGTAGCGTTGCCAATGATGTTACAGATGAGATGGTCAGACTAAACTGGCTGACGG
ATACCCTTCGGGCTACGCGGTCTCAACAAAGACTTTGTACCGTTTCCATCGCAACGGTTACTACAATGTCTACTCTACCAGTCTGATTTGACCGACTGCC
Y G K P D A P E L F L K H G K G S V A N D V T D E M V R L N W L T
kan
sdhA
AATTTATGCCTCTTCCGACCATCAAGCATTTTATCCGTACTCCTGATGATGCATGGTTACTCACCACTGCGATCCCCGGGAAAACAGCATTCCAGGTATT
TTAAATACGGAGAAGGCTGGTAGTTCGTAAAATAGGCATGAGGACTACTACGTACCAATGAGTGGTGACGCTAGGGGCCCTTTTGTCGTAAGGTCCATAA
E F M P L P T I K H F I R T P D D A W L L T T A I P G K T A F Q V L
kan
sdhA
AGAAGAATATCCTGATTCAGGTGAAAATATTGTTGATGCGCTGGCAGTGTTCCTGCGCCGGTTGCATTCGATTCCTGTTTGTAATTGTCCTTTTAACAGC
TCTTCTTATAGGACTAAGTCCACTTTTATAACAACTACGCGACCGTCACAAGGACGCGGCCAACGTAAGCTAAGGACAAACATTAACAGGAAAATTGTCG
E E Y P D S G E N I V D A L A V F L R R L H S I P V C N C P F N S
kan
sdhA
GATCGCGTATTTCGTCTCGCTCAGGCGCAATCACGAATGAATAACGGTTTGGTTGATGCGAGTGATTTTGATGACGAGCGTAATGGCTGGCCTGTTGAAC
CTAGCGCATAAAGCAGAGCGAGTCCGCGTTAGTGCTTACTTATTGCCAAACCAACTACGCTCACTAAAACTACTGCTCGCATTACCGACCGGACAACTTG
D R V F R L A Q A Q S R M N N G L V D A S D F D D E R N G W P V E
kan
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800
900
1000

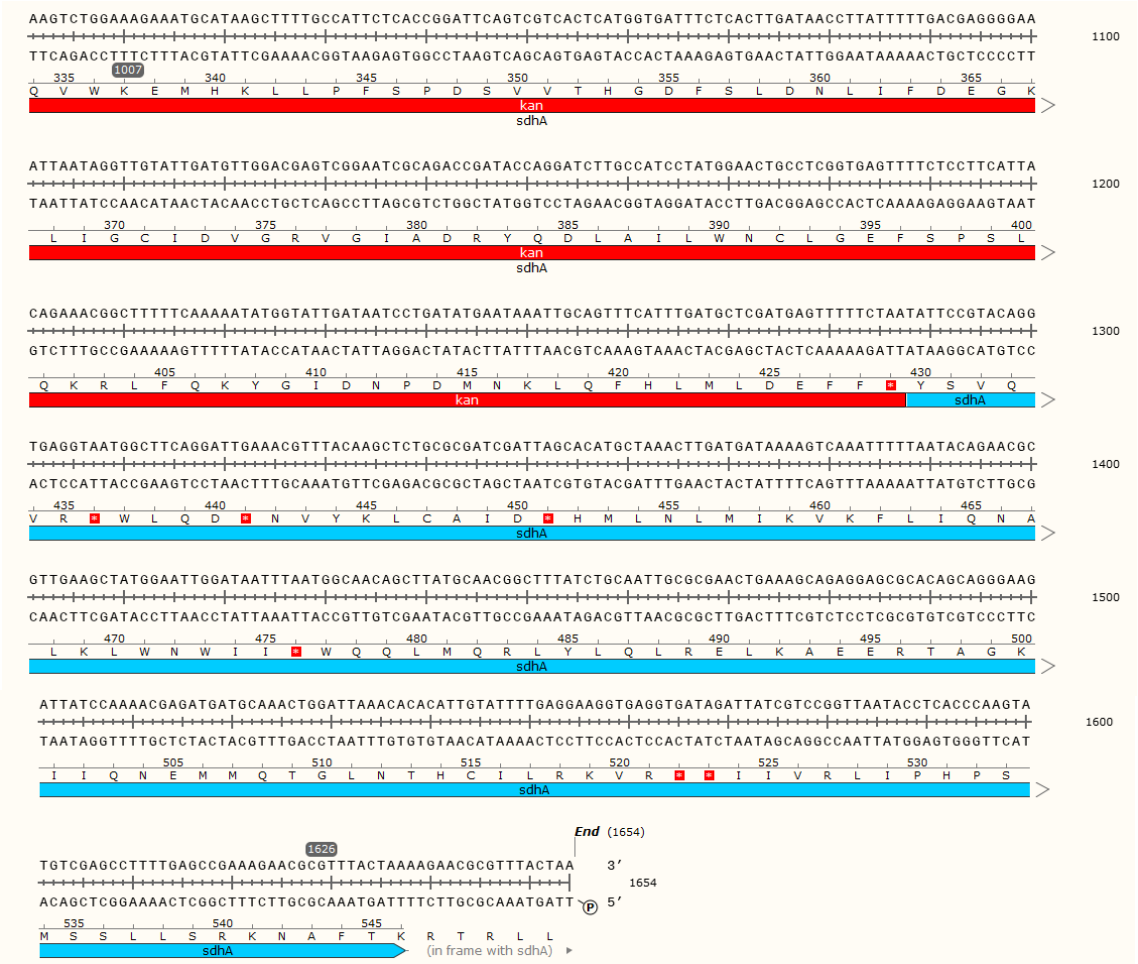

Figure 8. Δ*sdhA* mutant construction cassette sequence. 1 to 350 bp - initial portion of *L. pneumophila* will type *sdhA* gene (blue); 351 bp to 470 bp - promoter of kanamycin resistance gene (orange); 471 bp to 1287 bp - kanamycin resistance gene (red); 1288 bp to 1637 bp – final portion of L. pneumophila will type *sdhA* gene (blue).

3.1.2. Successful complementation of Δ*sdhA* strain with wild-type *sdhA* by homologous recombination

In order to perform Δ*sdhA* strain complementation with the wild-type *sdhA* (WT-*sdhA*), mutant strains were subjected to a natural transformation protocol (section 2.5.2.) that resulted in the growth of kanamycin-susceptible *L. pneumophila* strains. DNA extraction (section 2.4.2.1.) from the selected strains and a subsequent *sdhA* gene amplification by PCR (section 2.4.2.2.) were performed. To confirm that complementation of Δ*sdhA* strain was successfully accomplished, PCR amplification

products were subjected to a restriction analysis and the resulting fragments were analysed by agarose gel electrophoresis. The same principle described above (section 3.1.1) was used to discriminate between wild-type *sdhA* gene and Δ*sdhA* (Fig. 9).

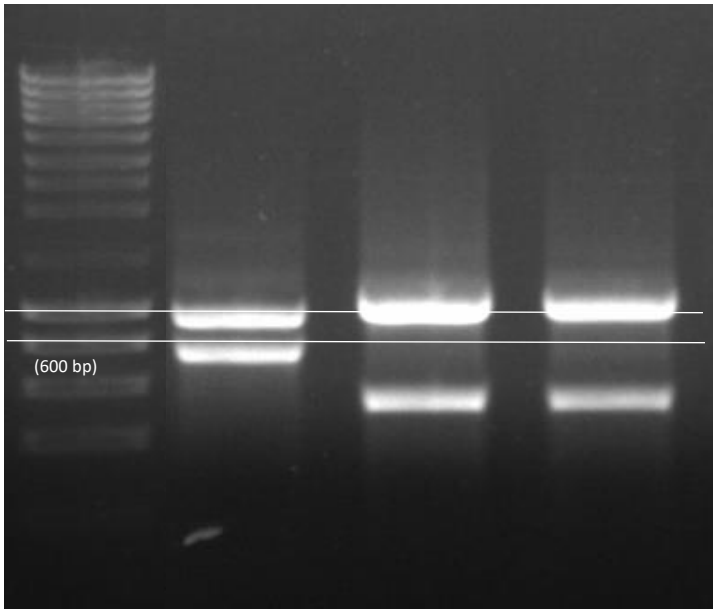

Figure 9. Analysis of the restriction profile of *sdhA* PCR amplification products by agarose gel (1%) electrophoresis. Lanes: 1, positive result for the complementation of Δ*sdhA* (first fragment: 859 bp; second fragment: 778 bp); 2 and 3, negative result for the complementation of Δ*sdhA* (first fragment: 1077 bp; second fragment 560 bp). A molecular marker (M) was added for comparison purposes.

Further confirmation procedures were adopted and the nucleotide sequence from each amplification product was determined by Sanger sequencing method (GATC Biotech). Comparison between the obtained sequences with Δ*sdhA* construction sequence and *sdhA* wild type sequence allowed to finally conclude which *L. pneumophila* strains were successfully complemented with the corresponding *sdhA* wild-type gene.

3.2. *sdhA* role in *Legionella pneumophila* pathogenesis in the *Galleria mellonella* infection model

3.2.1. *sdhA* role in disease-related *L. pneumophila* Philadelphia1 strain pathogenesis in *Galleria mellonella* infection model

3.2.1.1. Influence of *sdhA* in *L. pneumophila* Philadelphia1-infected *G. mellonella* survival

In order to determine if infection of *G. mellonella* with *L. pneumophila* Philadelphia1 strain induced a *sdhA*-dependent larval mortality, 10 larvae per replicate were infected with 10^7 CFU of Philadelphia1 wild-type, Δ*sdhA* or Δ*sdhA* + wt:*sdhA*. As control, 10 larvae were injected with PBS and another 10 larvae were not injected. *G. mellonella* larvae were observed at 18, 24, 48 and 72 hours post-infection (Fig. 10). The Mantel-Cox Test was performed to determine *P*-values using GraphPad Prism. Differences were considered statically significant if *P*-values were lower than 0.05.

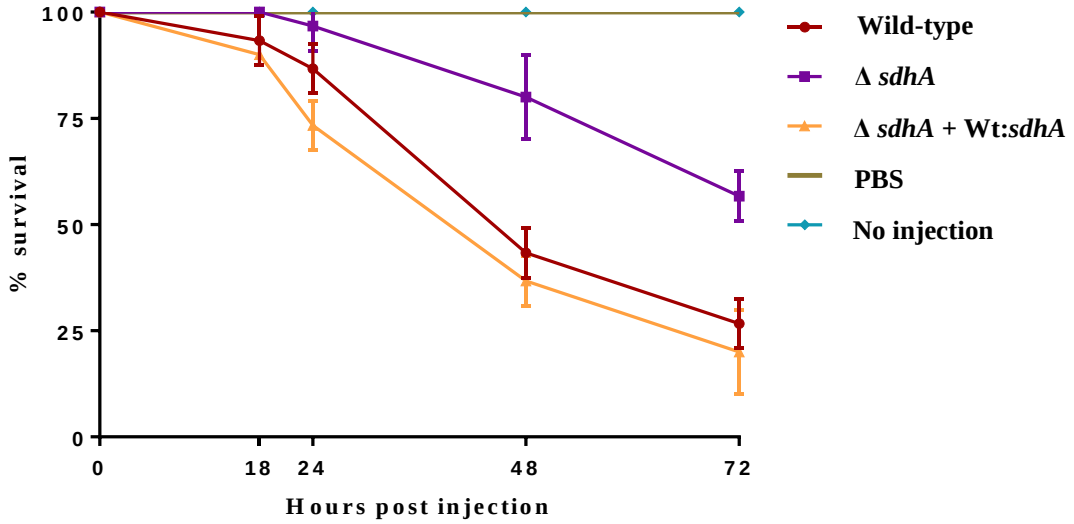

Figure 10. Infection with *L. pneumophila* Philadelphia1 strain induces *sdhA*-dependent larval mortality (*P*<0.05, Mantel-Cox Test). Survival (%) of *G. mellonella* infected with 10^7 CFU of Philadelphia1 wild type (WT) or Philadelphia1 Δ*sdhA* or Philadelphia1 Δ*sdhA* + WT:*sdhA*, incubated at 37 °C for 72 h. Negative controls with only PBS and no injection were used. Results are the mean of three separate experiments, ± standard deviation with ten larvae per condition.

No mortality events were observed in larvae that were either injected with PBS or not injected, demonstrating that injection did not caused mortality (Fig. 10).

In the course of the experiment, the Δ*sdhA* Philadelphia1 strain was significantly impaired for its ability to induce larval mortality when compared to the wild-type strain (*P* = 0.0061, Mantel-Cox Test). As expected, the complemented Δ*sdhA* Philadelphia1 strain with the WT-*sdhA* showed no significant differences on larval mortality when compared to the wild-type strain (*P* = 0.3578, Mantel-Cox Test), demonstrating that the mutation in *sdhA* in Philadelphia1 was responsible for the observed phenotype and that the WT- *sdhA* restored the ability to induce larval mortality (Fig. 10). From these results we can conclude that *sdhA* is an important gene for the pathogenicity of this disease-related strain in *G. mellonella* as it was previously described for the disease-related *L. pneumophila* strain 130b (Harding *et al.*, 2013).

3.2.1.2. Influence of *sdhA* in *L. pneumophila* Philadelphia1-infected *G. mellonella* health index

The Health Index (Tsai *et al*, 2016) for each larvae was also determined (Fig.11), along with the survival of *G. mellonella* infected with 10^7 CFU of Phildadelphia1 wild-type, Δ*sdhA* or Δ*sdhA* + wt:*sdhA*, based on the observation of four features: cocoon formation, survival, melanization and activity (Table XIV). Higher activity and more cocoon formation coincide with healthier larvae. Melanization of larvae as a result of an immune response against infection and, frequently, a complete melanization correlates with death of the larvae soon after (Fig. 11). The Mann-Whintey Test was performed to determine *P*-values using GraphPad Prism. Differences were considered statically significant if *P*-values were lower than 0.05.

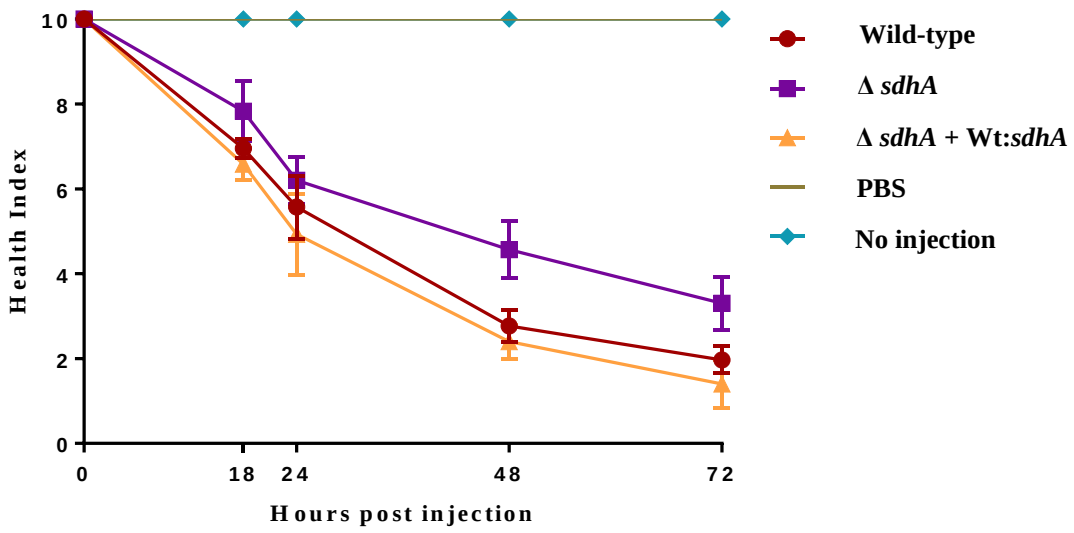

Figure 11. Infection with *L. pneumophila* Philadelphia1 strain induced *sdhA*-independent health index decrease (*P*>0.05, Mann-Whitney Test). Health Index of *G. mellonella* infected with 10^7 CFU of Philadelphia wild type (WT) or Philadelphia1 Δ*sdhA* or Philadelphia1 Δ*sdhA* + WT:*sdhA*, incubated at 37 °C for 72 hr. Negative controls with only PBS and no injection were used. Results are the mean of three separate experiments, ± standard deviation with ten larvae per condition. Health Index scores of *G. mellonella* infected with Philadelphia wild type (WT) or Philadelphia1 Δ*sdhA* or Philadelphia1 Δ*sdhA* + WT:*sdhA* are represented in appendix table A, B and C, respectively.

Both larvae with injection of PBS and no injection did not presented signals of decrease of health demonstrating that remained healthy in the course of experiment (Fig. 11).

During the experiment, the Δ*sdhA* Philadelphia1 strain did not had significantly differences in the health index when compared to the wild-type strain ($P = 0.1641$, Mann-Whitney Test). Likewise, the complementation of Δ*sdhA* Philadelphia1 strain with the WT-*sdhA* showed no significant differences in Health Index scores ($P = 0.6432$, Mann-Whitney Test). The absence of differences between Δ*sdhA* Philadelphia1 strain and the wild-type strain were consistent with those previously described for the disease-related *L. pneumophila* strain 130b (Harding *et al.*, 2013). That study demonstrated that, although *L. pneumophila* 130b Δ*sdhA* strain was not able to replicate in *G. mellonella*, it was able to persist in the insect. These evidences suggest that the higher melanization and the decrease in activity observed in larvae infected with Philadelphia 1 Δ*sdhA* strain resulted from the response of *G. mellonella* against the infection leading to a considerable decrease on the health index of the remaining larvae (Fig. 11).

3.2.1.3. *sdhA* influence in the kinetics of *L. pneumophila* Philadelphia1-infected *G. mellonella* mortality

In order to evaluate the effect of Philadelphia1 *sdhA* gene in the kinetics of *G. mellonella* infected mortality, the ability of Δ*sdhA* Philadelphia1 strain to induce dead was compared to the one from wild-type Philadelphia1 strain at 18 h, 24 h, 48 h and 72 h post infection (Fig. 12). The Mantel-Cox test to determine *P*-values was performed using GraphPad Prism software. Differences were considered to be statistically significant if the *P*-value was lower than 0.05

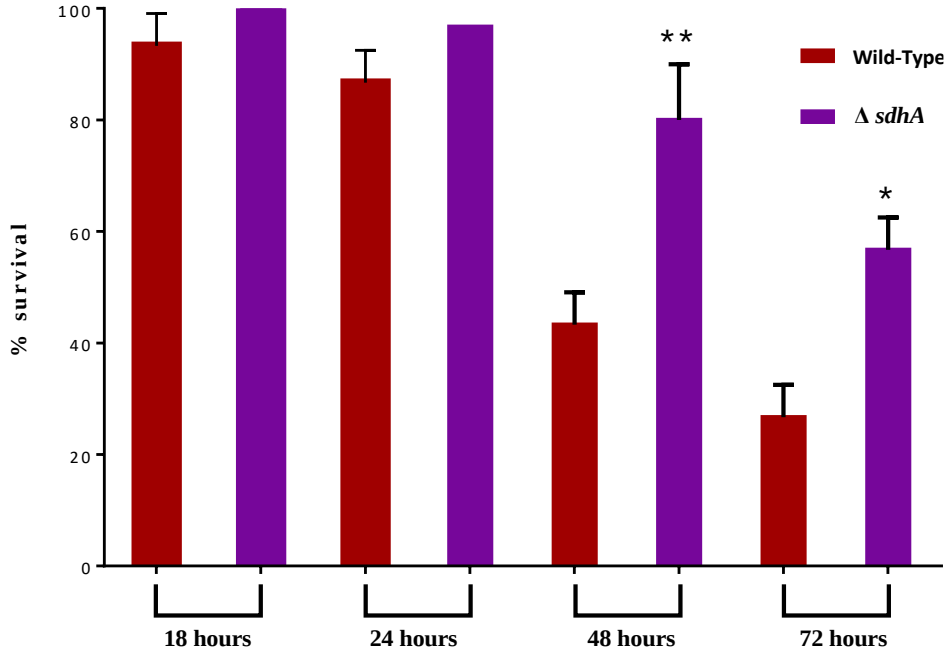

Figure 12. Comparison between the survival of infected *G. mellonella* with *L. pneumophila* Philadelphia1 wild-type and Philadelphia1 Δ*sdhA*. Survival (%) of *G. mellonella* infected with 10^7 CFU of Philadelphia1 wild-type (WT) or Philadelphia1 Δ*sdhA*, incubated at 37 ºC for 72 hours. Results are the mean of three separate experiments, ± standard deviation with ten larvae per condition. Differences were considered statically significant if $P < 0.05$ (*), highly significant if $P < 0.01$ (**), and extremely significant if $P < 0.001$ (***).

Philadelphia1 Δ*sdhA* strain was significantly impaired on its ability to induce larval mortality when compared to the wild-type strain at 48 hours ($P = 0.0052$, Mantel-Cox Test) and 72 hours ($P = 0.0493$, Mantel-Cox Test) (Fig. 12). Previous studies on the influence of SdhA in the virulence of *L. pneumophila* serogroup 1 strain 130b in *G. mellonella* (Harding *et al*., 2013), showed that in the absence of this effector a disruption of LCV membrane occurred with the consequent degradation of this bacteria by the larvae immune system. Furthermore, they showed that infection of *G. mellonella* with Δ*sdhA* strain resulted in a rapid hemocyte decrease however this decrease was transitory since 24 hours post infection the number of hemocytes increased, due to the loss of bacterial replication, proposing that hemocytes decrease was not sufficient to cause larval mortality (Harding *et al*., 2013) Since the survival of *G. mellonella* infected with Philadelphia1

Δ*sdhA* strain was impaired on its ability to induce larval mortality when compared to the wild-type strain, it is possible that, such as in *L. pneumophila* serogroup 1 strain 130b, the low number of bacteria (Philadelphia1 Δ*sdhA* strain) led to an incapacity to infect all remaining hemocytes due to an incomplete replication of bacteria. On other hand, an efficient replication of bacteria (wild-type) resulted in a larger destruction of all hemocytes that combined with high number of bacteria resulted in the dead of larvae (Harding *et al*., 2013).

3.2.2. *sdhA* role in natural environmental *L. pneumophila* NMex1 strain pathogenesis in *Galleria mellonella* infection model

3.2.2.1. Influence of *sdhA* in *L. pneumophila* N. Mex1-infected *G. mellonella* survival

In order to determine if infection of *G. mellonella* with *L. pneumophila* N.Mex1 strain induced an *sdhA*-dependent larval mortality (Fig. 13), it was used the same experimental design previously described in section 3.2.1.1.

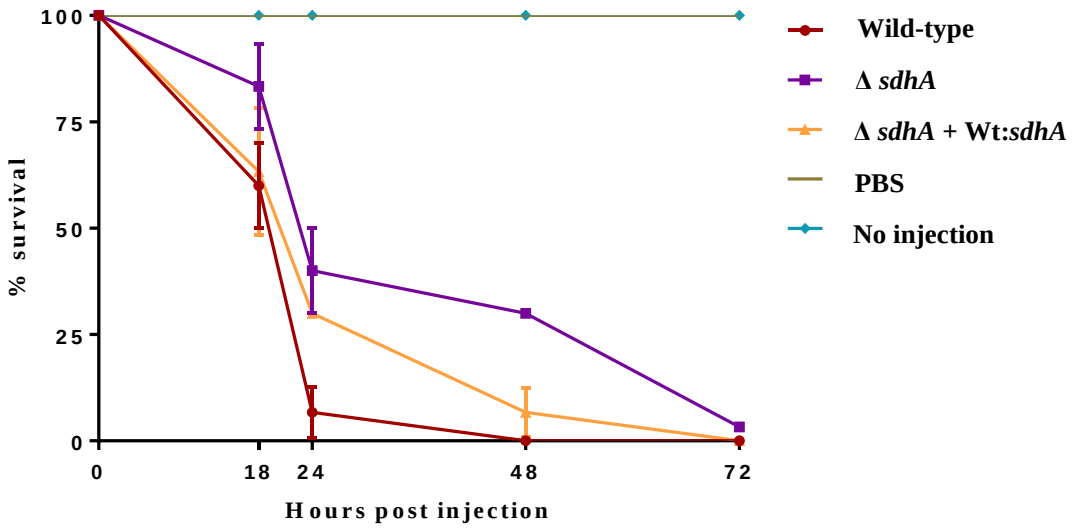

Figure 13. Infection with *L. pneumophila* NMex1 strain induces *sdhA*-dependent larval mortality (*P*<0.05, Mantel-Cox Test). Survival (%) of *G. mellonella* infected with 10^7 CFU of NMex1 wild type (WT) or NMex1 Δ*sdhA* or Nmex1 Δ*sdhA* + WT:*sdhA*, incubated at 37 °C for 72 h. Negative controls with only PBS and no injection were used. Results are the mean of three separate experiments, ± standard deviation with ten larvae per condition.

No mortality events were observed in larvae that were either injected with PBS or not injected, demonstrating that injection did not caused mortality (Fig. 13).

In the course of the experiment, the Δ*sdhA* N.Mex1 strain was significantly impaired for its ability to induce larval mortality when compared to the wild-type strain (*P* =0.0007, Mantel-Cox Test). As expected, the complemented Δ*sdhA* N.Mex1 strain with the WT-*sdhA* showed no significant differences on larval mortality when compared to the wild-type strain (*P* = 0.0816, Mantel-Cox Test), demonstrating that the mutation in *sdhA* in N.Mex1 was responsible for the observed phenotype and that the WT- *sdhA* restored the ability to induce larval mortality (Fig. 13). From these results we can conclude that *sdhA* is an important gene for the pathogenicity of this natural environmental strain in *G. mellonella* as it was previously described for the disease-related *L. pneumophila* strain 130b (Harding *et al.*, 2013).

3.2.2.2. Influence of *sdhA* in *L. pneumophila* NMex1-infected *G. mellonella* health index

The Health Index (Tsai et al., 2016) for each larvae was determined (Fig. 14) as described in section 3.1.2.2.

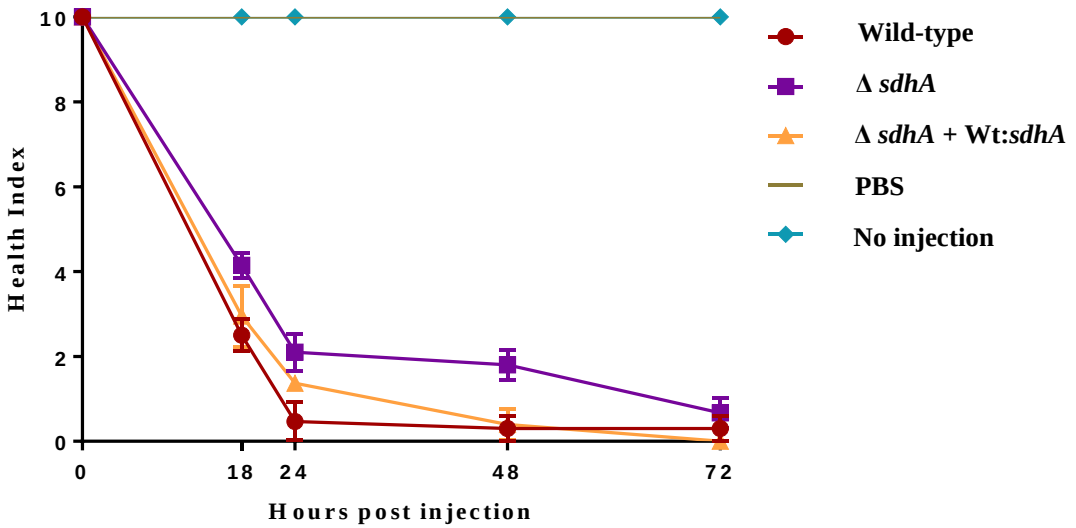

Figure 14. Infection with *L. pneumophila* NMex1 strain induced *sdhA*-dependent health index decrease (*P*<0.05, Mantel-Whitney Test). Health Index of *G. mellonella* infected with 10^7 CFU of NMex1 wild type (WT) or NMex1 Δ*sdhA* or Nmex1 Δ*sdhA* + WT:*sdhA*, incubated at 37 °C for 72 hr. Negative controls with only PBS and no injection were used. Results are the mean of three separate experiments, ± standard deviation with ten larvae per condition. Health Index scores of *G. mellonella* infected with NMex1 wild type (WT) or NMex1 Δ*sdhA* or NMex1 Δ*sdhA* + WT:*sdhA* are represented in appendix table D, E and F, respectively.

Both larvae with injection of PBS and no injection did not presented signals of decrease of health demonstrating that remained healthy in the course of experiment (Fig. 14).

During the experiment, the Δ*sdhA* NMex1 strain had significantly increase on the health index when compared to the wild-type strain (P = 0.0163, Mann-Whitney Test). On other hand, the complementation of Δ*sdhA* NMex1 strain with the WT-*sdhA* showed no significant differences in Health Index scores (P = 0.677, Man-Whitney Test)

demonstrating that NMex1WT-*sdhA* restored the ability to induce larval Health Index decrease.

3.2.2.3. *sdhA* influence in the kinetics of *L. pneumophila* NMex1-infected *G. mellonella* mortality

In order to evaluate the effect of NMex1 *sdhA* gene in the kinetics of *G. mellonella* infected mortality (Fig. 15) the same experimental design previously described in section 3.2.1.3. was used.

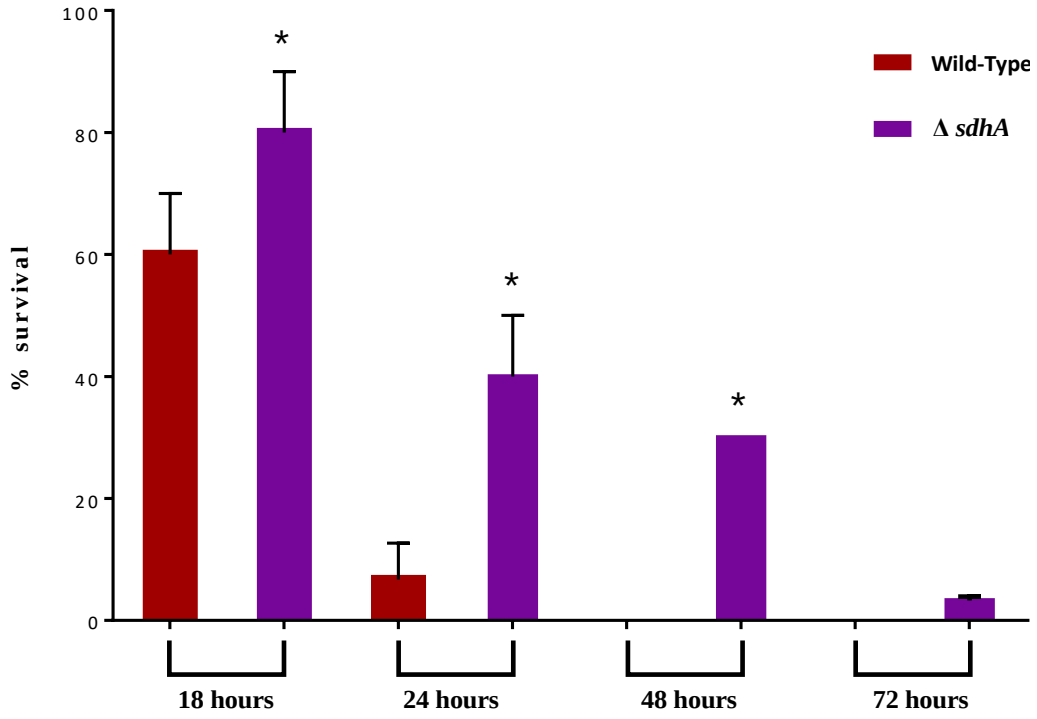

Figure 15. Comparison between the survival of infected *G. mellonella* with *L. pneumophila* NMex1 wild-type and NMex1 Δ*sdhA*. Survival (%) of *G. mellonella* infected with 10^7 CFU of NMex1 wild-type (WT) or NMex1 Δ*sdhA*, incubated at 37 ºC for 72 hours. Results are the mean of three separate experiments, ± standard deviation with ten larvae per condition. Differences were considered statically significant if $P < 0.05$ (*), highly significant if $P < 0.01$ (**), and extremely significant if $P < 0.001$ (***).

NMex1 Δ*sdhA* strain was significantly impaired on its ability to induce larval mortality when compared to the wild-type strain at 18 hours (P = 0.047, Mantel-Cox Test), 24 hours (P = 0.012, Mantel-Cox Test) and 48 hours (P = 0.048, Mantel-Cox Test) (Fig. 15). Previous studies on the influence of SdhA in the virulence of *L. pneumophila* serogroup 1 strain 130b in *G. mellonella* (Harding *et al*., 2013), showed that in the absence of this effector a disruption of LCV membrane occurred with the consequent degradation of the bacteria by the larvae immune system. Furthermore, they showed that infection of *G. mellonella* with Δ*sdhA* strain resulted in a rapid hemocyte decrease however this decrease was transitory since 24 hours post infection the number of hemocytes increased, due to the loss of bacterial replication, proposing that hemocytes decrease was not sufficient to cause larval mortality (Harding *et al*., 2013).

Since the survival of *G. mellonella* infected with NMex1 Δ*sdhA* strain was impaired on its ability to induce larval mortality when compared to the wild-type strain, it is possible that, such as previously observed in this study for Philadelphia1 strain (section 3.2.1.3.) and previously described in *L. pneumophila* serogroup 1 strain 130b, the low number of bacteria (NMex1 Δ*sdhA* strain) led to an incapacity to infect all remaining hemocytes due to an incomplete replication of bacteria. On other hand, an efficient replication of bacteria (wild-type) resulted in a larger destruction of all hemocytes that combined with high number of bacteria resulted in the dead of larvae (Harding *et al*., 2013).

3.2.3. *sdhA* role in disease-related *L. pneumophila* MicuB strain pathogenesis in *Galleria mellonella* infection model

3.2.3.1. Influence of *sdhA* in *L. pneumophila* MicuB-infected *G. mellonella* survival

In order to determine if infection of *G. mellonella* with *L. pneumophila* MicuB strain induced an *sdhA*-dependent larval mortality (Fig. 16), it was used the same experimental design previously described in section 3.2.1.1.

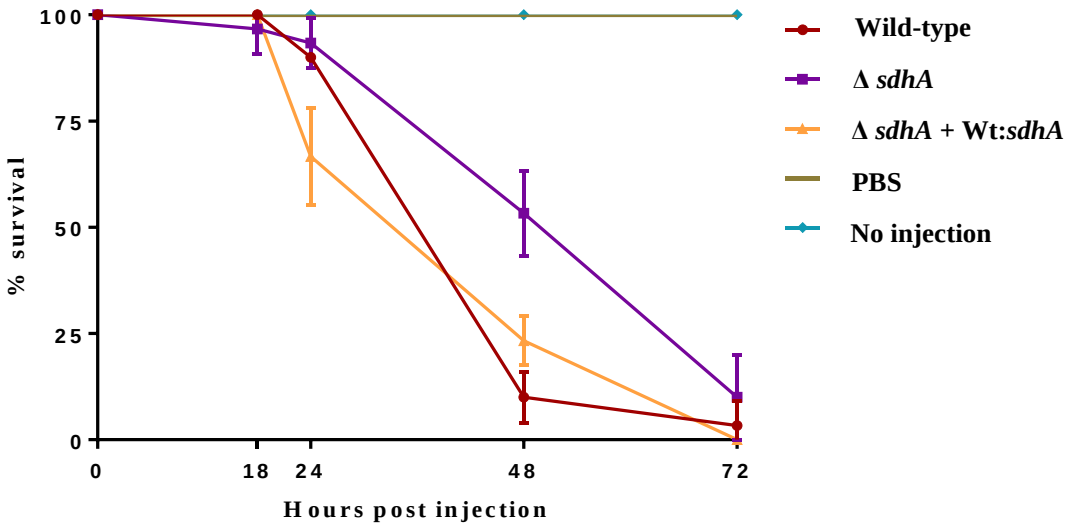

Figure 16. Infection with *L. pneumophila* MicuB strain induces *sdhA*-dependent larval mortality (P<0.05, Mantel-Cox Test). Survival (%) of *G. mellonella* infected with 10^7 CFU of MicuB wild type (WT) or MicuB Δ*sdhA* or MicuB Δ*sdhA* + WT:*sdhA*, incubated at 37 °C for 72 h. Negative controls with only PBS and no injection were used. Results are the mean of three separate experiments, ± standard deviation with ten larvae per condition.

No mortality events were observed in larvae that were either injected with PBS or not injected, demonstrating that injection did not caused mortality (Fig. 16).

In the course of the experiment, the Δ*sdhA* MicuB strain was significantly impaired for its ability to induce larval mortality when compared to the wild-type strain ($P = 0.0034$, Mantel-Cox Test). As expected, the complemented Δ*sdhA* MicuB strain with the WT-*sdhA* showed no significant differences on larval mortality when compared to the

wild-type strain (*P* = 0.505, Mantel-Cox Test), demonstrating that the mutation in *sdhA* in MicuB was responsible for the observed phenotype and that the WT- *sdhA* restored the ability to induce larval mortality (Fig. 16). From these results we can conclude that *sdhA* is an important gene for the pathogenicity of this disease-related strain in *G. mellonella* as it was previously described for the disease-related *L. pneumophila* strain 130b (Harding *et al.*, 2013).

3.2.3.2. Influence of *sdhA* in *L. pneumophila* MicuB-infected *G. mellonella* health index

The Health Index (Tsai *et al*., 2016) for each larvae was determined (Fig. 17) as described in section 3.1.2.2.

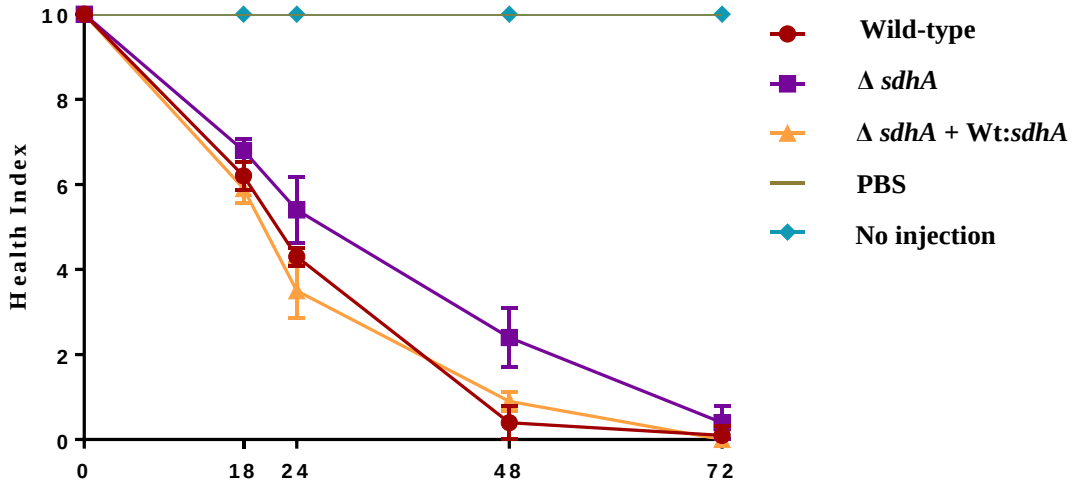

Figure 17. Infection with *L. pneumophila* MicuB strain induced *sdhA*-independent health index decrease (*P*>0.05, Mann-Whitney Test). Health Index of *G. mellonella* infected with 10^7 CFU of MicuB wild type (WT) or MicuB Δ*sdhA* or MicuB Δ*sdhA* + WT:*sdhA*, incubated at 37 °C for 72 hr. Negative controls with only PBS and no injection were used. Results are the mean of three separate experiments, ± standard deviation with ten larvae per condition. Health Index scores of *G. mellonella* infected with MicuB wild type (WT) or MicuB Δ*sdhA* or MicuB Δ*sdhA* + WT:*sdhA* are represented in appendix table G, H and I, respectively.

Both larvae with injection of PBS and no injection did not presented signals of decrease of health demonstrating that remained healthy in the course of experiment (Fig. 17).

During the experiment, the Δ*sdhA* MicuB strain did not had significantly differences in the health index when compared to the wild-type strain ($P = 0.2344$, Man-Whitney Test). Likewise, the complementation of Δ*sdhA* MicuB strain with the WT-*sdhA* showed no significant differences in Health Index scores ($P = 0.2338$, Man-Whitney Test). The absence of differences between Δ*sdhA* MicuB strain and the wild-type strain, also observed in Philadelphia1 strain (section 3.2.1.2.), were consistent with those previously described for the disease-related *L. pneumophila* strain 130b (Harding *et al.*, 2013). That study demonstrated that, although *L. pneumophila* 130b Δ*sdhA* strain was not able to replicate in *G. mellonella,* it was able to persist in the insect. These evidences suggest that the higher melanization and the decrease in activity observed in larvae infected with MicuB Δ*sdhA* strain resulted from the response of *G. mellonella* against the infection leading to a considerable decrease on the health index of the remaining larvae (Fig. 17).

3.2.3.3. *sdhA* influence in the kinetics of *L. pneumophila* MicuB-infected *G. mellonella* mortality

In order to evaluate the effect of NMex1 *sdhA* gene in the kinetics of *G. mellonella* infected mortality (Fig.18) the same experimental design previously described in section 3.2.1.3. was used.

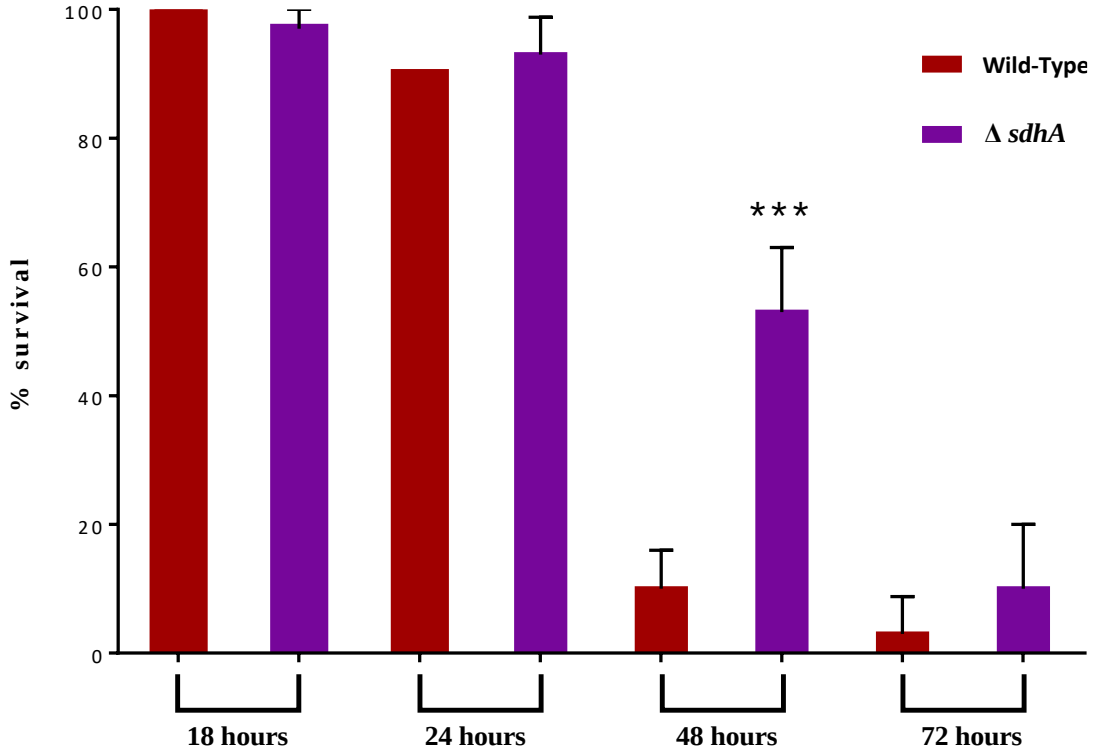

Figure 18. Comparison between the survival of infected *G. mellonella* with *L. pneumophila* MicuB wild-type and MicuB Δ*sdhA*. Survival (%) of G. mellonella infected with 10^7 CFU of MicuB wild-type (WT) or MicuB Δ*sdhA*, incubated at 37 ºC for 72 hours. Results are the mean of three separate experiments, ± standard deviation with ten larvae per condition. Differences were considered statically significant if P < 0.05 (*), highly significant if P < 0.01 (**), and extremely significant if P < 0.001 (***).

MicuB Δ*sdhA* strain was significantly impaired on its ability to induce larval mortality when compared to the wild-type strain at 48 hours (*P* = 0.0006, Mantel-Cox (Fig. 18). Previous studies on the influence of SdhA in the virulence of *L. pneumophila* serogroup 1 strain 130b in *G. mellonella* (Harding *et al*., 2013), showed that in the absence of this effector a disruption of LCV membrane occurred with the consequent degradation of this bacteria by the larvae immune system. Furthermore, they showed that infection of *G. mellonella* with Δ*sdhA* strain resulted in a rapid hemocyte decrease however this

decrease was transitory since 24 hours post infection the number of hemocytes increased, due to the loss of bacterial replication, proposing that hemocytes decrease was not sufficient to cause larval mortality (Harding *et al*., 2013).

Since the survival of *G. mellonella* infected with MicuB Δ*sdhA* strain was impaired on its ability to induce larval mortality when compared to the wild-type strain, it is possible that, such as previously observed in this study for Philadelphia1 strain (section 3.2.1.3.) and NMex1 (section 3.2.2.3.) and previously described in *L. pneumophila* serogroup 1 strain 130b, the low number of bacteria (MicuB Δ*sdhA* strain) led to an incapacity to infect all remaining hemocytes due to an incomplete replication of bacteria. On other hand, an efficient replication of bacteria (wild-type) resulted in a largedestruction of all hemocytes that combined with high number of bacteria resulted in the dead of larvae (Harding *et al*., 2013).

3.2.4. *sdhA* role in man-made environmental *L. pneumophila* Huc1 strain pathogenesis in *Galleria mellonella* infection model

3.2.4.1. Influence of *sdhA* in *L. pneumophila* Huc1-infected *G. mellonella* survival

In order to determine if infection of *G. mellonella* with *L. pneumophila* Huc1 strain induced an *sdhA*-dependent larval mortality (Fig. 19) the same experimental design previously described in section 3.2.1.1.

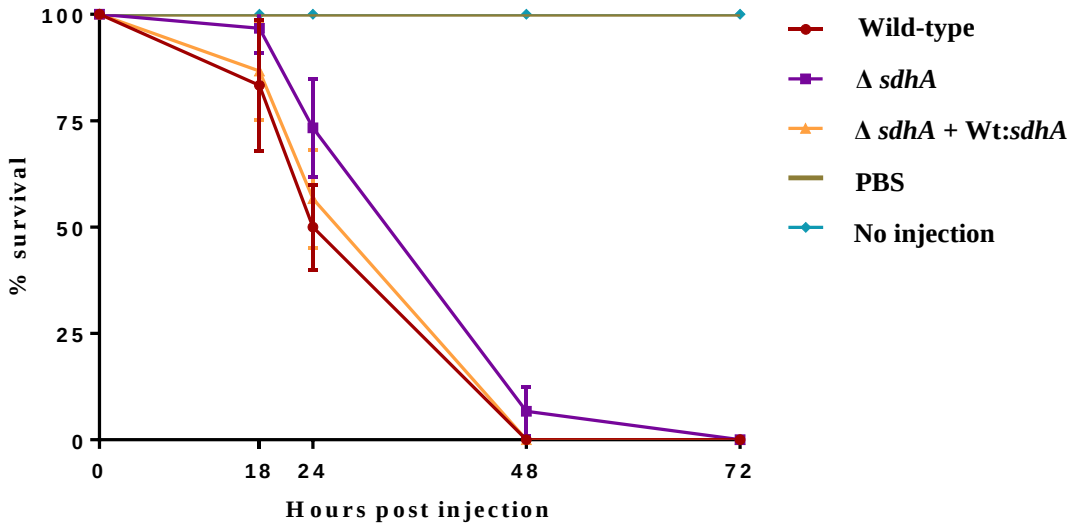

Figure 19. Infection with *L. pneumophila* Huc1 strain induces *sdhA*-dependent larval mortality (P<0.05, Mantel-Cox Test). Survival (%) of *G. mellonella* infected with 10^7 CFU of Huc1 wild type (WT) or Huc1 Δ*sdhA* or Huc1 Δ*sdhA* + WT:*sdhA*, incubated at 37 °C for 72 h. Negative controls with only PBS and no injection were used. Results are the mean of three separate experiments, ± standard deviation with ten larvae per condition.

No mortality events were observed in larvae that were either injected with PBS or not injected, demonstrating that injection did not caused mortality (Fig. 19).

In the course of the experiment, the Δ*sdhA* Huc1 strain was significantly impaired for its ability to induce larval mortality when compared to the wild-type strain (P = 0.0240, Mantel-Cox Test). As expected, the complemented Δ*sdhA* Huc1 strain with the WT-*sdhA* showed no significant differences on larval mortality when compared to the wild-type strain (P = 0.6707, Mantel-Cox Test), demonstrating that the mutation in *sdhA* in Huc1 was responsible for the observed phenotype and that the WT- *sdhA* restored the ability to induce larval mortality (Fig. 19). From these results we can conclude that *sdhA* is an important gene for the pathogenicity of this man-made strain in *G. mellonella* as it

was previously described for the disease-related *L. pneumophila* strain 130b (Harding *et al.*, 2013).

3.2.4.2. Influence of *sdhA* in *L. pneumophila* Huc1-infected *G. mellonella* health index

The Health Index (Tsai *et al.*, 2016) for each larvae was determined (Fig. 20) as described in section 3.1.2.2.

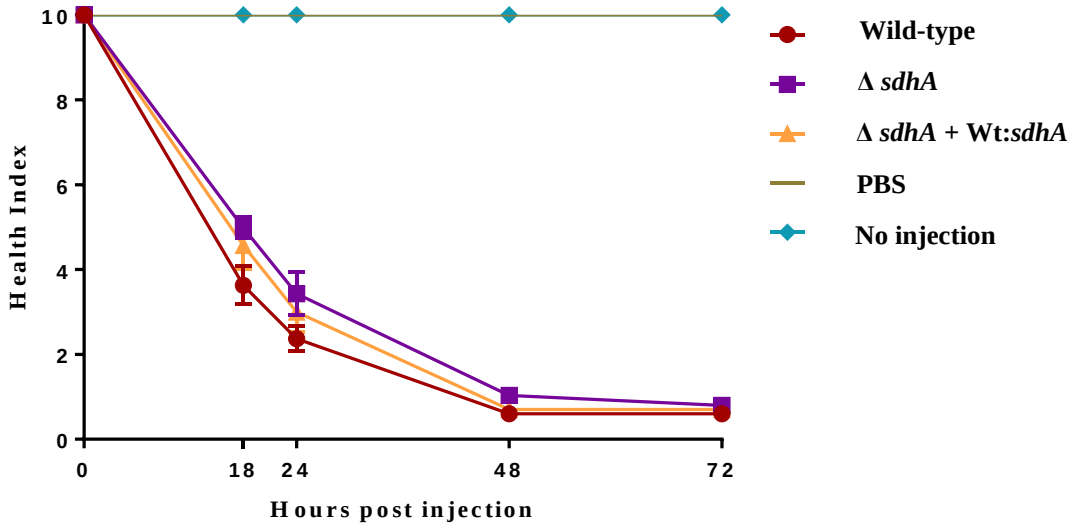

Figure 20. Infection with *L. pneumophila* Huc1 strain induced *sdhA*-independent health index decrease (*P*>0.05, Mann-Whitney Test). Health Index of *G. mellonella* infected with 10^7 CFU of Huc1 wild type (WT) or Huc1 Δ*sdhA* or Huc1 Δ*sdhA* + WT:*sdhA*, incubated at 37 °C for 72 hr. Negative controls with only PBS and no injection were used. Results are the mean of three separate experiments, ± standard deviation with ten larvae per condition. Health Index scores of *G. mellonella* infected with Huc1 wild type (WT) or Huc1 Δ*sdhA* or Huc1 Δ*sdhA* + WT:*sdhA* are represented in appendix table J, K and L, respectively.

Both larvae with injection of PBS and no injection did not presented signals of decrease of health demonstrating that remained healthy in the course of experiment (Fig. 20).

During the experiment, the Δ*sdhA* Huc1 strain did not had significantly differences in the health index when compared to the wild-type strain (*P* = 0.0968, Man-

Whitney Test). Likewise, the complementation of Δ*sdhA* Huc1 strain with the WT-*sdhA* showed no significant differences in Health Index scores (P = 0.3393, Man-Whitney Test). The absence of differences between Δ*sdhA* Huc1 strain and the wild-type strain, also observed in Philadelphia1 strain (section 3.2.1.2.) and MicuB (section 3.2.3.2.) , were consistent with those previously described for the disease-related *L. pneumophila* strain 130b (Harding *et al.*, 2013). That study demonstrated that, although *L. pneumophila* 130b Δ*sdhA* strain was not able to replicate in *G. mellonella*, it was able to persist in the insect. These evidences suggest that the higher melanization and the decrease in activity observed in larvae infected with Huc1 Δ*sdhA* strain resulted from the response of *G. mellonella* against the infection leading to a considerable decrease on the health index of the remaining larvae (Fig. 20).

3.2.4.3. *sdhA* influence in the kinetics of *L. pneumophila* Huc1-infected *G. mellonella* mortality

In order to evaluate the effect of Huc1 *sdhA* gene in the kinetics of *G. mellonella* infected mortality (Fig. 21) the same experimental design previously described in section 3.2.1.3. was used.

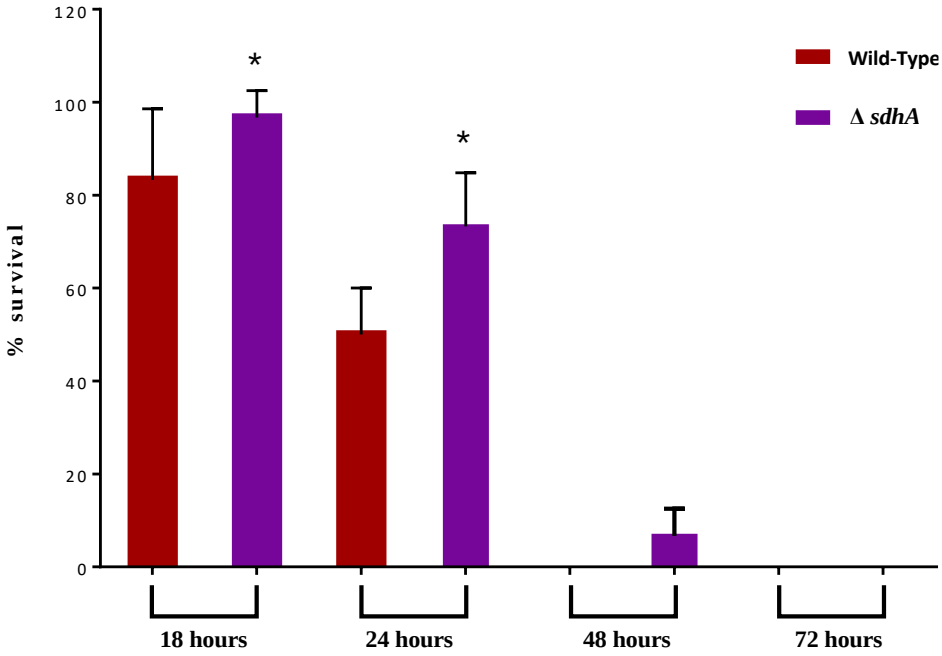

Figure 21. Comparison between the survival of infected *G. mellonella* with *L. pneumophila* Huc1 wild-type and Huc1 Δ*sdhA*. Survival (%) of *G. mellonella* infected with 10^7 CFU of Huc1 wild-type (WT) or Huc1 Δ*sdhA*, incubated at 37 ºC for 72 hours. Results are the mean of three separate experiments, ± standard deviation with ten larvae per condition. Differences were considered statically significant if $P < 0.05$ (*), highly significant if $P < 0.01$ (**), and extremely significant if $P < 0.001$ (***).

Huc1 Δ*sdhA* strain was significantly impaired on its ability to induce larval mortality when compared to the wild-type strain at 18 hours ($P = 0.0462$, Mantel-Cox) and at 24 hours ($P = 0.0488$) (Fig. 21). Previous studies on the influence of SdhA in the virulence of *L. pneumophila* serogroup 1 strain 130b in *G. mellonella* (Harding *et al.*, 2013), showed that in the absence of this effector a disruption of LCV membrane occurred with the consequent degradation of this bacteria by the larvae immune system. Furthermore, they showed that infection of *G. mellonella* with Δ*sdhA* strain resulted in a rapid hemocyte decrease however this decrease was transitory since 24 hours post infection the number of hemocytes increased, due to the loss of bacterial replication, proposing that hemocytes decrease was not sufficient to cause larval mortality (Harding *et al.*, 2013).

Since the survival of *G. mellonella* infected with Huc1 Δ*sdhA* strain was impaired on its ability to induce larval mortality when compared to the wild-type strain, it is possible that, such as previously observed in this study for Philadelphia1 strain (section 3.2.1.3.), NMex1 (section 3.2.2.3.), MicuB (section 3.2.3.3.) and previously described in *L. pneumophila* serogroup 1 strain 130b, the low number of bacteria (Huc1 Δ*sdhA* strain) led to an incapacity to infect all remaining hemocytes due to an incomplete replication of bacteria. On other hand, an efficient replication of bacteria (wild-type) resulted in a large destruction of all hemocytes that combined with high number of bacteria resulted in the dead of larvae (Harding *et al*., 2013).

3.2.5. *sdhA* role in natural environmental *L. pneumophila* Ice27 strain pathogenesis in *Galleria mellonella* infection model

3.2.5.1. Influence of *sdhA* in *L. pneumophila* Ice27-infected *G. mellonella* survival

In order to determine if infection of *G. mellonella* with *L. pneumophila* Ice27 strain induced an *sdhA*-dependent larval mortality (Fig. 22) the same experimental design previously described in section 3.2.1.1.

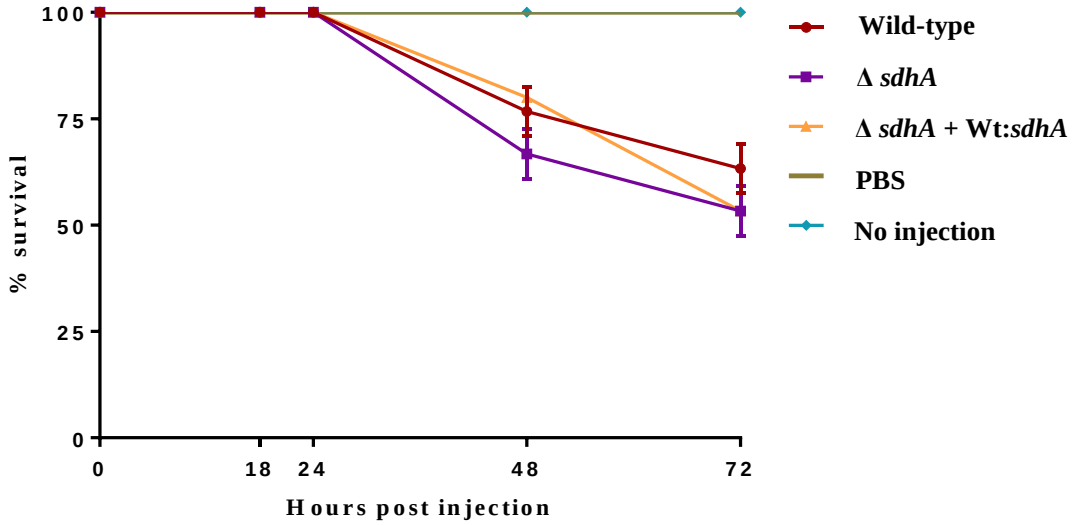

Figure 22. Infection with *L. pneumophila* Ice27 strain induces *sdhA*-independent larval mortality (*P*>0.05, Mantel-Cox Test). Survival (%) of *G. mellonella* infected with 10^7 CFU of Ice27 wild type (WT) or Ice27 Δ*sdhA* or Ice27 Δ*sdhA* + WT:*sdhA*, incubated at 37 °C for 72 h. Negative controls with only PBS and no injection were used. Results are the mean of three separate experiments, ± standard deviation with ten larvae per condition.

No mortality events were observed in larvae that were either injected with PBS or not injected, demonstrating that injection did not caused mortality (Fig. 22).

In the course of the experiment, the Δ*sdhA* Ice27 strain was not significantly impaired for its ability to induce larval mortality when compared to the wild-type strain ($P = 0.4146$, Mantel-Cox Test). Likewise, the complemented Δ*sdhA* Ice27 strain with the WT-*sdhA* showed no significant differences on larval mortality when compared to the wild-type strain ($P = 0.5341$, Mantel-Cox Test) (Fig. 22). From these results we can conclude that *sdhA* is an dispensable gene for the pathogenicity of this natural environmental strain in *G. mellonella* contrarily to what was previously described for the disease-related *L. pneumophila* strain 130b (Harding *et al.*, 2013).

3.2.5.2. Influence of *sdhA* in *L. pneumophila* Ice27-infected *G. mellonella* health index

The Health Index (Tsai *et al*., 2016) for each larvae was determined (Fig. 23) as described in section 3.1.2.2.

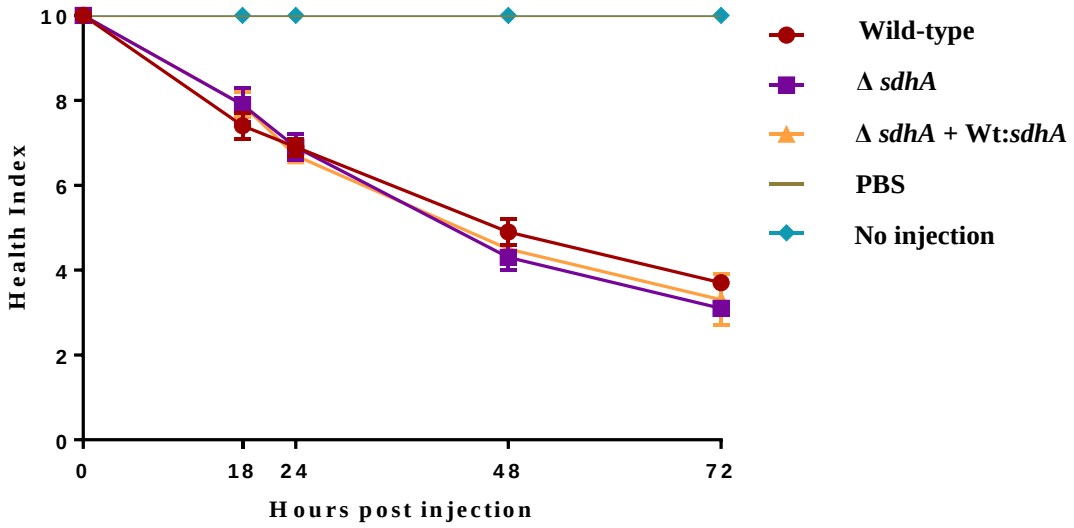

Figure 23. Infection with *L. pneumophila* Ice27 strain induced *sdhA*-independent health index decrease (*P*>0.05, MannWhitney Test). Health Index of *G. mellonella* infected with 10^7 CFU of Ice27 wild type (WT) or Ice27 Δ*sdhA* or Ice27 Δ*sdhA* + WT:*sdhA*, incubated at 37 °C for 72 hr. Negative controls with only PBS and no injection were used. Results are the mean of three separate experiments, ± standard deviation with ten larvae per condition. Health Index Scores of *G. mellonella* infected with Ice27 wild type (WT) or Ice27 Δ*sdhA* or Ice27 Δ*sdhA* + WT:*sdhA* are represented in appendix Table M, N and O, respectively.

Both larvae with injection of PBS and no injection did not presented signals of decrease of health demonstrating that remained healthy in the course of experiment (Fig. 23).

In the course of the experiment, the Δ*sdhA* Ice27 strain did not had significantly differences in the health index when compared to the wild-type strain (P = 0.9714, Man-Whitney Test). Likewise, the complementation of Δ*sdhA* Ice27 strain with the WT-*sdhA* showed no significant differences in Health Index scores (P = 0.9714, Man-Whitney Test). The absence of differences in the health index between Δ*sdhA* Ice27 strain and the

wild-type strain, were consistent with those previously observed in larvae mortality (Fig. 23).

3.2.5.3. *sdhA* influence in the kinetics of *L. pneumophila* Ice27-infected *G. mellonella* mortality

In order to evaluate the effect of NMex1 *sdhA* gene in the kinetics of *G. mellonella* infected mortality (Fig. 24) the same experimental design previously described in section 3.2.1.3. was used.

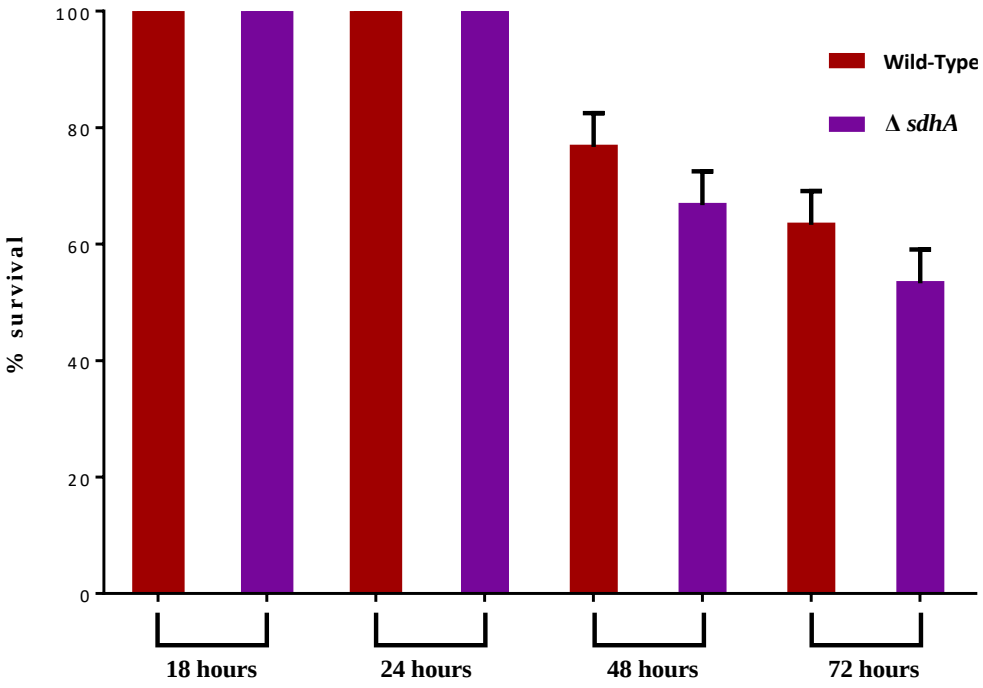

Figure 24. Comparison between the survival of infected *G. mellonella* with *L. pneumophila* Ice27 wild-type and Ice27 Δ*sdhA*. Survival (%) of *G. mellonella* infected with 10^7 CFU of Ice27 wild-type (WT) or Ice27 Δ*sdhA*, incubated at 37 ºC for 72 hours. Results are the mean of three separate experiments, ± standard deviation with ten larvae per condition. Differences were considered statically significant if $P < 0.05$ (*), highly significant if $P < 0.01$ (**), and extremely significant if $P < 0.001$ (***).

Ice27 Δ*sdhA* strain maintained its ability to induce larval mortality when compared to the wild-type strain at whole course of experiment (Fig. 24). These results contrast with those previously observed in this study for Philadelphia1 strain (section 3.2.1.3.), NMex1 (section 3.2.2.3.), MicuB (section 3.2.3.3.), Huc1 (section 3.2.4.3.) and previously described in *L. pneumophila* serogroup 1 strain 130b (Harding *et al*., 2013).

It was proved that SdhA protein is one of several effectors that is crucial for the intracellular growth of *L. pneumophila* in macrophages (Laguna *et al*., 2006) and *G. mellonella* (Harding *et al*., 2013), since in its absence a disruption of LCV membrane occurs with the consequent degradation of this bacteria by the macrophages and larvae immune system, respectively (Laguna *et al*., 2006; Harding *et al*., 2013) . The difficulty in detect alterations in Ice27 Δ*sdhA* mortality when compared to the wild-type could be due to the existence of functional redundancy of *sdhA* in this environmental natural, therefore other or others genes in Ice27 strain genome may carry out similar functions that *sdhA* in *G. mellonella.*

3.3. Importance of *sdhA* in *L. pneumophila* infection

In order to analyse the importance of *sdhA* in induction of different levels of virulence among unrelated *L. pneumophila* strains (Table I), a comparison between *sdhA* influences in the kinetics of mortality in *G. mellonella* infected with each one of studied strains was made (Fig. 25).

Strain	Hours			
	18	24	48	72
Philaldelphia1	0.1538	0.5164	0.0052	0.0493
MicuB	0.3173	0.3211	0.0006	0.134
NMex1	0.0467	0.0118	0.0483	0.7237
Huc1	0.0462	0.0488	0.2363	1.0
Ice27	1.0	1.0	0.3941	0.8284

Significance level

No significant	significant	Highly significant	Extremely significant

Figure 26. Heat map of significan level on comparison between the survival of infected *G. mellonella* with wild-type and Δ*sdhA* from each *L. pneumophila* studied strain. Differences were considered statically significant if $P < 0.05$ (*), highly significant if $P < 0.01$ (**), and extremely significant if $P < 0.001$ (***).

The natural environmental Ice 27 strain was the only strain with absence of differences in whole course of experiment, suggesting that this effector is dispensable to virulence of this strain (Fig. 25).

The natural environmental NMex1 and man-made environmental Huc1 strains presented differences in mortality between wild-type and Δ*sdhA* strains at early phases of *G. mellonella* infection (at 18h and 24h), however in late phase (at 72h), no differences were observed (Fig. 25). Moreover, this strains induced similar *G. mellonella* mortality in late phases, since that infection with both NMex1 and Huc1 wild-type resulted in 100% mortality within 48 hours post-infection (Figs. 15 and 21, respectively). We these data is possible correlate the ability of this strains in induce mortality in early phases of *G. mellonella* infection and the importance of *sdhA* in this infection phases.

On the other hand, the disease-related Philadelphia1 and MicuB strains presented differences in mortality between wild-type and Δ*sdhA* strains at late phases of *G. mellonella* infection (at 48h), however in early phases (at 18h and 24h), no differences were observed (Fig. 25). Furthermore, this strains induced similar *G. mellonella* mortality, since that infection with both Philadelphia1 and MicuB wild-type resulted in only 10-15% mortality at 24 hours post-infection (Figs. 12 and 18, respectively). We these data is possible correlate the ability of this strains to cause mortality in late phases of *G. mellonella* infection and the importance of *sdhA* in this infection phases.

Taken together, these data suggest that *sdhA* is important in induction of different levels of virulence, since in more virulent strains *sdhA* is necessary in induction of *G. mellonella* mortality at early phases of infection (NMex1 and Huc1 strains), and in the other hand, in less virulent strains the *sdhA* is necessary in induction of *G. mellonella* mortality at late phases of infection (Philadelphia1 and MicuB strains).

Chapter IV – Conclusion and Future Perspectives

Previous studies conclude that SdhA was a crucial effector for *L. pneumophila* virulence in *G. mellonella* (Harding *et al.*, 2013). The majority of *L. pneumophila* strains included in this study proved to induce *sdhA*-dependent larval mortality. Nevertheless, in the natural environmental strain Ice27 no significant differences were observed between the wild-type and Δ*sdhA* suggesting that the existence of functional redundancy.

In addition, relevant differences on the role on the *sdhA* were observed among the studied strains. In fact, more virulent strains, such NMex1 and Huc1, required *sdhA* to induce *G. mellonella* mortality in early phases of infection, while less virulent strains, such Philadelphia1 e MicuB, recruited *sdhA* in late phases of infection.

In the near future may be important to develop a strategy to confirm if indeed *L. pneumophila* Ice27 has functional redundancy for *sdhA* gene.

Moreover, *L. pneumophila* strains were selected from several others assuring the maximum genetic variability inferred from the complete sequence of Type IV virulence-related effectors *dotA, sdhA* and *sidJ* (Costa *et al.* 2010, 2014, 2017). In this study we determined that different strains with similar degrees of virulence required *sdhA* to induce *G. mellonella* mortality in different stages of infection. In order to understand if variations on the degree of virulence and the requirement of *sdhA* to induce *G. mellonella* mortality in different stages of infection are due to different allelic forms characteristics of each strain, it would be important to perform an allelic exchange of *sdhA* gene between the type strain Philadelphia1 wild-type strain, and the remaining *sdhA* studied strains. This approach will allow to correlate allelic forms with pathogenicity.

Chapter V – References

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Appendix

1. Health index values of *L. pneumophila* Philadelphia 1-infected *G. mellonella*

Table A. Health index values of wild-type Philadelphia 1-infected *G. mellonella*

Hours post injection	Survival		Melanization				Cocoon formation			Activity				Health index scores
	Dead	Alive	No	spots	Black spots	Black	Full	Partial	No	move without stimulation	move when stimulated	minimal movement on stimulation	No movement	
18 (R1)	1	9	1	9	0	1	0	0	10	9	0	0	1	6.7
18 (R2)	0	10	2	8	0	0	0	1	9	7	2	1	0	7.05
18 (R3)	1	9	3	5	2	0	0	2	8	8	2	0	0	7.1
24 (R1)	1	9	1	6	3	1	0	0	10	8	1	1	1	6.4
24 (R2)	1	9	0	6	4	0	0	0	10	4	2	3	1	5.3
24 (R3)	2	8	0	6	2	1	0	0	10	6	0	2	2	5.0
48 (R1)	5	5	0	4	4	2	0	0	10	1	2	3	5	3.2
48 (R2)	6	4	0	4	3	3	0	0	10	0	3	1	6	2.6
48 (R3)	6	4	0	3	4	3	0	0	10	0	3	1	6	2.5
72 (R1)	7	3	0	3	5	2	0	0	10	0	2	1	7	2.2
72 (R2)	7	3	0	3	4	3	0	0	10	0	2	1	7	2.1
72 (R3)	8	2	0	2	5	3	0	0	10	0	1	1	8	1.6

Table B. Health index values of Δ*sdhA* Philadelphia 1-infected *G. mellonella*

Hours post injection	Survival		Melanization				Cocoon formation			Activity				Health index scores
	Dead	Alive	No	spots	Black spots	Black	Full	Partial	No	move without stimulation	move when stimulated	minimal movement on stimulation	No movement	
18 (R1)	0	10	8	1	1	0	0	5	5	9	1	0	0	8.65
18 (R2)	0	10	3	4	3	0	0	4	6	9	1	0	0	7.4
18 (R3)	0	10	3	7	0	0	0	3	7	7	3	0	0	7.45
24 (R1)	0	10	0	9	1	0	0	0	10	5	4	1	0	6.3
24 (R2)	0	10	1	8	1	0	0	0	10	6	4	0	0	6.7
24 (R3)	1	9	0	6	2	1	0	0	10	6	3	0	1	5.6
48 (R1)	2	8	0	7	2	1	0	0	10	3	3	2	2	4.9
48 (R2)	1	9	0	7	2	1	0	0	10	0	7	2	1	5.0
48 (R3)	3	7	0	5	3	2	0	0	10	0	4	3	3	3.8
72 (R1)	4	6	0	6	3	1	0	0	10	0	5	1	4	3.8
72 (R2)	4	6	0	4	5	1	0	0	10	0	4	2	4	3.5
72 (R3)	5	5	0	2	5	3	0	0	10	0	2	3	5	2.6

Table C. Health index values of Δ*sdhA* + Wt:*sdhA* Philadelphia 1-infected *G. mellonella*

Hours post injection	Survival		Melanization				Cocoon formation			Activity				Health index scores
	Dead	Alive	No	spots	Black spots	Black	Full	Partial	No	move without stimulation	move when stimulated	minimal movement on stimulation	No movement	
18 (R1)	1	9	3	4	2	1	0	3	7	6	2	1	1	6.45
18 (R2)	1	9	4	5	0	1	0	2	8	7	2	0	1	7.0
18 (R3)	1	9	3	4	2	1	0	2	8	6	1	2	1	6.3
24 (R1)	3	7	1	7	1	1	0	0	10	4	3	0	3	5.1
24 (R2)	2	8	2	6	1	1	0	0	10	5	3	0	2	5.8
24 (R3)	3	7	0	5	2	3	0	0	10	1	4	2	3	3.9
48 (R1)	6	4	0	4	4	2	0	0	10	0	4	0	6	2.8
48 (R2)	6	4	0	4	3	3	0	0	10	0	1	3	6	2.4
48 (R3)	7	3	0	3	3	4	0	0	10	0	2	1	7	2.0
72 (R1)	8	2	0	0	8	2	0	0	10	0	1	1	8	1.5
72 (R2)	7	3	0	1	6	3	0	0	10	0	2	1	7	1.9
72 (R3)	9	1	0	1	3	6	0	0	10	0	0	1	9	0.8

2. Health index values of *L. pneumophila* NMex1-infected *G. mellonella*

Table D. Health index values of wild-type NMex1-infected *G. mellonella*

Hours post injection	Survival		Melanization				Cocoon formation			Activity				Health index scores
	Dead	Alive	No	spots	Black spots	Black	Full	Partial	No	move without stimulation	move when stimulated	minimal movement on stimulation	No movement	
18 (R1)	4	6	0	0	6	4	0	1	9	0	0	6	4	2.45
18 (R2)	5	5	0	0	5	5	0	3	7	0	0	5	5	2.15
18 (R3)	3	7	0	0	7	3	0	2	8	0	0	7	3	2.9
24 (R1)	10	0	0	0	0	10	0	0	10	0	0	0	10	0
24 (R2)	9	1	0	0	3	7	0	0	10	0	0	0	10	0.5
24 (R3)	9	1	0	0	6	4	0	0	10	0	0	1	9	0.9
48 (R1)	10	0	0	0	0	10	0	0	10	0	0	0	10	0
48 (R2)	10	0	0	0	3	7	0	0	10	0	0	0	10	0.3
48 (R3)	10	0	0	0	6	4	0	0	10	0	0	0	10	0.6
72 (R1)	10	0	0	0	0	10	0	0	10	0	0	0	10	0
72 (R2)	10	0	0	0	3	7	0	0	10	0	0	0	10	0.3
72 (R3)	10	0	0	0	6	4	0	0	10	0	0	0	10	0.6

Table E. Health index values of Δ*sdhA* NMex1-infected *G. mellonella*

Hours post injection	Survival		Melanization				Cocoon formation			Activity				Health index scores
	Dead	Alive	No	spots	Black spots	Black	Full	Partial	No	move without stimulation	move when stimulated	minimal movement on stimulation	No movement	
18 (R1)	2	8	0	1	8	1	0	3	7	0	3	5	2	3.85
18 (R2)	1	9	0	4	6	0	0	3	7	0	2	7	1	4.45
18 (R3)	2	8	0	3	6	1	0	5	5	1	4	2	3	4.35
24 (R1)	6	4	0	0	5	5	0	0	10	0	1	3	7	1.8
24 (R2)	7	3	0	0	10	0	0	0	10	0	0	3	7	1.9
24 (R3)	5	5	0	1	8	1	0	0	10	0	1	4	5	26
48 (R1)	7	3	0	3	5	2	0	0	10	0	0	3	7	2
48 (R2)	7	3	0	0	10	0	0	0	10	0	1	2	7	2
48 (R3)	7	3	0	0	9	1	0	0	10	0	2	1	7	2
72 (R1)	10	0	0	0	3	8	0	0	10	0	0	0	10	0.3
72 (R2)	10	0	0	0	7	3	0	0	10	0	0	0	10	0.7
72 (R3)	9	1	0	0	7	3	0	0	10	0	0	1	9	1

Table F. Health index values of Δ*sdhA* + Wt:*sdhA* NMex1-infected *G. mellonella*

Hours post injection	Survival		Melanization				Cocoon formation			Activity				Health index scores
	Dead	Alive	No	spots	Black spots	Black	Full	Partial	No	move without stimulation	move when stimulated	minimal movement on stimulation	No movement	
18 (R1)	4	6	0	0	6	4	0	3	7	1	0	5	4	2.75
18 (R2)	2	8	0	1	7	2	0	1	9	0	2	8	2	3.75
18 (R3)	5	5	0	2	3	5	0	1	9	0	1	4	5	2.35
24 (R1)	7	3	0	0	3	7	0	0	10	0	0	3	7	1.2
24 (R2)	7	3	0	0	6	4	0	0	10	0	0	3	7	1.5
24 (R3)	7	3	0	0	5	5	0	0	10	0	0	3	7	1.4
48 (R1)	9	1	0	0	4	6	0	0	10	0	0	1	9	0.7
48 (R2)	9	1	0	0	2	8	0	0	10	0	0	1	9	0.5
48 (R3)	10	0	0	0	0	10	0	0	10	0	0	0	10	0
72 (R1)	10	0	0	0	0	10	0	0	10	0	0	0	10	0
72 (R2)	10	0	0	0	0	10	0	0	10	0	0	0	10	0
72 (R3)	10	0	0	0	0	10	0	0	10	0	0	0	10	0

3. Health index values of *L. pneumophila* MicuB-infected *G. mellonella*

Table G. Health index values of wild-type MicuB-infected *G. mellonella*

Hours post injection	Survival		Melanization				Cocoon formation			Activity				Health index scores
	Dead	Alive	No	spots	Black spots	Black	Full	Partial	No	move without stimulation	move when stimulated	minimal movement on stimulation	No movement	
18 (R1)	0	10	0	8	2	0	0	1	9	4	4	2	0	6.05
18 (R2)	0	10	0	9	1	0	0	1	9	7	2	1	0	6.55
18 (R3)	0	10	0	5	4	0	0	4	6	5	3	2	0	5.9
24 (R1)	1	9	0	4	5	1	0	0	10	1	3	4	1	4.4
24 (R2)	1	9	0	5	3	1	0	0	10	0	5	4	1	4.5
24 (R3)	1	9	1	5	3	1	0	0	10	1	5	3	1	4.1
48 (R1)	8	2	0	0	2	8	0	0	10	0	0	2	8	0.8
48 (R2)	9	1	0	0	1	9	0	0	10	0	0	1	9	0.4
48 (R3)	10	0	0	0	0	10	0	0	10	0	0	0	10	0
72 (R1)	9	1	0	0	1	9	0	0	10	0	0	1	9	0.4
72 (R2)	10	0	0	0	0	10	0	0	10	0	0	0	10	0
72 (R3)	10	0	0	0	0	10	0	0	10	0	0	0	10	0

Table H. Health index values of Δ*sdhA* MicuB-infected *G. mellonella*

Hours post injection	Survival		Melanization				Cocoon formation			Activity				Health index scores
	Dead	Alive	No	spots	Black spots	Black	Full	Partial	No	move without stimulation	move when stimulated	minimal movement on stimulation	No movement	
18 (R1)	0	10	0	10	0	0	0	0	10	10	0	0	0	7
18 (R2)	0	10	2	5	3	0	0	1	9	5	3	2	0	6.45
18 (R3)	1	9	4	5	0	1	0	3	7	5	4	0	1	6.85
24 (R1)	0	10	0	10	0	0	0	0	10	4	5	1	0	6.3
24 (R2)	1	9	0	6	3	1	0	0	10	3	1	6	1	5
24 (R3)	1	9	0	5	3	2	0	0	10	4	2	2	2	4.9
48 (R1)	3	7	0	2	5	3	0	0	10	0	1	6	3	3.1
48 (R2)	6	4	0	1	3	6	0	0	10	0	0	4	6	1.7
48 (R3)	5	5	0	5	0	5	0	0	10	0	0	5	5	2.5
72 (R1)	9	1	0	0	1	9	0	0	10	0	0	1	9	0.4
72 (R2)	8	2	0	0	2	8	0	0	10	0	0	2	8	0.8
72 (R3)	10	0	0	0	0	10	0	0	10	0	0	0	10	0

Table I. Health index values of Δ*sdhA* + Wt:*sdhA* MicuB-infected *G. mellonella*

Hours post injection	Survival		Melanization				Cocoon formation			Activity				Health index scores
	Dead	Alive	No	spots	Black spots	Black	Full	Partial	No	move without stimulation	move when stimulated	minimal movement on stimulation	No movement	
18 (R1)	0	10	0	8	2	0	0	6	4	4	4	2	0	6.3
18 (R2)	0	10	0	6	4	0	0	3	7	4	2	4	0	5.75
18 (R3)	0	10	0	6	4	0	0	3	7	4	1	5	0	5.65
24 (R1)	2	8	0	4	4	2	0	0	10	3	0	5	2	4.2
24 (R2)	4	6	0	3	3	4	0	0	10	1	2	3	4	3.1
24 (R3)	4	6	0	4	2	4	0	0	10	0	3	3	4	3.1
48 (R1)	8	2	0	0	2	8	0	0	10	0	0	2	8	0.8
48 (R2)	8	2	0	0	2	8	0	0	10	0	0	2	8	0.8
48 (R3)	7	3	0	0	3	7	0	0	10	0	0	3	7	1.2
72 (R1)	10	0	0	0	0	10	0	0	10	0	0	0	10	0
72 (R2)	10	0	0	0	0	10	0	0	10	0	0	0	10	0
72 (R3)	10	0	0	0	0	10	0	0	10	0	0	0	10	0

4. Health index values of *L. pneumophila* Huc1-infected *G. mellonella*

Table J. Health index values of wild-type Huc1-infected *G. mellonella*

Hours post injection	Survival		Melanization				Cocoon formation			Activity				Health index scores
	Dead	Alive	No	spots	Black spots	Black	Full	Partial	No	move without stimulation	move when stimulated	minimal movement on stimulation	No movement	
18 (R1)	2	8	0	1	8	1	0	4	6	0	0	8	2	3.6
18 (R2)	3	7	0	1	9	0	0	0	10	0	0	7	3	3.2
18 (R3)	0	10	0	0	10	0	0	0	10	0	1	9	0	4.1
24 (R1)	4	6	0	1	7	2	0	0	10	0	0	6	4	2.7
24 (R2)	5	5	0	0	7	3	0	0	10	0	0	5	5	2.2
24 (R3)	6	4	0	0	10	0	0	0	10	0	0	4	6	2.2
48 (R1)	10	0	0	0	6	4	0	0	10	0	0	0	10	0.6
48 (R2)	10	0	0	0	5	5	0	0	10	0	0	0	10	0.5
48 (R3)	10	0	0	0	7	3	0	0	10	0	0	0	10	0.7
72 (R1)	10	0	0	0	6	4	0	0	10	0	0	0	10	0.6
72 (R2)	10	0	0	0	5	5	0	0	10	0	0	0	10	0.5
72 (R3)	10	0	0	0	7	3	0	0	10	0	0	0	10	0.7

Table K. Health index values of Δ*sdhA* Huc1-infected *G. mellonella*

Hours post injection	Survival		Melanization				Cocoon formation			Activity				Health index scores
	Dead	Alive	No	spots	Black spots	Black	Full	Partial	No	move without stimulation	move when stimulated	minimal movement on stimulation	No movement	
18 (R1)	0	10	0	1	9	0	0	6	4	0	7	3	0	5.1
18 (R2)	0	10	0	1	9	0	0	4	6	1	2	7	0	4.7
18 (R3)	1	9	1	1	8	0	0	5	5	2	3	5	0	5.15
24 (R1)	2	8	0	1	9	0	0	0	10	0	0	8	2	3.5
24 (R2)	4	6	0	1	8	1	0	0	10	0	1	5	4	2.9
24 (R3)	2	8	0	2	7	1	0	0	10	0	2	8	2	3.9
48 (R1)	10	0	0	0	9	1	0	0	10	0	0	0	10	0.9
48 (R2)	9	1	0	1	7	2	0	0	10	0	0	0	10	1.1
48 (R3)	9	1	0	1	6	3	0	0	10	0	0	1	9	1.1
72 (R1)	10	0	0	0	9	1	0	0	10	0	0	0	10	0.9
72 (R2)	10	0	0	0	8	2	0	0	10	0	0	0	10	0.8
72 (R3)	10	0	0	0	7	3	0	0	10	0	0	0	10	0.7

Table L. Health index values of Δ*sdhA* + Wt:*sdhA* Huc1-infected *G. mellonella*

Hours post injection	Survival		Melanization				Cocoon formation			Activity				Health index scores
	Dead	Alive	No	spots	Black spots	Black	Full	Partial	No	move without stimulation	move when stimulated	minimal movement on stimulation	No movement	
18 (R1)	2	8	0	2	7	1	0	3	7	2	1	5	2	4.15
18 (R2)	2	8	0	3	5	2	0	1	9	3	2	3	2	4.35
18 (R3)	0	10	0	3	7	0	0	0	10	3	3	4	0	5.2
24 (R1)	5	5	0	2	6	2	0	0	10	0	1	4	5	2.6
24 (R2)	5	5	0	3	5	2	0	0	10	0	3	2	5	2.9
24 (R3)	3	7	0	2	6	2	0	0	10	0	4	3	3	3.5
48 (R1)	10	0	0	0	8	2	0	0	10	0	0	0	10	0.8
48 (R2)	10	0	0	0	8	2	0	0	10	0	0	0	10	0.8
48 (R3)	10	0	0	0	5	5	0	0	10	0	0	0	10	0.5
72 (R1)	10	0	0	0	8	2	0	0	10	0	0	0	10	0.8
72 (R2)	10	0	0	0	8	2	0	0	10	0	0	0	10	0.8
72 (R3)	10	0	0	0	5	5	0	0	10	0	0	0	10	0.5

5. Health index values of *L. pneumophila* Ice27-infected *G. mellonella*

Table M. Health index values of wild-type Ice27-infected *G. mellonella*

Hours post injection	Survival		Melanization				Cocoon formation			Activity				Health index scores
	Dead	Alive	No	spots	Black spots	Black	Full	Partial	No	move without stimulation	move when stimulated	minimal movement on stimulation	No movement	
18 (R1)	0	10	2	8	0	0	0	7	3	10	0	0	0	7.75
18 (R2)	0	10	0	10	0	0	0	4	6	10	0	0	0	7.2
18 (R3)	0	10	2	8	0	0	0	3	7	8	2	0	0	7.35
24 (R1)	0	10	1	8	1	0	0	0	10	10	0	0	0	7.1
24 (R2)	0	10	0	9	1	0	0	0	10	10	0	0	0	6.9
24 (R3)	0	10	1	6	3	0	0	0	10	9	0	1	0	6.7
48 (R1)	2	8	0	7	3	0	0	0	10	3	5	0	2	5.2
48 (R2)	2	8	0	5	5	0	0	0	10	2	5	1	2	4.8
48 (R3)	3	7	0	7	2	1	0	0	10	3	3	1	3	4.6
72 (R1)	4	6	0	6	4	0	0	0	10	0	3	3	4	3.7
72 (R2)	3	7	0	5	4	1	0	0	10	0	2	5	3	3.7
72 (R3)	4	6	0	6	4	0	0	0	10	0	2	4	4	3.6

Table N. Health index values of Δ*sdhA* Ice27-infected *G. mellonella*

Hours post injection	Survival		Melanization				Cocoon formation			Activity				Health index scores
	Dead	Alive	No	spots	Black spots	Black	Full	Partial	No	move without stimulation	move when stimulated	minimal movement on stimulation	No movement	
18 (R1)	0	10	5	5	0	0	0	5	5	10	0	0	0	8.25
18 (R2)	0	10	4	6	0	0	1	2	7	9	1	0	0	7.9
18 (R3)	0	10	2	8	0	0	0	4	6	9	1	0	0	7.5
24 (R1)	0	10	2	7	1	0	0	0	10	9	1	0	0	7.2
24 (R2)	0	10	0	8	2	0	0	0	10	9	0	1	0	6.6
24 (R3)	0	10	1	8	1	0	0	0	10	9	1	0	0	7
48 (R1)	4	6	0	6	3	1	0	0	10	3	1	2	4	4
48 (R2)	3	7	0	6	2	2	0	0	10	3	2	2	3	4.3
48 (R3)	3	7	0	7	2	1	0	0	10	1	6	1	3	4.6
72 (R1)	5	5	0	3	7	0	0	0	10	0	2	3	5	3
72 (R2)	4	6	0	4	3	3	0	0	10	1	1	4	4	3.2
72 (R3)	5	5	0	3	6	1	0	0	10	1	3	1	5	3.2

Table O. Health index values of Δ*sdhA* + Wt:*sdhA* Ice 27-infected *G. mellonella*

Hours post injection	Survival		Melanization				Cocoon formation			Activity				Health index scores
	Dead	Alive	No	spots	Black spots	Black	Full	Partial	No	move without stimulation	move when stimulated	minimal movement on stimulation	No movement	
18 (R1)	0	10	4	6	0	0	0	2	8	10	0	0	0	7.9
18 (R2)	0	10	2	8	0	0	0	3	7	10	0	0	0	7.55
18 (R3)	0	10	5	5	0	0	0	3	7	10	0	0	0	8.15
24 (R1)	0	10	0	7	3	0	0	0	10	10	0	0	0	6.7
24 (R2)	0	10	0	9	1	0	0	0	10	9	1	0	0	6.8
24 (R3)	0	10	0	8	2	0	0	0	10	8	2	0	0	6.6
48 (R1)	2	8	0	5	5	0	0	0	10	2	1	5	2	4.4
48 (R2)	2	8	0	8	1	1	0	0	10	1	2	5	2	4.5
48 (R3)	2	8	0	8	2	0	0	0	10	1	2	5	2	4.6
72 (R1)	5	5	0	3	6	1	0	0	10	0	1	3	6	2.7
72 (R2)	5	5	0	5	5	0	0	0	10	0	4	2	4	3.5
72 (R3)	4	6	0	7	3	0	0	0	10	0	3	3	4	3.8