AMERICAN UNIVERSITY OF BEIRUT

CHARACTERIZING *ANOPHELES GAMBIAE* SUSCEPTIBILITY TO ORAL INFECTIONS WITH A PANEL OF HUMAN BACTERIAL PATHOGENS

by
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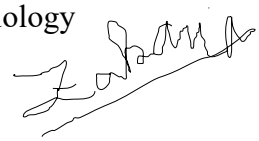

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ABSTRACT

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The mosquito midgut is a key organ associated with multiple physiological functions including development, digestion, immunity and reproduction. In mosquito vectors of diseases, all transmitted microbes including viruses and parasites start their journey in the midgut where they interact with several chemical and physical barriers that function to restrain microbial invasion of the host. In the malaria vector *Anopheles gambiae,* the physiological responses of the midgut epithelium as well as local barriers, such as the peritrophic matrix and barrier epithelial, are known to impact the development of early stages of *Plasmodium* parasites. However, the midgut responses to bacterial pathogens are not very well understood as those to malaria parasites, and have mainly focused on the use of the Gram-negative bacterium *S. marcescens* (*Sm*) as an oral pathogen to establish midgut infections.

In this project, we aim to study mosquito susceptibility to oral infections with human bacterial pathogens known to cause gut infections, in order to determine whether these are virulent to the insect and, hence, can be used as tools to deepen our understanding of the mosquito gut physiologic responses to infection. The results show that several human pathogens including *Pseudomonas aeruginosa*, *Salmonella* Typhi, *Klebsiella pneumoniae* and *Escherichia coli* can indeed cause mosquito mortality when acquired through the oral route, however none of the tested human pathogens persisted in the mosquito midgut beyond 4 days from feeding suggesting that the midgut microenvironment is not permissive for colonization by these bacteria. We also show that oral infections with a particularly virulent *P. aeruginosa* sequence type 309 and the insect gut pathogen *Erwinia carotovora carotovora* significantly increased the number of cells undergoing enhanced endoreplication in the proventriculus but not in the midgut (anterior and posterior), further supporting the recognized immune defensive role of the proventriculus. None of the oral infections triggered significant cell division in midgut regenerative cells in PH3 staining, supporting previous studies in *A. gambiae* showing that chemical and microbial damage of the midgut does not trigger significant proliferation of regenerative cells. In summary, our data reveal that human bacterial pathogens can cause mosquito mortality when acquired orally despite their transient colonization of the midgut indicating that they might be causing irreversible damage. A genome wide transcriptomic approach is underway to identify the functional groups of genes whose expression may be altered by these oral pathogens, in order to gain better insight into mosquito midgut physiological responses to infection.

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CHAPTER I

INTRODUCTION

A. Mosquitoes as vectors of disease

Bloodsucking insects are important vectors of pathogens that cause a variety of severe diseases worldwide, with a strong impact on human and animal health [1, 2]. Concern about vector-borne diseases has increased in the last decade due to the geographical spread of several insect vectors caused by intense trade and climate changes [3, 4].

Vector-borne diseases are human illnesses caused by parasites, viruses and bacteria that are transmitted by vectors. In particular, mosquitoes are major vectors of pathogens, including protozoa (e.g., *Plasmodium spp*. which causes malaria), nematodes (e.g., filariae), and viruses (e.g., dengue, chikungunya, West Nile, and Zika). Over 3,500 species of mosquitoes have been described, but only a limited number of them can function as disease vectors, and varying levels of specificity are observed for different types of pathogens. Overall, mosquito-borne pathogens are estimated to cause around 500,000 deaths each year, with billions of people exposed to the risk of contracting these infectious agents. Since 2014, major outbreaks of dengue, malaria, chikungunya, yellow fever and Zika have afflicted populations, claimed lives, and overwhelmed health systems in many countries (WHO report 2020).

Aedes mosquitoes are vectors of chikungunya, dengue, yellow fever and zika viruses whereas *Anopheles* mosquitoes are carriers of malaria caused by *Plasmodium* parasites. There are around 530 recognized species of *Anopheles* [5] with *An. gambiae s.s.* being the best studied as it is the major malaria vector in Africa with a prominent

role in the transmission of *P. falciparum*, and the first mosquito vector whose genome was sequenced [6, 7]. *An. gambiae* belongs to *Anopheles gambiae s.l.* species complex which contains at least 9 species, including 3 of the most important vectors in sub-Saharan Africa: *An. gambiae s.s.*, *An. coluzzi* and *An. arabiensis* [8]. Although mosquitoes can transmit several human pathogens, they are not considered passive vectors but rather mount multiple innate immune responses against the various microbes they encounter.

B. Mosquito Innate Immune Responses to Infection

1. Cellular Defenses

a. Hemocytes and Phagocytosis

Phagocytosis is a rapid, evolutionary conserved cellular response through which phagocytic cells clear microbes they encounter in the host tissue. Mosquito hemocytes "analogous to vertebrate white blood cells" are classified into 3 types, oenocytoids, prohemocytes and granulocytes based on their morphology [9].Granulocytes, the professional phagocytic cells, are further divided into several subpopulations based on their transcriptional profile [10]. In adult mosquitoes, approximately 75 % of hemocytes are in circulation while 25 % are sessile [11]. In *An. gambiae*, hemocytes tend to aggregate at the periosteal regions of the heart that witness the highest hemolymph flow in response to all systemic infections, where they are actively engaged in phagocytosis [12]. In *Ae. aegypti*, circulating and sessile hemocytes were shown to phagocytose microbes within seconds of their introduction into the hemolymph [13]. Mosquito hemocytes have the capacity to phagocytose hundreds of bacteria at any given time [14]. Unlike the hemocyte-mediated melanization and encapsulation responses that

are detrimental to hemocytes, phagocytosis is non-destructive to hemocytes, which perhaps explains why small arthropods harboring a small number of hemocytes have a higher proportion of phagocytic granulocytes than larger arthropods [15].

Phagocytosis is initiated when cell-surface pattern recognition receptors (PRRs) or soluble PRRs bind pathogen-associated molecular patterns (PAMPs) on microbial surfaces. PRRs that have been empirically shown to be involved in phagocytosis include thioester-containing proteins, Nimrod proteins, DSCAM, β-integrins, and PGRPs [16-20]. Different PRRs have different specificities. For example, *D. melanogaster* PGRP-LC mediates the phagocytosis of *E. coli* but not *S. aureus* [17], while NimC1 mediates the phagocytosis of *S. aureus* and to a lesser extent *E. coli* [19]. The intracellular signaling pathways that drive or enhance phagocytosis remain poorly understood in insect models of immunity, but in mosquitoes the cell death abnormal CED6 pathway regulates the internalization of bacteria (phagocytosis) that is mediated by the putative PRRs, TEP1, TEP3, LRIM1 and LRP1 [21].

b. Gut Epithelial Immunity Against Parasites

To successfully establish an infection, *Plasmodium* ookinetes need to invade and traverse the midgut epithelial cells reaching the basal lamina where they transition into oocysts between the basal side of the epithelium and the basal lamina. This midgut traversal by ookinetes is considered a very critical step and the most severe bottleneck in the *Plasmodium* lifecycle as the transition to oocysts is accompanied by dramatic losses in parasite numbers [22, 23]. *Plasmodium* ookinetes cause cellular damage when they invade the mosquito midgut epithelium; consequently, hemocytes are recruited to the infected cells where they release vesicles for complement activation against

Plasmodium [24]. *Plasmodium* ookinetes also cause irreversible damage to the cells they invade and trigger a strong caspase-mediated nitration response [25, 26]. When hemocytes come in contact with a nitrated midgut surface, they undergo apoptosis and release hemocyte-derived microvesicles (HdMv) [24]. Local HdMv release promotes activation of thioester containing-protein 1 (TEP1) [24], a major final effector of the mosquito complement-like system that binds to the parasite's surface and forms a complex that lyses the ookinete [27]. In *An. gambiae*, it has been reported that Toll over-activation induces hemocyte differentiation which enhances the immune response against *Plasmodium*. Toll signaling promotes hemocyte differentiation into the megacyte lineage, resulting in a dramatic increase in the proportion of circulating megacytes and enhanced midgut megacyte recruitment [28]. Megacytes are effector cells with hemocytes as their predecessors before differentiation.

Moreover, Silencing Cactus, a negative regulator of Toll signaling in *A. gambiae* mosquitoes, elicits a very strong TEP1-mediated immune response that eliminates *Plasmodium berghei* ookinetes [29]. This phenotype can be rescued by co-silencing Cactus with either TEP1 or the Rel1 transcription factor, indicating that parasite elimination is mediated by activation of Toll signaling, with TEP1 as a final effector [29]. Later studies showed that hemocytes mediate this enhanced immune response, as transfer of Cactus-silenced hemocytes into naïve mosquitoes recapitulates the phenotype of systemic Cactus silencing [30]. Furthermore, Cactus silencing also increases HdMv release in response to ookinete midgut invasion [24], indicating that hemocytes are more reactive to *Plasmodium* infection.

2. Humoral Defenses

a. Anti-microbial Peptides

Antimicrobial peptides (AMPs) are secreted as low molecular weight proteins that were initially identified for their antimicrobial activity in vitro. In *Drosophila*, there are seven well-characterized AMP gene families, Attacins, Cecropins, Defensins, Diptericins, Drosomycins, Drosocins and Metchnikowins [31], and a loss of function mutation in Imd compromises the induction of most of these AMPs except Drosomycin that is controlled by the Toll pathway [32]. Systematic CRISPR-mediated gene depletion of these various AMP genes revealed that AMPs function mainly against Gram-negative bacteria and fungi, acting either synergistically or additively [33]. Interestingly, certain *Drosophila* AMPs reveal remarkable specificity against certain pathogens, such as the two Diptericins (A and B) that seem to have evolved in Dipterans in response to two species of gut commensal bacteria, *Providencia* and *Acetobacter* spp. [34]. Besides AMPs, two families of peptides that are regulated by the Toll pathway, Bomanins and Baramycin A, have been identified to mediate protection against Gram-positive bacteria and fungi, respectively, as well as against toxins produced by Gram-positive bacteria and fungi [35-38].

Defensins, cecropins, diptericins, gambicins and attacins comprise the five main AMP gene families in mosquitoes, with Holotricins being found in *Aedes* but not *Anopheles* [39, 40]. *In vitro* analyses of the antimicrobial spectra of *A. gambiae* AMPs showed that Cecropin1 and Gambicin are cytotoxic primarily against Gram-negative bacteria; Defensin1, Cecropin1 and Gambicin are cytotoxic primarily against Gram-positive bacteria; Cecropin1, Defensin1 and Gambicin are cytotoxic against filamentous fungi [41-43]. *In vivo* silencing of *An. gambiae* Defensin1 increased mosquito

susceptibility to *S. aureus* infections [44], whereas RNAi based silencing of *Ae. aegypti* Defensin A had no effect on mosquito survival following challenge with three bacterial species [45]. Gambicin seems to exhibit some activity against *P. berghei in vitro* and *in vivo* [42, 46]. In *Ae. aegypti*, the transgenic overexpression of Cecropin A and Defensin A blocked the development of *P. gallinaceum* [47].

The AMPs produced by mosquitoes are regionalized with one study showing the transcriptional patterning of AMPs within the *Ae. aegypti* gut, where over 95 % of all AMP/putative AMP transcripts are derived from the proventriculus and anterior midgut, whereas the posterior midgut contributes to less than 3 % [48]. In the same study, disproportionate high expression of AMPs transcripts was also noted in the proventriculus and anterior midgut of *An. gambiae* as compared to the posterior midgut. In both species of *Ae. aegypti* and *An. gambiae*, the posterior midgut's investment in AMPs was negligible. Altogether, the expression of digestive and defensive genes as well as a GOEA (Gene Ontology Enrichment Analysis) of the *An. gambiae* midgut regions confirm that the midgut structure-function relationship is well conserved between the two mosquito species [48].

b. Melanization

Melanization is an enzymatic reaction used by insects for cuticle hardening, wound healing, and immunity [49, 50]. In the realm of immunity, melanization is an immune effector mechanism involved in the killing of bacteria, fungi, protozoan parasites, and nematode worms. When this process also involves the aggregation of hemocytes, it is known as nodulation or encapsulation. Melanization involves a series of reactions that include the conversion of tyrosine to melanin precursors and the cross-

linking of proteins on microbial surfaces to form a layer of melanin that surrounds and sequesters an invading pathogen [49, 50]. Melanization is phenotypically manifested as a darkened proteinaceous capsule that surrounds the invading pathogen, and the death of the pathogen presumably occurs via either oxidative damage or via starvation, as the foreign agent becomes isolated from the nutrient-rich hemolymph [49, 50]. Melanization also assists in the clearing of dead or dying pathogens [51, 52]. The process of melanization involves the coordinated interaction of pattern recognition receptors, cascades of clip domain serine proteases, serine protease inhibitors, and the enzymes that drive the production of melanin [53, 54].

c. Complement-like Responses

The complement-like system in *An. gambiae* has emerged as a key anti-plasmodial defense mechanism. The main component of this system is TEP1 which shares significant sequence similarity and structural organization with the mammalian complement factor C3 [16, 55]. TEP1 is produced by hemocytes and functions as a phagocytosis enhancer. It is secreted in the hemolymph as a single chain peptide that is inactive and is activated by proteolytic cleavage [16]. The activated TEP1 protein is then stabilized by the formation of a leucine-rich repeat complex containing LRIM1 and APL1C proteins. After this complex is formed, TEP1 gains the ability to bind to bacteria in the hemolymph and *Plasmodium* (ookinetes) in the midgut leading to their destruction [56, 57].

The two LRR proteins are unstable individually and require the presence of each other to persist in circulation where a single knockdown of either LRIM1 or APL1C is sufficient to entirely remove both proteins from circulation which is phenotypically

equivalent to a double knockdown [56]. In addition, silencing of either of the two LRR encoding genes leads to deposition of cleaved TEP1 on self-tissues, resulting in depletion of the protein from circulation and to the abolishment of TEP1 binding to ookinetes and their subsequent lysis during *Plasmodium* infection. This shows that the TEP1cut/LRIM1/APL1C complex functions as a complement-like system for parasite killing.

C. Mosquito Immune Defenses in the Gut

The basic structure of the digestive tract is similar across insects although they possess a diversity of modifications associated with adaptation to different feeding modes (Figure 1). The gut has three primary regions: foregut, midgut (or ventriculus), and hindgut [58].

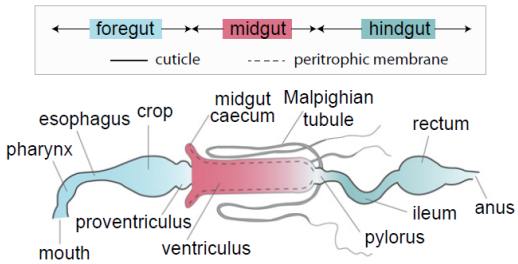

Figure 1. Generalized Gut Structure of Insects. The foregut and hindgut are lined by a cuticle layer (thick black line), and the midgut secretes a peritrophic matrix (dashed line).

The foregut and hindgut originate from embryonic ectoderm and are lined with exoskeleton made up of chitin and cuticular glycoproteins. This exoskeleton separates the gut lumen from the epidermal cells and is shed at each ecdysis. Foregut or hindgut may be subdivided into functionally distinct subsections, with the foregut often having a separate crop or diverticula for temporary food storage and the hindgut encompassing discrete sections such as fermentation chambers and a separate rectum for holding feces before defecation. The midgut is the primary site of digestion and absorption in many

insects; it lacks the exoskeletal lining and has a different developmental origin, arising from endodermal cells.

The midgut is composed of a single layer of polarized epithelial cells, with either pole displaying different morphological adaptations for increasing the surface area involved in the exchange of molecules. The distinct microvillous apical surface is exposed to the lumen, and its primary role is the secretion of digestive enzymes and absorption of nutrients. The basal pole is characterized by intricate convolutions of the basolateral membrane, forming the basal labyrinth that functions in ion and water transport and provides spaces for molecular exchanges [59]. Exterior to the labyrinth and connected to the basal membrane is the basal lamina, which encloses the midgut and separates it from the hemolymph and other hemocoel contents. In addition, a web like arrangement of muscles encircle the midgut on the hemocoel side to allow distention following the blood meal [60]. The posterior midgut, in particular, is vulnerable to invasion as this is where the blood meal is often stored during the digestive process and where parasites (such as *Plasmodium*) have the opportunity to attach to and invade the midgut epithelium.

In many insects, the midgut epithelial cells secrete a layer composed of chitin and proteins, called the peritrophic matrix (or peritrophic membrane). The peritrophic matrix divides the midgut into the endo- and ectoperitrophic space, and microorganisms are usually confined to the former, preventing their direct contact with midgut epithelium [61]. The peritrophic matrix serves a variety of functions, including providing a barrier that protects the epithelium from mechanical damage by food particles, from exposure to large toxin molecules present in food, and from microbial invasion, and also concentrating food and digestive enzymes [62-65].

The midgut represents an effective barrier for the entrance of pathogens into insect body cavity. The midgut plays complex roles in immune resistance and tolerance as it has to preserve the beneficial microbiota and mount effective responses against harmful pathogens that are based particularly on the production of antimicrobial peptides (AMPs) and reactive oxygen species [66-69]. In many insects, the midgut is characterized by a complex functional regionalization. In some flies (i.e., non-hematophagous brachycerous *Diptera*), where this specialization is particularly evident, the different midgut regions (anterior, middle, and posterior) are characterized by peculiar features, such as columnar cell morphology, presence of atypical cell types, expression of genes encoding for cell proteins and digestive enzymes, luminal pH, and even microbiota load and composition [70-78].

Columnar cells (CCs) also termed "enterocytes" are the predominant cell type in the insect midgut and are responsible for digestive enzyme production and absorption of nutrients. In 1979, Cioffi performed one of the first detailed morphological and ultrastructural analysis on the insect midgut. The author examined the midgut of a lepidopteran larva and observed that the fine structure of CCs gradually changes from the anterior to the posterior end of the midgut. In particular, in the anterior region of the midgut, the microvilli are irregular and vesicles form from their membrane; in the posterior region, the microvilli consist of long, thin, and regular projections of the apical membrane [79].

Endocrine cells (ECs) are also present in the insect midgut. They produce and secrete bioactive peptides which have important regulatory activities that are fundamental for midgut physiology and insect homeostasis. In addition, the bioactive peptides released by ECs play several important roles such as in coordinating insect

growth with nutrition availability, regulating midgut peristalsis, digestive enzyme release in the midgut lumen, and stem cell proliferation [79].

Stem cells (SCs), also known as regenerative cells, play a key role in maintaining the overall integrity of the gut to guarantee insect homeostasis. The gut must cope with mechanical food abrasion, interact with resident bacteria and act as a barrier against ingested toxic compounds and pathogens [68]. SCs can undergo asymmetric division (i.e., formation of a SC and another cell that undergoes terminal differentiation) to assure the maintenance of a constant number of SCs, or symmetric division that results in the generation of two SCs or two daughter mature cells [80]. The remarkable capacity of SCs to proliferate is fundamental to ensure the growth of the midgut during larval development. During metamorphosis, the midgut is completely replaced by a newly forming epithelium that originates from proliferation and differentiation of larval SCs [81, 82]. Studies from *Drosophila* revealed the importance of intestinal stem cell proliferation in gut homeostasis and protection from infection [83, 84]. In contrast, mosquitoes seem to exhibit very minimal intestinal stem cell proliferation in response to microbial insult and chemical damage, when compared to *Drosophila* [85, 86].

Insect excretory organs are the Malpighian tubules, which are extensions of the anterior hindgut that extend into the body cavity and absorb wastes, such as uric acid, which are delivered to the anterior hindgut (Figure 1). Thus, the hindgut contains a combination of nitrogenous waste and food waste, probably creating a different nutritive environment for insect gut bacteria than for gut bacteria of animals, in which these two waste products are separated [61].

1. Role of Imd Pathway in Gut Immunity

a. Overview of *Drosophila* Imd Pathway

The role of the Imd pathway in insect immunity was initially characterized and studied in *Drosophila* [32], and since then it has been found to play an essential role in the immune system of insects including mosquitoes [87]. Invasion of the *Drosophila* gut by Gram-negative bacteria [and few Gram-positives with DAP-type peptidoglycan (PGN)] activates the immune-deficiency (Imd) pathway to produce AMPs, through the sensing of DAP-PGN by two peptidoglycan recognition proteins, the transmembrane receptor PGRP-LC [17, 88, 89] and the intracellular soluble receptor PGRP-LE [90, 91]. Sensing of PGN by PGRPs triggers functional amyloid formation in the adaptor protein Imd that is essential for signal propagation downstream to the NF-κB-like transcription factor Relish that controls the inducibility of several classes of antimicrobial peptides-encoding genes (AMPs) [92].

Full activation of Relish requires its phosphorylation by the Iκ-B Kinase complex (IKK) and its cleavage by the caspase DREDD [93-95].

In *Drosophila*, the IMD pathway is the main pathway controlling AMPs expression in the midgut, and its activity is strongly constrained by expression of amidase PGRPs [83], while the Toll pathway is expressed mostly in the ectodermal portions of the gut. A similar pattern was found in the gut of Ae. aegypti with orthologs of Toll pathway recognition proteins most strongly expressed in the crop while IMD-activating PGRPs (PGRPLC and PGRP-LE) and IMD pathway components were enriched in the midgut. Investment in these IMD-activating PGRPs was highest in the anterior midgut, while immune-modulating PGRPs showed more divided expression. Altogether, the expression patterns of these key genes suggested enhanced immune

vigilance in the anterior portion of the midgut, with hallmarks of immune tolerance prominent in the posterior midgut [48].

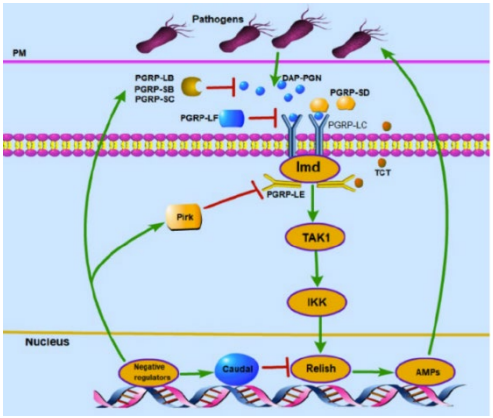

Figure 2. Imd signaling pathway and its negative regulators in insect intestinal immunity. When pathogens invade the gut, DAP-PGN in the cell wall is recognized by the PGRP-LC in the cell membrane, activating the intranuclear transcription factor Relish.

b. Mosquito Imd Pathway in Immune Defense

The activation of the Imd pathway in mosquitoes has been reported to be triggered by bacteria and *Plasmodium*, and an indirect effect of the Imd pathway has been shown on viral loads in *Aedes* mosquitoes [96]. Over-expression of the gene encoding Rel2 transcription factor confers complete resistance against laboratory cultured *P. falciparum* in *An. gambiae*, *Anopheles stephensi*, and *Anopheles albimanus* mosquitoes [97]. A study conducted on *An. gambiae* infected with isolates of *P. falciparum* revealed the requirement of the PGRP-LC receptor which activates the IMD pathway, thereby emphasizing the role of the IMD pathway in *Anopheles* immunity against *Plasmodium* [97].

The majority of the intracellular components of the Imd pathway in *Drosophila* are conserved in the mosquito with few exceptions [39]. The mosquito orthologue of

Drosophila Relish is Rel2 that plays a central role in Imd signaling but little is known about the gene targets of the Imd/Rel2 pathway in mosquitoes [87].In vitro studies provide evidence for the regulation of Cecropin1 and Gambicin by Rel2 [98], but a more recent study conducted in vivo proposed dual regulation of mosquito AMPs by Imd and Toll pathways [97]. In contrast to *Drosophila*, Rel2 in the mosquito *Aedes aegypti* is encoded by 3 alternatively-spliced isoforms [99], whereas in *A. gambiae* by 2 alternatively spliced isoforms [98].

c. Mosquito Imd Pathway and Control of Gut Microbiota

One of the distinct features of the IMD pathway is its activation, which is regulated by the endogenous bacterial flora of the mosquito midgut [96].These bacteria exhibit a physiological role in the development, digestion, nutrition, and reproduction of the mosquito [61]. According to recent studies, mosquito microbiota have been found to have a profound effect on the immune system [100]. Host-microbe interactions between mosquitoes and their bacterial and fungal symbionts are bi-directional: microbes alter mosquito physiology and vector competence; reciprocally, mosquitoes employ resistance and immune tolerance to shape their associated microbial communities [101]. In a study conducted on *Ae. aegypti* mosquitoes infected with the DENV, a reciprocal tripartite interaction between the microbiota, immune system, and dengue virus infection was reported after the blood intake [100]. It is hypothesized that this kind of interaction between the three players may not be restricted only to DENV but could be a general feature of other arboviral interactions as well [102].

Moreover, PGRPs play essential roles in regulating the gut microbiota of mosquitoes. In one study, silencing PGRP-LC led to a significant 2-fold increase of the

bacterial load in sugar-fed *An. gambiae* mosquitoes and a 6-fold increase following a bloodmeal (compared to a 4-fold increase in control) [103]. It was also demonstrated, in the same study, that the effect of PGRPLC on *Plasmodium* survival is directly related to the bacteria residing in the mosquito midgut. That was evident through a 3-fold increase in oocyst numbers in PGRP-LC kd mosquitoes which was comparable to gentamycin treated mosquitoes.

In another study, PGRPLB knockdown in *An. stephensi* upregulated AMPs and ROS which resulted in a ~500 times reduction of culturable microbes [104]. Therefore, it was concluded that PGRP-LD helps protect commensal bacteria by preventing overactivation of host immune responses. In addition, gut microbiota promote PM structural integrity and their reduction in numbers, after antibiotic treatment (similar expression profiles with dsRNA treated mosquitoes), prevented PM formation and blood was dispersed within the entire gut lumen after a bloodmeal [104].

Moreover, knockdown of PGRP-LA in *An. stephensi* resulted in around 6-fold increase in bacteria CFU (Colony Forming Units) and a significant reduction in the expression of four immune genes (attacin, tep1, nos and defension) compared to dsGFP control [105]. These results showed that PGRP-LA control the abundance of gut microbiota through regulating the synthesis of downstream immune effectors.

2. Role of Peritrophic Matrix

The midgut in insects is protected by a tightly arranged semi-permeable membrane structure of chitin and protein, known as the peritrophic membrane (PM) [106]. It is the first physical barrier of the insect intestinal immune system, which prevents damage caused by pathogens, food particles and bacterial toxins ingested by

insects and coming into direct contact with intestinal epithelial cells [107, 108]. Therefore, the PM is a defense outpost for pathogenic microbial infections that occur in invertebrates via food. As the PM has a role in protecting against pathogens, its thickness and integrity are particularly important in defense. In *Drosophila*, Drosocrystallin (dcy) protein is a major component of the PM, and mortality is significantly increased in dcy mutant flies following intestinal infection with *Pseudomonas entomophila* [109, 110]. The integrity of the PM has been shown to be regulated through the Wnt (Wingless/Integrated) signaling pathway in tsetse flies [111]. In *An. coluzzii*, the synthesis and the integrity of the PM were shown to be microbiota-dependent [112]. Altogether, these studies highlight the key role of the PM in intestinal homeostasis and in providing protection from systemic infections through midgut invasion.

The structural integrity of PM is necessary for a proper response against pathogens: for example, silencing of PGRP-LD in *An. stephensi* causes a dysbiosis, as a consequence of the altered expression of genes that codify for structural components of the PM and thus for its integrity [104]. Noteworthy, the fragmentation of the PM consequent to silencing increases the vectorial potential of the mosquito thanks to the enhanced susceptibility to *P. berghei* infections [104].

In *An. gambiae* mosquitoes in addition to PM, the formation of a mucin-barrier lining the epithelium in the ectoperitrophic space has been proposed [113]. In particular, upon the increase of microbiota load induced by blood meal, Duox enzyme mediates the crosslinking between mucins that are secreted on the cell surface. This cross-linked mucin-barrier seems to reduce the access of immune elicitors secreted by gut bacteria to the epithelium, hence limiting inflammatory responses by gut epithelial cells [114].

3. Role of DUOX

Activation of the *Drosophila* intestinal nicotinamide adenine dinucleotide phosphate oxidase Duox by pathogenic microorganisms produces ROS that can directly destroy pathogenic bacteria [115], thus the DUOX-ROS system plays an important role in insect intestinal immunity. In addition to its involvement in the clearance of pathogenic microorganisms, the Duox-ROS system plays an essential role in maintaining intestinal homeostasis in *Bactrocera dorsalis* [116]. A recent study also showed that serotonin in the gut of *B. dorsalis* and *Aedes aegypti* affects the homeostasis of gut microbiota by regulating the expression of Duox [117].

Many studies have shown that insect intestinal Duox activation produces ROS through two mechanisms, one regulating Duox gene expression in the nucleus and the other activating Duox enzyme activity. The specific ligand that activates Duox enzymatic activity is uracil, which is secreted by most pathogenic bacteria but not intestinal commensal microbes [118]. It regulates the Hedgehog (Hh) signaling pathway while being recognized by the G-protein coupled receptor (GPCR), activating the formation of Cad99C/PLCβ/PKC endosomes, leading to Ca2+ release from the endoplasmic reticulum and activating the enzymatic activity of Duox [119, 120].

Peptidoglycan-dependent activation of the Duox transcriptional pathway is negatively regulated by p38 activation, which itself is regulated by PLCβ, Calcineurin B (CanB) and MAP kinase phosphatase 3 (MKP3) [121]. This negative regulation ensures that transcriptional Duox is activated only when stimulated by large amounts of peptidoglycan, thus creating a balance between clearing harmful pathogens and tolerating the beneficial microbiota.

In mosquitoes, DUOX is involved in the generation of a dityrosine network in the ectoperitrophic space of the midgut, by cross-linking proteins in the mucin layer, that reduces the permeability of immune elicitors generated in the midgut lumen, hence, reducing the midgut immune responses to bacteria and *Plasmodium* parasites [113]. Silencing Duox in *An. stephensi* mosquitoes enhances the suppression of *Plasmodium* parasites by promoting the thioester-containing protein 1 (TEP1) pathway [122], which is the hallmark of mosquito immunity to malaria parasites [27].

ROS scavenging mechanisms exist in the insect gut, as excess ROS cause oxidative stress damage to intestinal epithelial cells. ROS enzymes are mainly involved in regulating normal levels of ROS that maintain normal oxidative reactions in the intestine and avoid cell damage by the excess of ROS such as catalase, long-oxide dehydrogenase, thioredoxin peroxidase, and glutathione peroxidase [106]. Upon invasion of exogenous pathogens, DUOX regulates various peroxidases in vivo to clear overexpressed ROS and maintain their levels within the threshold of host damage [106].

D. Human Bacterial Pathogens Identified in Insects

Insects are hosts to a wide variety of microorganisms, including bacteria, fungi, viruses, and parasites, several of which can cause infections and diseases in humans. Understanding the role of insects as reservoirs and vectors for human bacterial pathogens is crucial for public health, as it can aid in the development of effective prevention and control strategies.

Despite their importance, little is known about the origins of many emerging human pathogens. However, given the ancient evolution and current predominance of invertebrates, it is likely that bacteria–invertebrate interactions may not only constitute a

source of new human pathogens but also shape bacterial evolution such as fostering the spread of novel virulence factors into existing human commensal or pathogenic bacteria [123]. The interactions between insects and human bacterial pathogens are complex. Insects can serve as both reservoirs and vectors (mechanical and biological) for these pathogens, contributing to their survival and transmission. In some cases, the bacteria establish long-term relationships with their insect hosts, while in others, the insects act as accidental carriers. The mechanisms by which these pathogens adapt to insect hosts and transition to human hosts are subjects of ongoing research. Studying human bacterial pathogens isolated from insects is vital for understanding the epidemiology and ecology of these diseases. It helps in identifying the factors influencing pathogen transmission, host range, and the development of effective control measures. Furthermore, this knowledge can guide the surveillance and monitoring of insect populations, especially those with a high potential for transmitting pathogens to humans.

Among insects that serve as sources of bacterial human pathogens is the housefly *Musca domestica* which is considered as pest of human, poultry and livestock surroundings and facilities. The housefly can transmit several pathogenic bacteria to humans including *Vibrio cholerae*, members of the *Enterobacteriaceae*, *S. aureus*, *Pseudomonas spp*. and others [124, 125]. The mode of bacterial transmission is mechanical either through regurgitation or excretion [124, 126]. Furthermore, several studies reported bacteria isolated from flies that were resistant to multiple antibiotics including *E. coli* [127, 128], *Klebsiella pneumoniae* [125, 129], and *Pseudomonas aeruginosa* [125, 130]. Most of the antibiotic resistant bacteria were isolated from flies caught in and around hospital environments and animal farms (where there is an

extensive use of antibiotics as growth promoters), suggesting that house flies may also play a role in the dissemination of antibiotic resistant bacteria to different environments [131].

A systemic review and meta-analysis were conducted in a study to estimate the occurrence of various bacterial species of medical and veterinary importance harbored by the housefly *Musca domestica* around the world [132]. The most frequent reported isolated bacterial species by various studies were the following: (a) *E. coli*, the bacterium that causes nosocomial infections and bloody diarrhea and hemorrhagic colitis in humans [127, 133, 134]; (b) *Enterococcus faecium*, the most important nosocomial pathogen of humans, causing urinary tract infections, bloodstream infections, endocarditis and wound infections [135, 136]; (c) *K. pneumoniae*, which causes infections of the respiratory tract and urinary tract, as well as post-operative wound infections [127, 137]; (d) *P. aeruginosa*, cause disease in humans and other animals]207]; and (e) *S. aureus* which is capable of causing human illness and food poisoning]207]. The pathogenic Gram-negative bacteria were by far the most frequently identified in most of these studies as compared to Gram-positive bacteria. In another study, Gram negative bacteria isolated from the external surfaces of houseflies were identified as *Escherichia coli* (36.8 %), *Salmonella* species (26.3 %), *Pseudomonas* species (5.3 %), *Shigella* species (26.3 %) and *Klebsiella* species (5.3 %) in Illorin, Kwara state [206]. Moreover, Gram negative bacteria isolated from the internal surfaces of houseflies were identified as *Escherichia coli* (50 %), *Klebsiella* species (25 %) and *Proteus* species (25 %). *E. coli* seems to be frequently carried by flies and in one study, *E. coli* O157:H7 was identified in *Musca domestica* at a cattle

farm in Japan [133]. Bacterial species identified on houseflies seem to depend on the location from where the flies were captured [138].

In addition to flies, domestic cockroaches exist in many human habitats, such as hospitals, restaurants, offices, homes, markets and the urban community together with the bacteria they harbor. The role of cockroaches in disseminating and increasing the persistence of pathogens in residential environment remains unknown. Therefore, studies have been conducted to assess the presence of culturable microflora and pathogens in cockroaches. In a report by Newell & Fernley (2003), insects which include flies, darkling beetle and cockroaches found in and around chicken farms carried Campylobacter and the bacteria can survive on or within these insects for a few days. Cockroaches harbor a diverse range of bacterial genera including multiple drug-resistant strains, such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, Shiga toxin-producing *E. coli*, *Klebsiella spp.*, and *Salmonella* Typhi, in addition to a range of fungi and viruses [139, 140]. The German cockroach, *Blatella germanica*, is a common pest in built environments worldwide. They host diverse microbial communities within their gut [141]. They frequently harbor microorganisms with the potential to cause human disease, including enteric bacterial pathogens that are acquired by feeding on contaminated substrates in unsanitary habitats. For instance, cockroaches harboring *Salmonella spp.*, frequent agents of gastroenteritis, have been collected in China, Ethiopia, Ghana, India, Iran, Nigeria, Pakistan, Spain, and Thailand [142].

A recent meta-analysis determined that across multiple studies approximately 20 % of German cockroaches tested harbored *Salmonella spp.*, indicating that these insects are relevant environmental reservoirs [142]. Epidemiological evidence also suggests that German cockroaches contribute more directly to the spread of several bacterial and

viral enteric infections among human hosts. In one study, cockroaches could acquire a strain of *Salmonella typhimurium* from a contaminated food source and then transfer them to food, water and uninfected cockroaches [143]. Another study demonstrated that *Salmonella typhimurium* undergoes replication in the cockroach gut at multiple timepoints following ingestion [144]. In addition, *S. typhimurium* undergoes a lifestyle shift from individual motile rods to aggregated biofilm-like forms in the cockroach foregut unlike *Escherichia coli* which is frequently used as a non-colonizing control in *S. typhimurium* infection experiments [144].

A study was performed to estimate the prevalence of the external bacterial flora of two domestic cockroaches (*Blattella germanica* and *Blatta orientalis*) collected from households in Tebessa (northeast Algeria) [145]. Three major bacterial groups were cultured (total aerobic, enterobacteria, and staphylococci) from 14 specimens of cockroaches, and antibiotic susceptibility was tested for both *Staphylococcus* and *Pseudomonas* isolates. Culturing showed that the total bacterial load of cockroaches from different households were comparable ($P<0.001$) and enterobacteria were the predominant colonizers of the insect surface, with a bacterial load of (2.1×105 CFU/insect), whereas the staphylococci group was the minority [145].

The ability of mosquitoes to transmit bacterial pathogens is poorly understood and has not received significant attention. Although mosquitoes are mainly biological vectors of parasites and viruses there are few reports that highlight their ability to act as mechanical vectors for bacteria pathogenic to humans.

A study in Australia suggested a role for mosquitoes in *Mycobacterium ulcerans* transmission between possums and humans. *M. ulcerans* causes Buruli ulcer which is a necrotizing infection of skin and subcutaneous tissue. An extensive 4-month structured

mosquito field survey was conducted in an area endemic for Buruli ulcer. *M. ulcerans* was almost exclusively associated with *Aedes notoscriptus* with them being a possible intermediary between infected possums and humans [146] aiding the uprise of Buruli ulcer cases in the region of Mornington Peninsula.

In Sweden and Finland, clinical experience and epidemiological data indicate that mosquitoes are the main transmission route of human tularemia [147-149].Tularemia is a bacterial zoonotic disease of the northern hemisphere, endemic in certain geographical areas where it affects a wide range of mammals [150]. In one study, *Aedes aegypti* larvae exposed to a fully virulent *Francisella tularensis holarctica* strain for 24 hours, were allowed to develop into adults when they were individually homogenized. Mice infected with PCR-positive homogenates developed clinical signs of disease within five days similar to the positive controls. However, attempts to culture PCR-positive homogenates were unsuccessful. Interestingly, the bacteria are associated with the mosquito in a passive, non-replicating quiescent state, and are resuscitated upon contact with the mammalian host, a process which represents a novel transmission cycle for a bacterial pathogen [151].

E. Insect Models to Study the Virulence of Human Pathogens

The use of insects as model hosts for studying the virulence of human bacterial pathogens has a number of benefits. Insects can be reared in large numbers and are easy to manipulate, resulting in minimal time and cost of maintenance. The infection process relative to mammals is much quicker, yielding results more rapidly. Also, there are fewer ethical issues associated with inoculating insects with pathogens than mammalian species. Moreover, results obtained using insects as model hosts can easily be

confirmed using mammalian models, alleviating concerns over unwarranted extrapolation of the results [152].

The similarities between insect and mammalian pathogenesis, to a certain extent, indicate that insects may represent good model hosts for the study of human pathogens [152]. However, the prerequisite for utilizing such a system is a significant positive correlation between the virulence of the pathogen in insect and mammalian hosts. The human opportunistic pathogens *Candida albicans* and *Pseudomonas aeruginosa* have been bio-assayed against both types of hosts, demonstrating astonishing similarities in their responses. For instance, bioassays with *P. aeruginosa* mutant strains showed a significant positive correlation between the percent survival of mice and the LD50 value (50 % lethal dose) in *Galleria mellonella* larvae [153].

Several studies have reported on the virulence of human bacterial pathogens in insect models. In one study, a set of 44 environmental *P. aeruginosa* isolated from various (e.g. hydrocarbon-contaminated) environmental sources such as groundwater and soil, compost, industrial wastewater effluents of Hungarian oil refineries and municipal sewage were examined for their virulence in *G. mellonella* [154]. In the applied *G. mellonella* virulence model, the majority (65.9 %) of the examined environmental isolates was virulent with a mortality rate of 75-100 %. Five of the examined 44 environmental *P. aeruginosa* isolates were proved to be moderately virulent (50-75 %), five isolates were semi-virulent (25-50 %) and five strains were avirulent (0-25 % mortality) [154].

In another study involving *G. mellonella* collected from a commercial farm, two bacteria species were isolated and identified as *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. *P. aeruginosa* effectively killed *Trichoplusia ni* larvae by

either feeding or by surface-application of the bacterium, and total insect mortality was 83.33 % and 81.66 %, respectively. In contrast, the effective pathogenicity of *K. pneumoniae* for *T. ni* was 50 % for feeding and 0 % for surface application [155]. It was hypothesized that *K. pneumonia* is a secondary and opportunistic pathogen of insects that requires the primary pathogenicity of stronger microbes such as *P. aeruginosa* to manifest virulence. The virulence of 50 strains of *Klebsiella* was investigated in *Galleria* at an infectious dose of 1x105 c.f.u. The clinical strains showed a range of virulence, with the majority of strains (68 %) causing greater than 50 % mortality at an infectious dose of 1x105 c.f.u [156]. Moreover, infection of *G. mellonella* by *K. pneumoniae* via injection, resulted in dose-dependent larval death due to bacterial replication in the hemolymph, increased PO activity (at 12h post-infection) and hemocyte depletion [157]. When infected with *Salmonella typhimurium* at various doses, the wax moth larvae showed a clear dose-dependent response, with a 50 % lethal dose of 3.6 x 103 CFU [158].

In addition to *G. mellonella*, other insects proved to be good models also to assess the virulence of human bacterial pathogens. In the life cycle of the house fly, larvae consume bacteria that are necessary for their development. When larvae were artificially fed with *E. coli*, the average rate of larval survival dropped to 62 % within 48 h after ingestion [159]. In addition, *Drosophila melanogaster* flies infected by injection of 104 CFU of *Salmonella typhimurium* succumbed to infection within 7 to 9 days compared to controls [160].

F. Specific Aims

Innate immunity is the first line of defense against pathogen infection in all animals, including insects. It is a non-specific immune response that is activated immediately upon pathogen detection and does not require prior exposure to the pathogen. Innate immunity in insects involves both physical and chemical barriers, as well as cellular and humoral responses. Physical barriers in insects include the exoskeleton, which provides a mechanical barrier to prevent pathogen entry, and the peritrophic matrix, a protective layer found in the gut that prevents pathogens from coming into contact with the gut epithelium. The peritrophic matrix is produced by the mosquito midgut which is a key organ associated with multiple physiological functions including development, digestion, immunity and reproduction.

The physiological responses of the midgut epithelium to bacterial pathogens are not very well understood as those to malaria parasites, and have mainly focused on the use of the Gram-negative bacterium *S. marcescens* (*Sm*) as an oral pathogen to establish midgut infections. A study combining SNP phenotyping with transcriptomic and functional genetic analysis identified 3 genes encoding type III fibronectin domain proteins and a gustatory receptor that control the load of the *Sm* in the midgut of *A. gambiae* mosquitoes (Stathopoulos, Neafsey et al. 2014). *Sm* oral infections were shown to trigger a more pronounced transcriptional response in the midguts compared to abdomens that is characterized by an abundance of differentially expressed transcripts belonging to metabolism and a paucity of transcripts associated with immunity. Interestingly, transcripts that uniquely responded to *Sm* feeding belonged to processes related to protein translation, cell cycle, and DNA repair suggesting an investment in cellular damage repair (Dekmak, Yang et al. 2021). The response of the mosquito

midgut to damage appears to vary between mosquito species; chemical and *Sm*-induced damage trigger the proliferation of regenerative cells in the midguts of *Aedes albopictus* and *Culex pipiens* but only marginally in those of *A. gambiae*, suggesting distinct adaptations to stress responses (Janeh, Osman et al. 2017). Although *Sm* is able to colonize the guts of mosquitoes, it does not seem to be particularly virulent as it is commonly identified as member of the normal microbiota of mosquitoes and is well tolerated in the gut (Wang, Gilbreath et al. 2011, Chen, Zhao et al. 2016, Scolari, Casiraghi et al. 2019, Saab, Dohna et al. 2020). However, under certain conditions *Sm* may compromise the survival of *A. gambiae* mosquitoes, such as when ingested in the context of a blood meal containing *P. falciparum* gametocytes (Bahia, Dong et al. 2014) or when fed to mosquito in extremely large numbers (Janeh, Osman et al. 2019). Hence, a better understanding of the mosquito gut physiologic responses to infection will strongly benefit from the use of a broad panel of bacterial pathogens including the insect gut entomopathogens *Pseudomonas entomophila* (Vodovar, Vinals et al. 2005) and *Erwinia carotovora* (Basset, Khush et al. 2000), as distinct pathogens exhibit distinct virulence factors and vary in their capacity to efficiently colonize the gut.

Specific aims of my proposal:

• In Specific Aim 1, we will assess the virulence of selected human bacterial gut pathogens to *A. gambiae* mosquitoes through oral infections as well as their ability to colonize the mosquito midgut.

• In Specific Aim 2, we will assess whether bacterial oral infections trigger the proliferation of regenerative cells in the mosquito midgut and induce epithelial cell polyploidy.

CHAPTER II

MATERIALS AND METHODS

A. *Anopheles* gambiae rearing

All experiments were performed with 1-3 day old adult female *Anopheles gambiae* G3 strain mosquitoes, reared in a dedicated insectary in the Department of Biology at the American University of Beirut. Mosquitoes were maintained at 27 (±1) °C and 75 (±5) % humidity with a 12-hour day-night cycle. Larvae were reared in 752 cm^2 plastic pans at a density of approximately 150 larvae per pan and given Tetra® tropical fish food. Freshly emerged adult mosquitoes were collected from larval pans using a vacuum collector and fed on sugar pads containing 10 % sucrose. To maintain the cycle, adult mosquitoes were given a mouse blood meal once per week to lay eggs; BALB/c mice were anesthetized with a solution of ketamine and xylazine, then placed on top of mosquito cages allowing the starved mosquitoes to feed on mice blood for approximately 15 minutes in total darkness.

B. Bacteria Cultures

All bacterial species were pre-cultured in LB broth medium for approximately 16-18 hours in an incubator at 37°C with moderate shaking. *Ecc* pre-cultures were incubated at 29°C with moderate shaking. Bacterial stocks were previously prepared and stored in cryogenic vials at -80°C. In a BSL2 lab and under the hood, pre-cultures were prepared at a standard of 5 u of bacteria from cryogenic stock into 10 mL LB broth. Pre-cultures were prepared in either falcon tubes or Erlenmeyer flasks such that 1/3 of volume was occupied with LB.

After the incubation period, pre-cultures were centrifuged at 4000 rpm for 5 minutes and supernatant was discarded. Then, two rounds of washing and centrifuging at 4000 rpm with 1xPBS were done with the bacterial pellet for 1 minute/round. At the end, a final resuspension of the pellet was performed with 1x PBS. This final suspension was used to feed adult female mosquitoes in the experiments of this project.

C. Mosquito Survival Assays

Sixty (1-2 day old) female mosquitoes were fed with a suspension of pre-cultured bacteria and 3 % sucrose. The suspension was applied on cotton pads for 24 hours and then replaced with sterilized cotton pads containing 10 % sucrose. The survival of bacteria-fed mosquitoes was scored over a period of two weeks. The first batch of bacteria (*St-Kp-Pa* ST:639) were fed to female mosquitoes on different OD levels 1, 2 and 4. The remaining bacterial species were fed at OD:4. The concentration of bacteria was determined using a spectrophotometer (at λ=600 nm) by diluting the bacterial pellet (D.F=20). The suspension fed was a mixture of washed bacteria, water and sucrose (Final concentration: 3 %). At least 2 assays were performed for each bacteria using different batches of mosquitoes.

D. Microbial Proliferation Assays

To determine the extent of bacterial colonization in the mosquito midgut, female mosquitoes were fed with bacterial suspensions (as mentioned above) for 24 hours. After that, mosquitoes were dissected on ice and the midgut, with the proventriculus, were placed into Eppendorf tubes containing sterile 1x PBS. For each bacteria, 6 groups of 6 midguts each were grinded for 45 seconds in sterile 1x PBS. After that, serial

dilutions were plated onto either LB or Macconkey agar plates supplemented with the corresponding antibiotic (Table 1) and the CFUs were counted after 14-16 hours. Statistical significance was calculated using the Mann-Whitney test. Medians were considered significantly different if $P<0.05$.

Bacteria	ID	ST	Plate Medium	Antibiotic	Breakpoint (ug/mL)
Salmonella typhi	S1	-	LB + Antibiotic	Chloramphenicol	32
Klebsiella pneumoniae	097	383	LB + Antibiotic	Tetracycline	16
Pseudomonas aeruginosa	639	309	Macconkey + Antibiotic	Meropenem	8
Pseudomonas aeruginosa	548	111	Macconkey + Antibiotic	Meropenem	10
Pseudomonas aeruginosa	637	-	Macconkey + Antibiotic	Meropenem	10
Escherichia coli	006	648	LB + Antibiotic	Gentamicin	10
Escherichia coli	166	131	LB + Antibiotic	Gentamicin	6
Escherichia coli	020	167	LB + Antibiotic	Tetracycline	12

Table 1. Bacteria species and their corresponding antibiotics. The ID column shows the last three digits of the complete internal IDs.

E. Endoreplication Assay

This experiment was performed using the Click-iT® EdU Imaging Kit from Invitrogen. Three independent trials were executed and representative images were then chosen. This assay utilizes EdU (5-ethynyl-2′-deoxyuridine) which is a nucleoside analog of thymidine that is incorporated into DNA during active DNA synthesis. DNA synthesis is detected via a click reaction: a copper-catalyzed covalent reaction between an azide (Alexa Fluor®) and an alkyne (incorporated in the EdU). The sequence performed is as follows:

1) Two-day old mosquitoes were fed with 10 % sucrose containing 50/100 ug/mL of EdU for 48 hours.
2) After 48 hours, EdU was removed and mosquitoes were fed with a suspension of bacteria and 3 % sucrose for 24 hours.

3) After 24 hours, mosquitoes were dissected and midguts were extracted (with proventriculus) and fixated with 4 % PFA in PBS for 30 minutes.
4) After fixation, midguts were washed three times with 300 uL of 2 % BSA in PBS for 10 minutes each.
5) Midguts were blocked and permeabilized with 300 uL of 2 % BSA, 0.1 % Tx100 in PBS for 90 minutes.
6) Midguts were washed two times with 300 uL of 2 % BSA in PBS for 10 minutes each.
7) 300 uL of Click-iT reaction cocktail was added and midguts were incubated at RT for 30 minutes on a rocking plate in the dark.
8) Reaction cocktail was removed and midguts were washed once with 300 uL of 2 % BSA in PBS.
9) Midguts were then stained with Anti-PH3 (1:250) added in 2 % BSA, 0.1 % Tx100 in PBS (PBST) and incubated overnight at 4°C in the dark.
10) The following day, midguts were washed three times with 300 uL of PBST. After washing, 300 uL of Anti-Rabbit 568 Ab was then added for 2 hours at RT in the dark.
11) Midguts were washed three times with 300 uL of PBST. After washing, Hoescht stain was added for 5 minutes.
12) A final wash with 300 uL of 1x PBS was done before mounting the midguts on a slide using a mounting solution. A cover slip was added and sealed with nail polish. Samples were stored at -20°C.

CHAPTER III

RESULTS

A. Mosquito Susceptibility to Oral Infections with Human Bacterial Pathogens

Human bacterial pathogens have been associated with insects in a variety of settings. Recent studies suggest that the ability of an organism to survive an infection is determined by more than just the capacity to demonstrate physiological resistance by eliminating the invading microorganism [161]. Rather, tolerance, defined as the ability to limit the health impact and fitness effects caused by an infection, may be used in conjunction with resistance to promote host survival to a given infection. The survival rate of an organism following an infection is considered one of the key indicators of tolerance. In this project, we aimed to assess the survival rate of mosquitoes when challenged with different strains of human bacterial pathogens. *An. gambiae* female mosquitoes were orally fed with a suspension containing the bacteria and 3 % sucrose and their survival rates were scored over a period of 2 weeks. Mosquitoes fed with 3 % sucrose alone were used as control. At low OD (OD 1 and 2), *Salmonella* Typhi (*St*) and *Klebsiella pneumoniae* (*Kp*) slightly decreased the survival rate of mosquitoes whereas *Pseudomonas aeruginosa* (*Pa*) (ST:309) caused significant mortality (Fig. 3A-B). At higher OD (OD:4), all strains caused significant mortality except for *St* and the survival rate of mosquitoes fed with human bacterial pathogens were comparable to mosquitoes challenged with *Erwinia carotovora* (Ecc) of OD:1 which is an established entomopathogen (Fig. 3C).

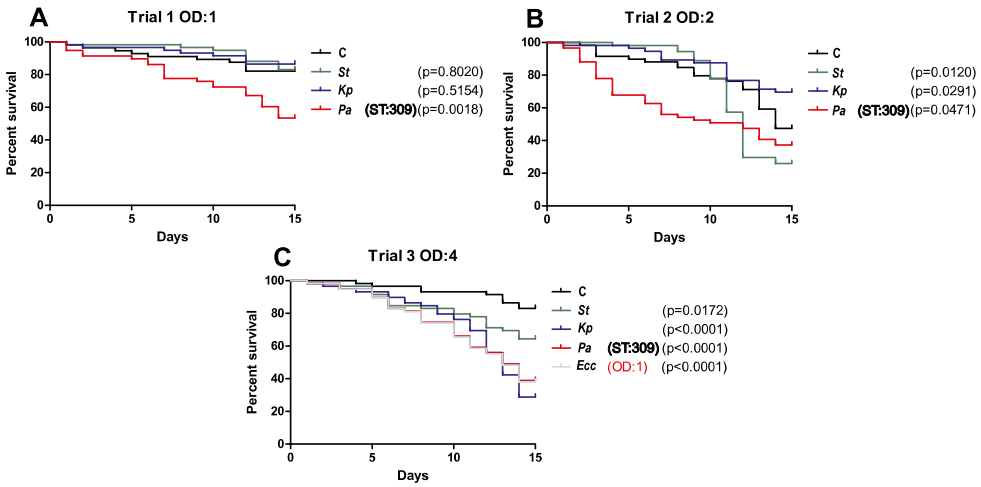

Figure 3. Challenging *An. gambiae* female mosquitoes with human bacterial pathogens reduced their survival rate. (A-C) Survival assays after oral infection with *S.t*, *K.p*, *P.a* (ST:309) of different ODs and *Ecc* (OD:1). Three independent biological experiments are shown. The Kaplan-Meier survival test was used to calculate the percent survival. Statistical significance of the observed differences was calculated using the Log-rank test.

To further assess this phenomenon, *An. gambia* mosquitoes were challenged with other human bacterial species such as *Escherichia coli* (*E. coli*). In addition, different sequence types of the same species were utilized to determine whether the phenomenon observed differs among the same species. At high OD, several species tested were able to cause significant mortality. *E. coli* (ST:131) caused significant mortality whereas *E. coli* (ST:648) seemed to be of moderate virulence (Fig. 4A-B). Thus, different strains of a certain species (of ≠ STs) have different virulence patterns.

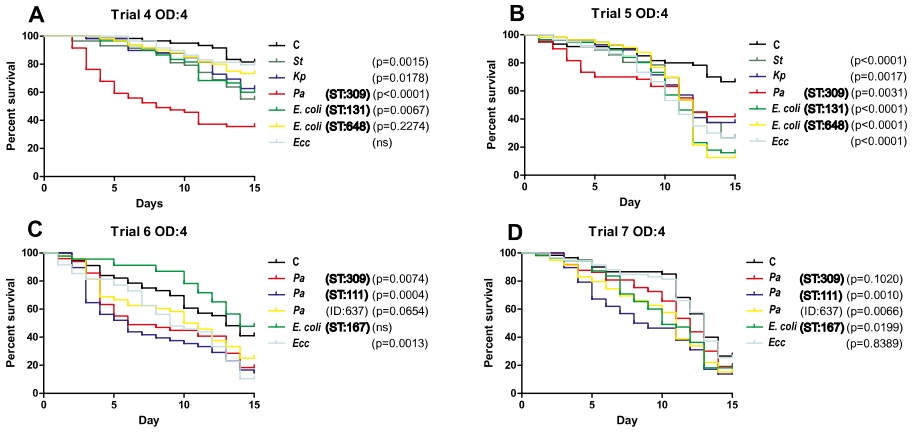

Figure 4. Challenging *An. gambiae* female mosquitoes with human bacterial pathogens (OD:4) reduced their survival rate. (A-D) Survival assays after oral infection with *S.t*, *K.p*, *P.a* (ST:309), *P.a* (ST:111), *P.a* (ID:637), *E. coli* (ST:131), *E. coli* (ST:648), *E. coli* (ST:167) and *Ecc*. Two independent biological experiments are shown. The Kaplan-Meier survival test was used to calculate the percent survival. Statistical significance of the observed differences was calculated using the Log-rank test.

To confirm this, we challenged mosquitoes with different STs (i.e different strains) of *P.a*. The survival rates showed that there is variation in virulence patterns among species and among sequence types of the same species (Fig. 4C-D). Moreover, *P.a* seemed to be particularly virulent when compared to other tested bacterial species.

V. cholerae and arthropods has been documented and is likely more frequent than that between *V. cholerae* and humans [162, 163]. In fact, environmental studies have demonstrated that common house flies carry *V. cholerae* in endemic areas [164]. In one study, it was hypothesized that *V. cholerae* may have evolved for an arthropod rather than for humans [165]. In addition, in one study, the infection frequency of *Acinetobacter* to *Ae. albopictus* was particularly high which may indicate that there is a symbiotic relationship between the bacterium and its host [166]. Therefore, *An. gambiae* female mosquitoes were also challenged with *Vibrio cholerae* (*Vc*) and

Acinetobacter baumannii (*Ab*). Both species displayed virulence towards their host throughout the trial particularly *V. cholerae* (Fig. 5).

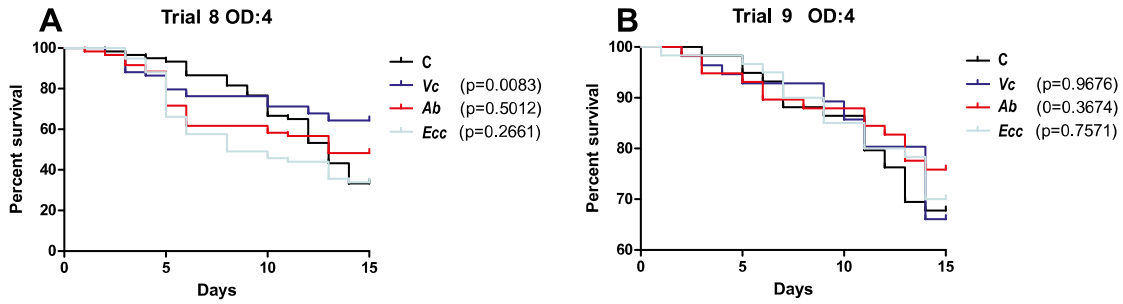

Figure 5. Challenging *An. gambiae* female mosquitoes with human bacterial pathogens (OD:4) reduced their survival rate. (A-B) Survival assays after oral infection with *V. cholerae* (*Vc*), *A. baumannii* (*Ab*) and *Ecc*. Two independent biological experiments are shown. The Kaplan-Meier survival test was used to calculate the percent survival. Statistical significance of the observed differences was calculated using the Log-rank test.

B. Human Bacterial Pathogens Cannot Colonize the Mosquito Midgut

Microorganisms, indeed, colonize different organs and tissues in mosquitoes, including gut, salivary glands and reproductive tissues [167-169]. They influence many aspects of the mosquito biology, including reproduction, development, adult survival and, overall, immunity [170]. After observing positive phenotypes of various degrees of virulence from different human bacterial pathogens, we opted to assess whether these pathogens do colonize the mosquito midgut. Female *An. gambiae* mosquitoes were challenged with bacteria for 24 hours and were dissected at different timepoints (Day 1, 4/5 and 8) for microbial proliferation assays. In addition, *Serratia marcescens*, a part of the mosquito gut flora, was used as control and proved capable of colonizing the mosquito midgut (Fig. 6C). At low OD, *St*, *K.p* and *P.a* (ST:309) colonies in the midgut were significantly reduced from Day 1 to Days 4 and 8 (Fig. 6 A-B-D). Thus, these

human bacterial pathogens couldn't efficiently colonize the mosquito midgut. Since *P.a* is of particular virulence, we next compared the CFUs of two different sequence types of *P.a* at higher OD (OD:4). The results were reciprocated as both sequence types of *P.a* showed significant reduction in colony numbers from Day 1 to Days 4 and 8 (Fig. 6 D-E). We hypothesize that *P.a* inflicts damage to *An. gambiae* female mosquitoes at early stages after challenging before it is cleared from the mosquito midgut. It is estimated that individual granulocytes can phagocytose approximately 1500 *E. coli [171]*. We challenged mosquitoes with high OD (OD:4) of *E. coli* of different sequence types. The clearance of *E. coli* from the midgut was much more significant than the remaining bacterial species (Fig. 6 F-G-H). In brief, *An. gambiae* female mosquitoes have low vector potential to carry the different species of human bacterial pathogens.

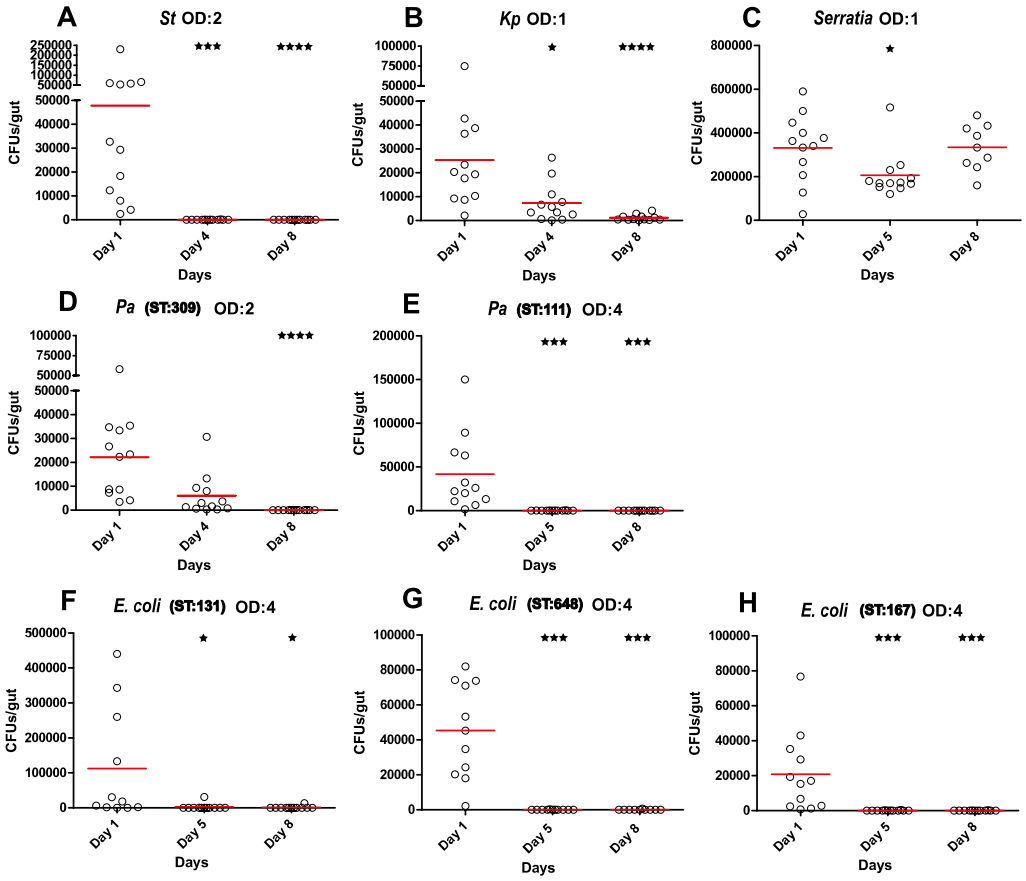

Figure 6. Human bacterial pathogens cannot efficiently colonize the mosquito midgut. Microbial proliferation assays in midguts at Days 1, 4/5 and 8 after oral infection. The results are based on two independent experiments pooled together and shown as mean values (Red line). Statistical significance was calculated using the Kruskal-Wallis test and means were considered significantly different (****) if $p < 0.05$.

C. Polyploidy Assessment in Response to Oral Infection

It is known that in insects, there are no adaptive immune responses. However, recently, there is contributing evidence that during the interaction with non-infective pathogens, an adaptive immune response can be developed inducing immune memory (priming). The immune response would be more intense and robust after a second encounter with an infective form of the pathogen. In the midgut of *Anopheles albimanus* and *Aedes aegypti,* significant DNA synthesis and endoreplication happen during immune priming [172-174]. There are cell cycle variations, a “cell cycle plasticity”,

which can generate polyploid cells using endoreplication [175]. Polyploid cells possess multiple copies of the complete genome [176], and they tend to support higher growth capacity for homeostasis and tissue differentiation. In addition, in *Ae. albopictus*, adult mosquito midgut cells can divide after damage induced by chemicals and bacterial ingestion [85]. Therefore, we sought whether the ingested human bacterial pathogens stimulate endoreplication in female *An. gambiae* midguts. Endoreplications assays were performed using the Click-iT® EdU Imaging Kit. Phosphorylation in histone 3 at serine 10 (PH3) is associated with mitosis in several systems, and it is used to determine the division activity [177] including adult mosquitoes. PH3 staining was established using Alexa Fluor® (Red) and DNA synthesis was evaluated by EdU (Green) incorporation into cell nuclei in mosquito midgut cells. In naïve *An. gambiae* mosquitoes, it was clear that EdU signals were concentrated in the midgut as opposed to the remaining compartments (Fig. 7A). Specifically, they were most concentrated at the entry site of the midgut. This observation comes in line with the fact that over 95 % of all AMP/putative AMP transcripts are derived from the proventriculus and anterior midgut [48]. Moreover, when mosquitoes were challenged with either *P.a* (ST:309) or *Ecc*, EdU signals were more pronounced in the proventriculus when compared to naïve mosquitoes (Fig. 7B-C). In addition, the number of cells undergoing endoreplication in the midgut (posterior and anterior) were similar between *A. gambiae* mosquitoes orally fed with human bacterial pathogens and sugar fed. However, endoreplicating cells were more pronounced in the proventriculus of orally infected mosquitoes than in sugar fed (Fig. 7D). In addition, regenerative cells in the midgut were rarely detected by PH3 staining, after oral infection with *Ecc* or *Pa 309* (Fig. 7E). We therefore hypothesize

that the proventriculus is a hallmark of the mosquito innate immunity in terms of AMP production.

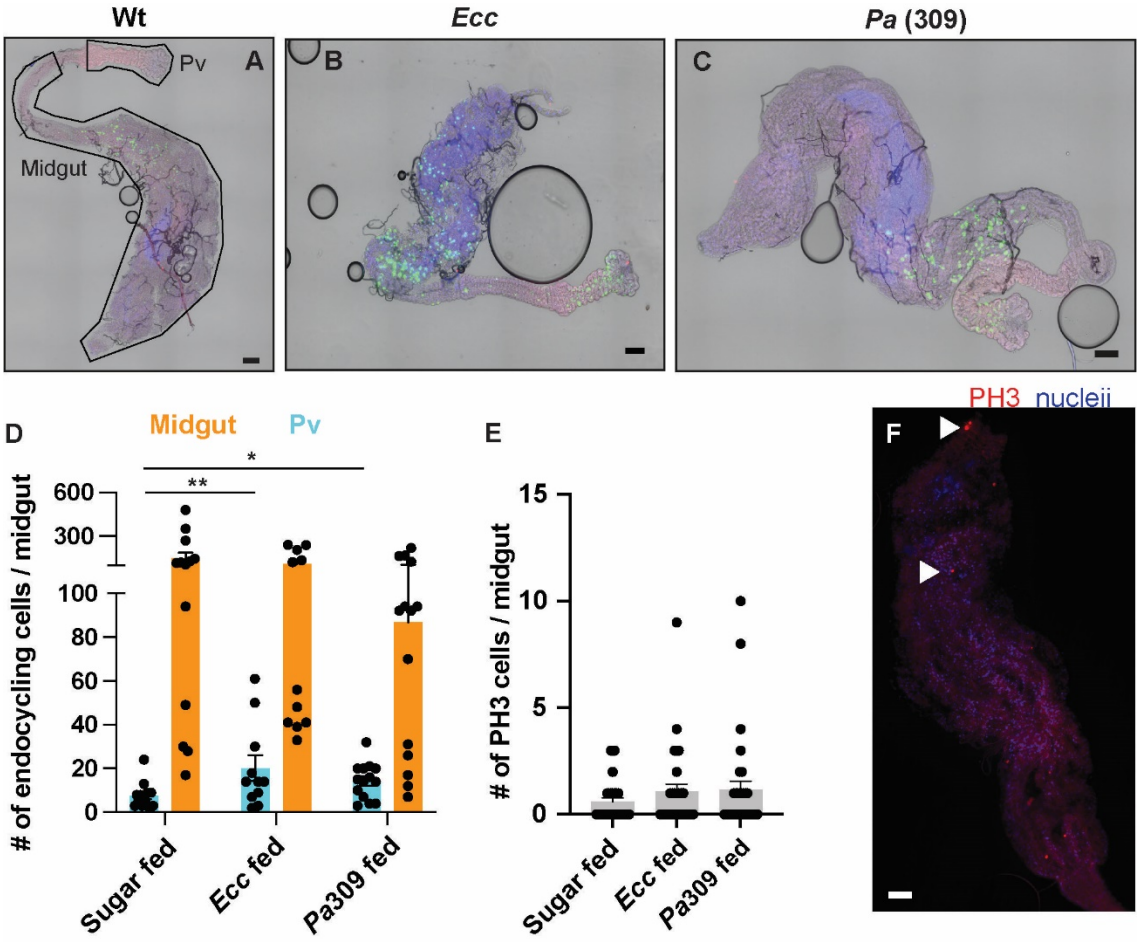

Figure 7. *Pseudomonas* and *Erwinia* oral infections increase the numbers of endocycling cells in the proventriculus. (A-C) sample images of full midguts captured from EDU-treated (A) sugar fed, (B) *Ecc* fed and (C) *Pa309* fed mosquitoes using tiling. (D) Numbers of endocycling cells in each condition. (E) Numbers of PH3 positive cells per condition. (F) Sample fluorescent image of a mosquito midgut fed with PSA (ST: 309) and stained with anti-PH3 (red) and Hoechst stain (blue). Arrow heads point to the rare regenerative cells. Statistical analysis was performed using the Mann-Whitney non-parametric test, and differences were considered significant if $P<0.05$. *, $P<0.05$; **, $P<0.005$.

CHAPTER IV

DISCUSSION

Mosquitoes are important vectors of disease that impose a significant burden on human health due to the plethora of pathogens they transmit including viruses and parasites. The mosquito midgut constitutes the first barrier for the entry of pathogens into the insect body cavity. It is also populated with bacteria communities that play important roles in shaping the immune response of the vector and its capacity to transmit human pathogens [103, 178, 179], in addition to vector susceptibility to certain insecticides [180]. In some cases, gut bacteria such as *S. marcescens* were found to promote dengue virus transmission by *Aedes aegypti* [181]. Bacterial communities change and shift with mosquito life stage and nutritional status, geography, and phenology [182].

The physiological responses of the midgut epithelium to bacterial pathogens are not very well understood as those to malaria parasites, and have mainly focused on the use of the Gram-negative bacterium *S. marcescens* as an oral pathogen to establish midgut infections. However, *S. marcescens* does not seem to be particularly virulent as it is commonly identified as member of the normal microbiota of mosquitoes and is well tolerated in the gut. Hence, a better understanding of the mosquito gut physiologic responses to infection will strongly benefit from the use of a broad panel of bacterial pathogens including the insect gut entomopathogens *Pseudomonas entomophila* [183] and *Erwinia carotovora carotovora* [184], as distinct pathogens exhibit distinct virulence factors and vary in their capacity to efficiently colonize the gut. In this project, we assessed the competency and fitness of female *An. gambiae* mosquitoes

after oral infection with a panel of human bacterial pathogens. The panel was diverse highlighting pathogens that are mainly causative agents of gut infections. These include *Salmonella* Typhi [185], *Pseudomonas aeruginosa* [186], *Klebsiella pneumoniae* [187], *Escherichia coli* [185], *Vibrio cholerae* [188] and *Acinetobacter baumannii* [189].

Among the human pathogens, *P. aeruginosa* seemed to be the most virulent as it caused significant mortality when fed to mosquitoes at a low OD. At higher OD, other species also caused significant mosquito mortality including *K. pneumoniae*, *S.* Typhi and *E. coli*. The virulence of *P. aeruginosa* was not unexpected as this bacterium is virulent to a wide range of insects and nematodes [190-193]. Among *P. aeruginosa* virulence factors in invertebrates are phenazines, pigments secreted by the bacteria causing the generation of the ROS in the host [190] and effector molecules secreted by the Type-2 and Type-3 secretion systems [194, 195]. On the contrary *V. cholerae* and *A. baumannii* did not cause mortality through feeding suggesting that their virulence mechanisms are more relevant to mammalian cells. Interestingly, *V. cholerae* was virulent to *Drosophila* through oral infections exhibiting several of the characteristics of the human disease. However, in contrast to mammalian infections, the cholera toxin alone did not cause disease in the fly in the absence of the bacterial cells, suggesting that *V. cholerae* may contain factors that exhibit virulence in the fly but not the mammalian host [196]. Of note, oral infections with *Ecc*, an entomopathogen, were not lethal to mosquitoes, and the same was observed in *Drosophila* [197].

Despite the mortality caused by the human bacterial pathogens, none of them was able to colonize the gut and they were all cleared by day 8, in contrast to *S. marcescens* which colonized the gut efficiently as expected, since, it is frequently isolated from the gut microbiota [198]. These results suggest that oral infections with

the human pathogenic bacteria are causing some irreversible damage to the midgut causing the observed mortality. It would be interesting to observe whether changes in the mosquito gut morphology are triggered by these oral infections using scanning electron microscopy. Also, it would be interesting to see whether RNAseq analysis of infected midguts (which is underway) reveals enrichment of functions associated with cellular damage that could explain the observed mortalities.

Measurement of polyploidy in midgut epithelial cells after feeding on *P. aeruginosa*309 and *Ecc* revealed that the number of cells undergoing endoreplication was similar in the midgut (anterior + posterior) of orally infected and the sugar fed control, however more endoreplicating cells were counted in the proventriculus of the former group. This observation is interesting since the proventriculus in *A. gambiae* mosquitoes was shown to be enriched in transcripts encoding anti-microbial peptides and anti-*Plasmodium* factors suggesting that it is a special defensive compartment against orally acquired pathogens [199]. The enrichment of the proventriculus in defensive genes was also observed in the gut of *Ae. aegypti* mosquitoes [48]. It is important to note that despite the fact that the midguts of orally infected mosquitoes have similar numbers of endoreplicating cells as those fed on sugar, this does not necessarily mean that they have the same levels of endoreplication. Future experiments should focus on quantitating the mean level of EdU fluorescence per midgut from both groups to determine if more endocycling is taking place in cells of orally infected mosquitoes. Midguts of *A. gambiae* mosquitoes did not exhibit significant numbers of regenerative cells, as deduced from PH3 staining, in response to oral infections with *Ecc* or *P. aeruginosa*309. These results are in agreement with a previous study showing that feeding *A. gambiae* mosquitoes with sucrose solution containing the chemical SDS

or *S. marcescens* did not result in an increase in the number of mitotic cells in the midgut [86]. However, it seems that different mosquito species have different gut physiologies since regenerative cells have been detected in the midguts of *Culex pipiens* and *Ae. albopictus* mosquitoes in response to gut damage [85, 86].

Our findings conclude that *An. gambiae* mosquitoes have no colonization capacity for human bacterial pathogens as they are rapidly cleared from the midgut. However, these bacteria seem to cause significant damage to the host as deduced from the increased mortality rate and levels of endoreplication in the proventriculus. It would be interesting to test whether these pathogens are virulent to mosquitoes when injected directly into the hemolymph to establish systemic infections, which could inform whether mosquitoes are good models to score and characterize the virulence of human bacterial pathogens. It would be also interesting to test the effects of the bacterial species and sequence types used in this study on other mosquito species that have a broader geographical distribution, such as *C. pipiens* mosquitoes and that are more likely to encounter human bacterial pathogens, specifically in their larval habitats.

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