Faculty of Pharmaceutical, Biomedical and Veterinary Sciences

Faculty of Engineering Technology

The black soldier fly as a source of new antimicrobials

De zwarte soldatenvlieg als een bron voor nieuwe antimicrobiële middelen

Dissertation submitted in partial fulfilment of the requirements for the degree of doctor of pharmaceutical sciences (University of Antwerp) and doctor of engineering technology (KU Leuven)

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Preface / voorwoord

Liefste lezer,

Nu ik nog je volle aandacht heb, zou ik eerst een hele waslijst aan mensen willen bedanken. Dit doctoraat is niet alleen door mij tot stand gekomen, maar is een resultaat van intensieve samenwerking tussen verschillende universiteiten, partners en collega's.

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coronalockdowns voor een verandering van omgeving: van de kempische campus naar het Lintse Home Office.

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Bedankt ook aan de juryleden voor de tijd en moeite die jullie in deze thesis gestoken hebben!

Het schrijfproces was er één met veel ups en downs (zie ook p. 225), maar de laatste woorden staan nu (eindelijk) op papier. Over naar het echte werk!

Juni 2024 *Laurence*

Abbreviations

A

AA	Amino acid
ATCC	American Type Culture Collection
APD	Antimicrobial peptide database
AMP	Antimicrobial peptide
AMR	Antimicrobial resistance

B

BC	Bodipy TR Cadaverine
BCCM	Belgian Coordinated Collections of Microorganisms
BSF	Black soldier fly
BSFL	Black soldier fly larvae

C

CF	Cystic fibrosis
CFU	Colony forming units

D

diSC3(5)	3,3′-dipropylthiadicarbocyanine iodide
DMSO	Dimethyl sulfoxide

E

ELISA	Enzyme-linked immunosorbent assay
EUCAST	European Committee for Antimicrobial Susceptibility Testing

F

FIC	Fractional inhibitory concentration
FOB	Functional observational battery

H

HC1	*Hermetia illucens* cecropin 1/Hill-Cec1
HC10	*Hermetia illucens* cecropin 10/Hill-Cec10
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic
HPLC	High-performance liquid chromatography

I

IC50	50% inhibitory concentration
iFCS	Inactivated foetal calf serum
IM	Inner membrane

L

LAL	Limulus ameubocyte lysate
LB	Luria Bertani
LPS	Lipopolysaccharide

M

MALDI-TOF	Matrix assisted laser desorption ionization time-of-flight
MDR	Multi-drug resistant
MEM	Minimum essential medium
MHA	Mueller Hinton agar

MHB	Mueller Hinton broth
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*

N

NPN	N-phenyl-naphthylamine

O

OD	Optic density
OM	Outer membrane

P

PBS	Phosphate buffered saline
PCA	Plate count agar
PCR	Polymerase chain reaction
PI	Propidium iodide
Pi	Isoelectric point

Q

qPCR	quantitative PCR

R

RAPD	Random amplification of polymorphic DNA
RMPI	Roswell Park Memorial Institute

T

TFA	Trifluoroacetic acid
TSA	Tryptic soy agar

W

VAP	Ventilator-associated pneumonia
WHO	World Health Organization

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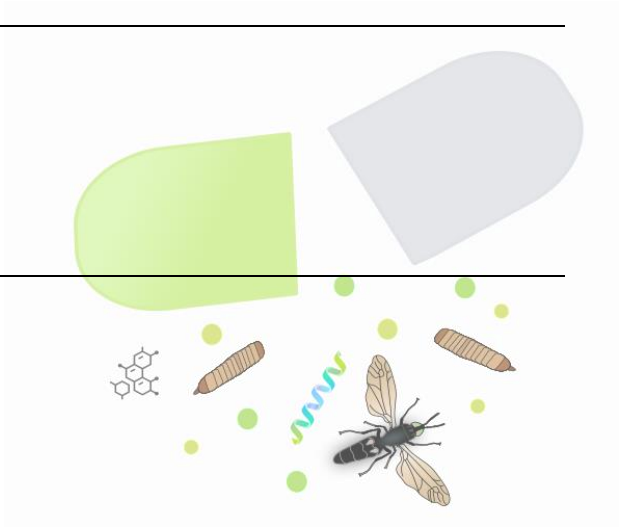
CHAPTER I

Research objectives



I.1 Research objectives

With the surge of antimicrobial resistance (AMR), many drugs routinely used in clinical practice are failing to treat critical infectious diseases [1]. Tackling this AMR emergency requires a multifaceted approach, including the discovery and development of new antimicrobial agents that are active against drug-resistant pathogens [2]. Nature has proven to be a valuable source of many, structurally diverse antimicrobial compounds discovered over the past decennia. Most antibiotics of natural origin or derived from a natural scaffold are produced by microbes, such as bacteria and fungi [2]. Recently, however, there has been increased interest in alternative natural niches such as insects [3-5]. Insects are remarkably resilient to microbial infections and possess a wide range of antimicrobial defense tactics [6]. In this thesis, the black soldier fly (BSF, *Hermetia Illucens*) specifically will be explored as a source of new antimicrobial compounds. Active agents can be found in both the insect itself, as well as in its microbiome (**Figure I.1**).

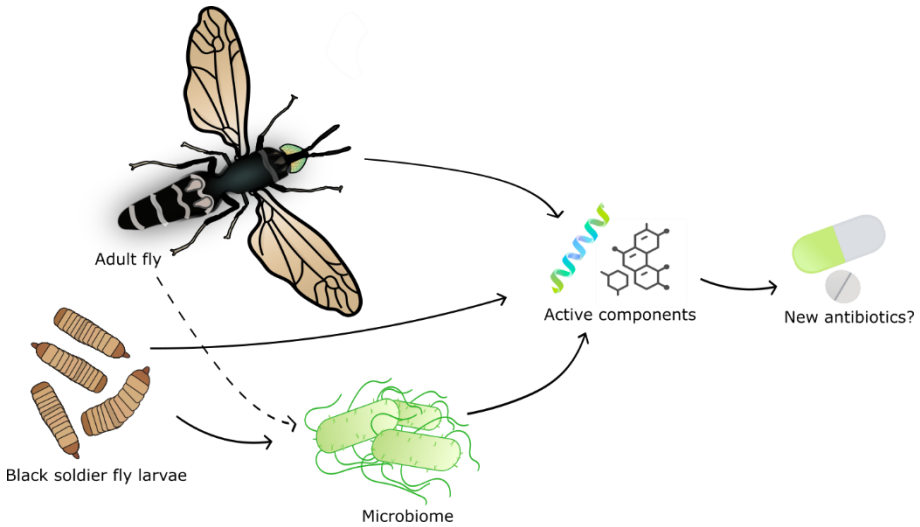


Figure I.1 Antimicrobial compounds can be produced by the insect itself, as well as by the microbes living in a close relation with the insect.

Within this work, a large focus is placed on the collection of BSF antimicrobial peptides (AMPs). AMPs are involved in the innate immune reaction of all living organisms and have gathered attention as alternatives to conventional antibiotics [7]. At the start of this

thesis, the antimicrobial profile of the BSF peptide library was not yet investigated [8]. Biological characterization of these peptides is an essential first step in unravelling their antimicrobial potential and any further optimization requirements. Hence, the main research objective of this thesis can be summarized as follows: **Antimicrobial evaluation and biological characterization of black soldier fly antimicrobial peptides** (**Figure I.2**). *Pseudomonas aeruginosa* was selected as the main pathogen of interest for the *in vitro* and *in vivo* characterization experiments as (**i**) high anti-pseudomonal activity was found for a set of peptides (CHAPTER III), and (**ii**) *P. aeruginosa* remains a priority pathogen of the World Health Organization, for which new antibiotics are urgently needed [9]. Apart from the BSF peptide library, the antimicrobial activity of the fly's dominant gut micro-organisms and larval extracts is investigated as well, leading to a second research objective: **Beyond antimicrobial peptides: exploring the antimicrobial potential of the black soldier fly's larval gut symbionts and larval extracts** (**Figure I.2**).

I.2 Outline of the thesis

CHAPTER II introduces the current AMR epidemic, AMPs, and the BSF. In CHAPTER III, the evaluation of the antimicrobial activity and cytotoxicity of the BSF peptide library is described. Two peptides with promising activity (HC1 and HC10) were chosen for further experiments, and some key characteristics, including presence of hemolysis, primary and secondary structure, and membrane permeabilizing activity are additionally explored in CHAPTER III. In CHAPTER IV, the anti-pseudomonal activity of HC1 and HC10 is described in more detail. Specifically, characteristics that are typically described as 'advantages of antimicrobial peptides' such as synergy with conventional antibiotics, lower resistance development, and anti-biofilm activity are investigated here. CHAPTER V discusses the lipopolysaccharide (LPS)-binding activity of HC1 and HC10 and its implications on the activity against *P. aeruginosa* strains with LPS mutations, *in vitro* macrophage activation, as well as the peptide activity in physiological salt conditions. CHAPTER VI describes the *in vivo* activity of HC10 against *P. aeruginosa*. Three different animal models have been used to study the activity of HC10: A *Galleria mellonella* model, a *P. aeruginosa* mouse

infection model, and an LPS-induced murine lung injury model. In CHAPTER VII, the antimicrobial activity of dominant gut microorganisms of the black soldier fly is evaluated, as well as the activity of black soldier fly larval extracts. Finally, CHAPTER VIII presents a general discussion of the work in this thesis, including future perspectives.

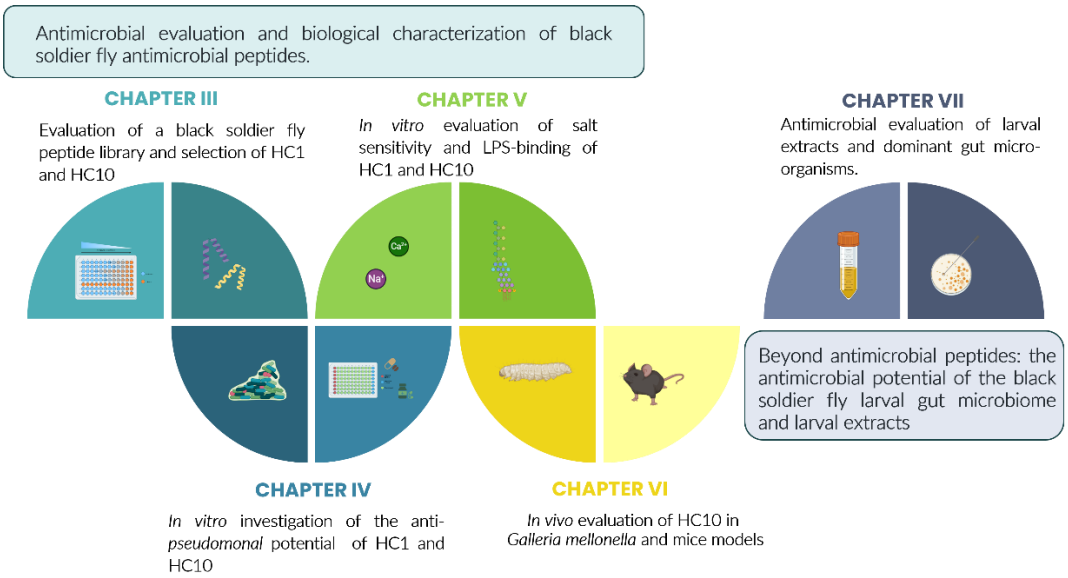
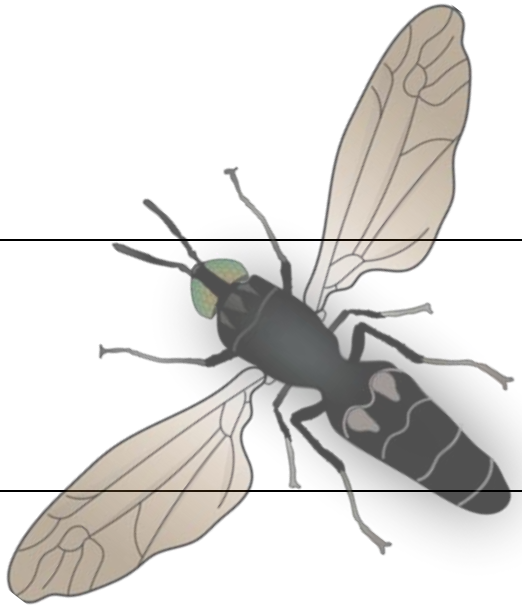


Figure I.2: Graphical overview of the research chapters and their respective goals.

CHAPTER II

Introduction



Sections II.3 “Insects as a source for novel antibiotics” and II.3.3 “Insect symbionts as a source for novel antibiotics” are partially adapted from: Van Moll, L., De Smet, J., Cos, P., Van Campenhout, L., Microbial symbionts of insects as a source of new antimicrobials: a review. Critical Reviews in Microbiology, 2021, 47(5):562-579, DOI: 10.1080/1040841X.2021.1907302

II.1 Antibiotics: from golden age to dark age

The emergence of modern day antibiotics has had an unprecedented impact on global health and is one of the greatest breakthroughs in 20th century medicine. Although there is no universal consensus, mycophenolic acid, originating from *Penicillium* fungi, is often recognized as the first antimicrobial agent, although it was never commercialized as such [10, 11]. It wasn't until the discovery of Paul Erlich's so-called "magic bullet" arsphenamine in 1909 that an antibiotic was clinically available to a broad group of patients [10, 12]. In 1928, Alexander Fleming discovered the penicillins, the oldest class of antibiotics still used up to this day, from a *Penicillium* fungus [13, 14]. Selman Waksman's findings that bacteria dispose of their own arsenal of antimicrobial compounds to combat other bacteria ushered in the golden age of antibiotics in the 1940s. In the following decades, the repertoire of available antibiotics was expanded considerably, largely due to the screening of soil *Streptomyces* bacteria [15]. In 1943 the aminoglycoside streptomycin was found to be active against a wide range of pathogens, making it the first broad-spectrum antibiotic and the first antibiotic used successfully in the treatment of tuberculosis [10, 15, 16]. Amphotericin B, also from *Streptomyces* origin, became the first antifungal antibiotic in 1958 [17]. The gramicidins, produced by *Bacillus* species, became the first clinically available peptide antibiotics [10, 11]. Along with the exploitation of bacteria as a source for novel antibiotics, synthetic design gained increased popularity as well. Some of the early synthetic antibiotics include metronidazole and nalidixic acid, a prototype fluoroquinolone [10]. Over time, the depletion of natural microbial resources slowed down the rate of antibiotic discovery significantly, and with the emergence of widespread antibiotic resistance, the thriving age of antibiotics came to an end.

The first report of AMR was of arsphenamine and predates the discovery of penicillin [18]. At first, the main strategy at hand was the modification of known antibiotics to evade the existing resistance mechanisms. Soon, however, resistance began to outpace this approach of molecular modification and the need for entirely new antibiotic classes

became evident [19]. Nevertheless, the majority of newly approved antibiotics as well as those currently in the clinical development pipeline, are based on earlier antibiotic scaffolds [20]. Daptomycin, linezolid, and lefamulin are one of the few recent antibiotics belonging to new classes [21-23]. Currently, AMR has grown to be one of the biggest challenges to both the global health and economic system. Uncontrolled AMR could lead to a death toll of 10 million people each year by 2050, undoing decades of medical progress [24]. Apart from increased investment in antibiotic research, other strategies are necessary to stop the continuous tug of war between new antibiotic development and the surfacing of drug-resistance. These strategies include the responsible prescription and usage of antibiotics, lowering the prevalence of infectious diseases with more preventive measures such as vaccines and better sanitation, and the optimization of antibiotic use, including therapeutic combination of multiple antibiotics [25, 26]. Overall, a joint effort will be needed to halt the emergence of a post-antibiotic era [24].

II.1.1 Current epidemiology of antimicrobial resistance

Many infectious diseases still rank among the leading causes of death worldwide [27]. The whole of lower respiratory tract infections, such as influenza and pneumococcal infections, remain the most deadly transmittable diseases [27]. When it comes to a single infectious agent, *Mycobacterium tuberculosis* is the leading cause of death, causing over 1.8 deaths annually [28]. Widespread AMR has fueled the lethality of many of these infectious diseases. It remains difficult to accurately estimate the global health and mortality burden of AMR due to insufficient accessible data on its prevalence and incidence, especially in low- and middle-income countries [29, 30]. Nevertheless, it is clear that AMR is a dire issue all over the world. A 2022 study (Murray et al.) estimated that in 2019, 1.27 million deaths were directly attributable to AMR, while another 2.68 million deaths were associated with AMR [1]. In the European region, around 670.000 infections with AMR occur annually, leading to a mortality of 33.000 people and an annual cost of approximately 1.5 billion euros [31, 32]. The majority of these AMR related deaths are seen in lower respiratory infections, bloodstream infections, and abdominal

infections [1]. A total of six bacteria accounts for over 73% of AMR mortality: *Escherichia coli, Staphylococcus aureus*, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* (Figure II.1) [1]. Especially alarming are the increasing reports on bacteria that have acquired resistance to multiple antibiotic groups (multidrug resistance or extensive drug resistance) or even to all clinically used antibiotic classes (pan drug resistance) [33, 34]. In the latter case, a combinatory treatment of synergystic antibiotics is often required to cure patients [33].

In 2008, the ESKAPE acronym was introduced to describe a group of bacterial pathogens with critically established antibiotic resistance (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanni*, *Pseudomonas aeruginosa*, and *Enterobacter* species) [35]. Years later, these pathogens are still prioritized by the World Health Organization (WHO) when it comes to antibiotic drug research and development [36]. The list now also includes other bacteria such as *Escherichia coli* and *Helicobacter pylori* [37]. Apart from the prevalence of resistance, criteria that are used to prioritize certain pathogens and infections are overall mortality, clinical burden, transmissibility, available preventive measures and treatment options, and antibiotics in the development pipeline [36, 38].

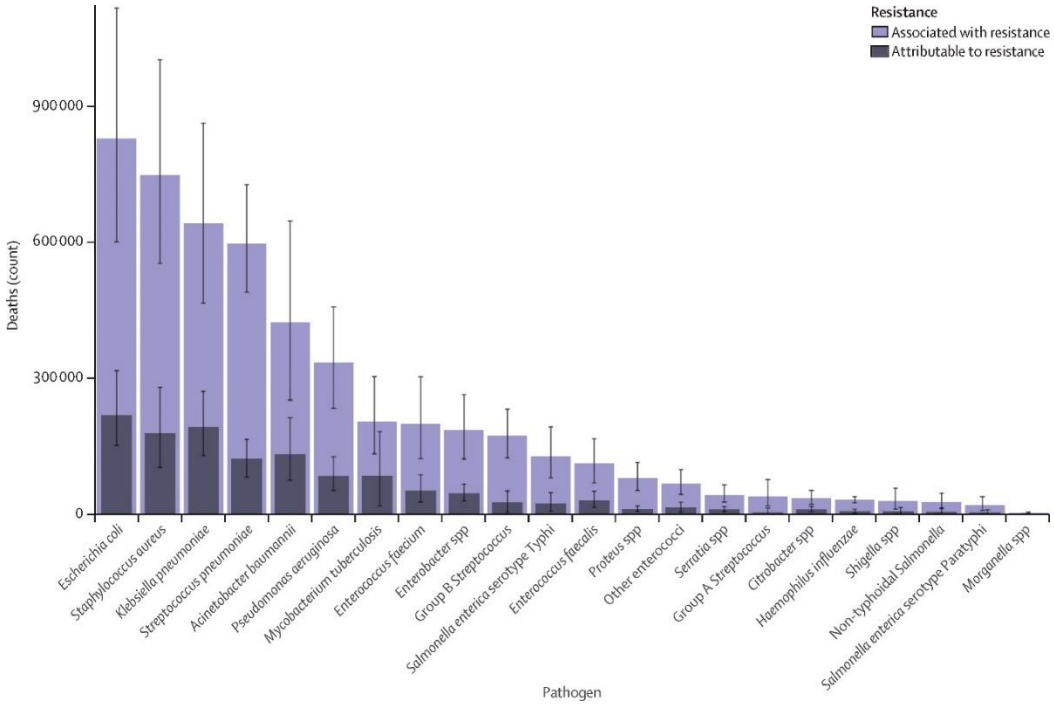


Figure II.1 Bacterial pathogens and their share in the global death toll of antimicrobial resistance (Murray et al., 2019). The graph shows the deaths directly related to the occurrence of a resistant pathogen (e.g. due to treatment failure), and those that were caused by an infection with a resistant pathogen, but where the resistance is not directly related to the infection outcome [20].

The Gram-negative bacteria *A. baumanni*, *P. aeruginosa* and the Enterobacterales bacteria are among the most critical pathogens due to established carbapenem-resistance. *A. baumannii* is an important cause of lung and bloodstream hospital-acquired infections, although the growing prevalence of community-acquired infections is concerning as well [39, 40]. *P. aeruginosa* is another frequent cause of nosocomial infections, including bloodstream, lung and urinary tract infections. Immunocompromised people, including cystic fibrosis patients, are specifically vulnerable to *P. aeruginosa* [41, 42]. Species within the Enterobacterales can lead to a variety of infections. *Escherichia coli*, for example, is the most common cause of both septicemia and hospital-acquired urinary tract infections [43]. Other pathogens that have been assigned high priority include methicillin-resistant *S. aureus* (MRSA), vancomycin-

resistant *E. faecium*, fluoroquinolone-resistant *Campylobacter* species and clarithromycin-resistant *H. pylori* [36]. Although less widespread than antibacterial resistance, antifungal resistance is an emerging threat as well, especially to immunocompromised people (e.g. HIV patients). Resistance can be found in a variety of fungi and yeasts, including azole-resistance in *Aspergillus fumigatus* and *Candida glabrata* [44, 45]. In parallel to bacteria and fungi, clinically relevant resistance has been observed for viruses as well. For example, resistance to antiretroviral medicines has been increasing exponentially in recent years and is an important cause of treatment failure in HIV patients [46]. An example of resistance in parasites, is artemisinin-resistance in *Plasmodium falciparum* and chloroquine-resistance in *Plasmodium vivax*, both of which have negatively impacted the morbidity and mortality of malaria considerably [47].

II.1.2 Behind the resistance

When microorganisms no longer respond to antimicrobial agents they were previously susceptible to, the term “acquired resistance” is used. In contrast, “intrinsic resistance” refers to the natural increased tolerance that some microbes possess against certain antibiotics [48]. Although acquired AMR occurs naturally over time, the widespread and often careless use of antibiotics ever since their first introduction has accelerated its occurrence [29, 49, 50]. To this day, misuse of antibiotics remains a dire issue, both in healthcare and in non-human applications [24, 51]. Many environmental reservoirs of resistant microbes exist outside of humans, including farm and wildlife animals, crops in agriculture, waste water, and soil [52]. Fifty percent of the global antibiotic consumption is attributed to usage in livestock [53]. Resistant microorganisms of animal origin are mainly transmitted to humans through the food chain [51]. When exposed to antibiotics, microbial populations undergo the pressure of natural selection. Strains that are less sensitive to the antibiotic due to mutational differences are selected and maintained [51, 52]. Mutations that confer protection against antibiotics can be passed on to other microbes through vertical and horizontal gene transmission, usually by means of plasmid conjugation [38]. Apart from spontaneous mutations, this horizontal gene transfer is the

driving force behind the rapid emergence and spread of multidrug resistance, enabling different microorganisms to interchange their resistance mechanisms [54]. On a molecular level, many different mechanisms can cause drug resistance (**Figure II.2**). These include (**i**) increased efflux of the antibiotic, (**ii**) decreased permeability and antibiotic uptake, (**iii**) changes in the antibiotic target, (**iv**) bypassing of the target and (**v**) inactivation of the antimicrobial agent itself [55]. The last option can be illustrated by β-lactamase, a bacterial enzyme that inactivates antibiotics of the β-lactam group such as penicillins [48, 55].

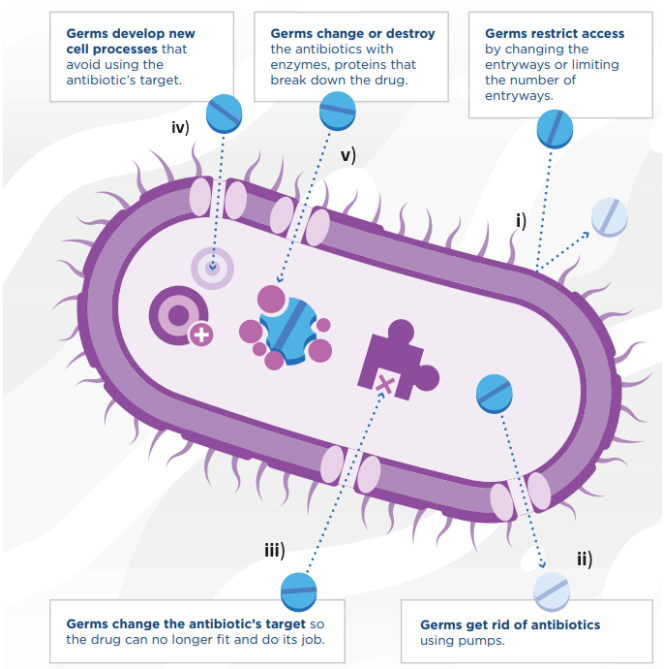


Figure II.2 Molecular mechanisms of antibiotic resistance. Acquired antibiotic resistance can result from a variety of molecular mechanisms, including: (**i**) decreased uptake of the antibiotic, (**ii**) increased efflux, (**iii**) modification of the target rendering the antibiotic ineffective, (**iv**) bypassing the of the antibiotic target, and (**v**) active break down of the administered antibiotic. Adapted from 'Centers for disease control and prevention, How antimicrobial resistance happens, 2022."

II.1.3 Combatting the drug resistance

To overcome the rise of AMR and its impact on the infectious disease burden, a multidisciplinary approach is required. Apart from sustainable antibiotic use and

increased antimicrobial surveillance, investment in innovative research and development of antimicrobials can help combat the ongoing drug resistance [56, 57]. In general, the incentive for pharmaceutical companies to develop new antimicrobial agents is low. On top of the short treatment duration of an acute infection, most newly approved antibiotics will be reserved as a last resort treatment for multidrug resistant microbes, leading to a low return on investment [2, 58]. Early-stage development, from hit identification to lead optimalization, is mostly driven by academic research, but allocated funds are often low [2]. Over the past few years, several organizations have joined forces in an attempt to stimulate the development of new antimicrobial treatments. One such organization is COMBACTE, a European private-public partnerships aiming to tackle the challenge of AMR [32, 56]. Apart from increased financing and research collaborations, innovative research techniques can help in the design and/or discovery of new antimicrobials. *In silico* tools such as artificial intelligence, for example, are gaining increasing traction in the scientific community [59]. Machine-learning guided exploration of existing compound databases recently led to the discovery of the antimicrobial activity of halicin and abaucin [60]. Nevertheless, the global efforts to stop AMR are so far not reflected by the amount of new, innovative antibiotics reaching the market.

II.1.3.1 Antibiotic pipeline

In the period of 2017-2022, only 13 antibacterial therapeutics were approved by either the Food And Drug Administration and/or the European Medicine Association [23, 61]. Most of these compounds are structural derivatives of known antibiotic classes, such as the tetracyclines or fluoroquinolones. Only two are based on a new antibiotic pharmacophore: vaborbactam, a boronic acid based β-lactamase inhibitor, and lefamulin, a pleuromutilin that inhibits bacterial protein translation [22, 23]. So far, no innovative compound with a new mode of action has gained market authorization in the past few years [23]. Nevertheless, the pre-clinical antibiotic pipeline contains a wide range of antimicrobial agents from various different classes (Figure II.3) [62]. Noticeably, the vast majority of these compounds are researched by small to medium-sized

enterprises, while large pharmaceutical companies are underrepresented in the preclinical pipeline [62, 63]. Most preclinical antibiotics are being developed against Gram-negative bacteria, and the most common application route is an intravenous injection [62, 63]

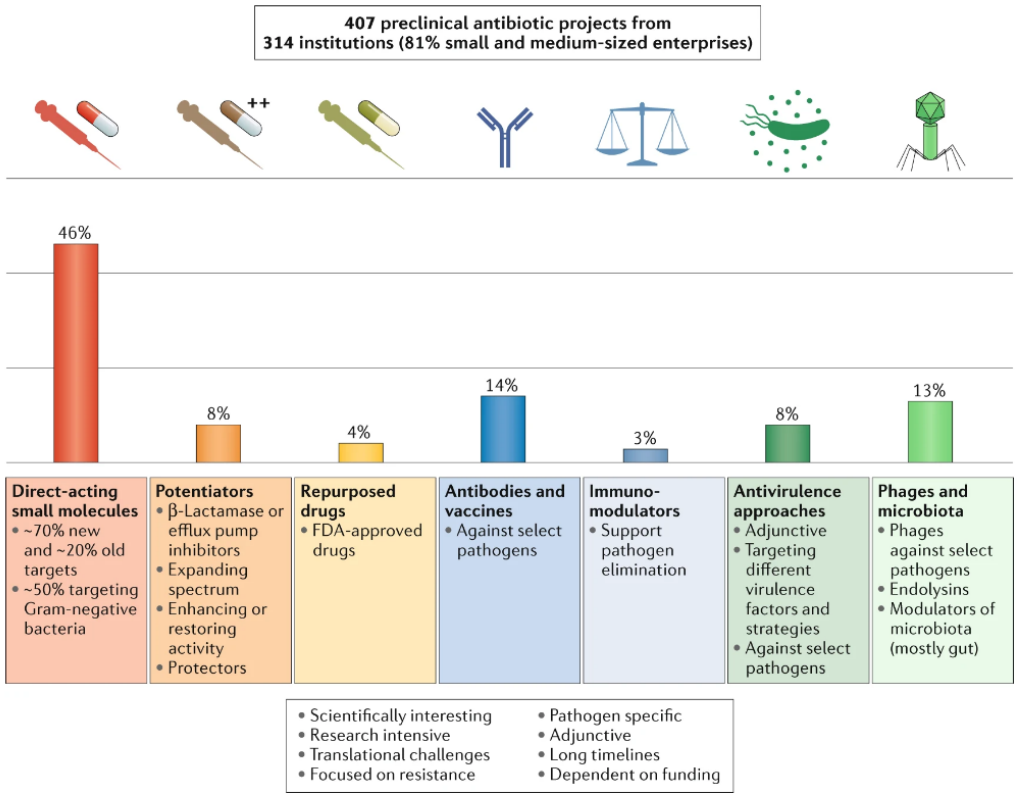


Figure II.3 The preclinical antibiotic pipeline (Theuretzbacher et al., 2020). Various strategies are being explored in preclinical development to target bacterial infections. The most popular therapeutics remain the traditional, direct-acting compounds [62].

The largest group of antimicrobials in pre-clinical development, which includes compounds at least present in the hit-to-lead phase but not yet tested in humans, is that of the direct-acting small molecules [62]. This group contains compounds that directly kill or inhibit bacteria by acting on one or more drug targets, much in line with the traditional antibiotics. They are either entirely new compounds working on new bacterial

targets, or modified versions of existing antibiotics [62]. Other groups that are well represented are those of the antibacterial vaccines, most of them targeting *S. aureus*, and phage therapies or phage-derived therapeutics, such as endolysins [62, 64]. Other innovative antibacterial strategies that are gaining popularity are antivirulence therapies and potentiators [62]. These therapies selectively target pathogen virulence factors or their underlying regulatory mechanisms to decrease the severity of an infection or increase the effect of a traditional antibiotic [65]. Toxin-neutralization or biofilm disruption are examples of possible methods of virulence reduction [65]. Compounds that can aid the effect of traditional antibiotics are potentiators, either by heightening the effect of the antibiotic itself, or by reversing existing bacterial resistance, such as β-lactamase inhibitors [66]. To this day, no comprehensive literature review of all antimicrobial agents in the clinical pipeline exists. Most reports and reviews focus on (**i**) the antibiotics targeting WHO priority pathogens, and (**ii**) antibiotics with systemic application [67, 68]. 37% of these antibiotics are innovative compounds with either new modes of action, new chemical structures or the absence of antibiotic cross-resistance [67, 68]. It is expected that over the following years, more of these innovative agents will find their way through the development steps and clinical trials to eventually surface on the market. A popular molecular group of antibiotics in research and development, often classified as non-traditional antimicrobials, are the peptide antibiotics [23].

II.1.3.2 Peptide antibiotics

Peptide-based antibiotics are a group of molecules situated between the small, direct-acting antibiotics and the large protein therapeutics [69]. Currently, most peptide antibiotics on the market are membrane-active compounds, such as gramicidin, colistin, and daptomycin; they are able to kill bacteria by damaging the membrane bilayer by, for instance, pore-formation [70]. Others, such as the glycopeptide vancomycin, do not work through mechanical membrane disruption, but through the inhibition of cell wall formation [70]. Some peptide antimicrobials, including dityromycin, target different non-membrane related cell processes, such as DNA, RNA, or protein synthesis [70]. However,

no peptide antibiotic of that last category is currently available on the market. Development of new peptide antibiotics has been challenged by intrinsic peptide limitations, such as poor stability and bioavailability. Moreover, the current generation of peptide antimicrobials often causes acute or chronic toxic effects, such as nephrotoxicity. Hence, the use of many of these peptide drugs is limited to either local use, or as a last-resort treatment of resistant pathogens [70, 71]. Nevertheless, there is growing interest in peptide antibiotics, including AMPs, as a generation of new antimicrobials that can help tackle the current resistance crisis [72].

II.2 Antimicrobial peptides: the future of peptide antibiotics?

Although the first peptide antibiotic found its way to the market in 1939 when gramicidin D was isolated from *Bacillus brevis*, the research on AMPs, a subset of peptide antibiotics, is much more recent and began to take off in the 1980s, when many AMPs were discovered independently from different animal sources [73, 74]. So far, however, no AMPs are commercially available, in contrast to the regular peptide antibiotics from which a select set are used in clinical practice today (see II.1.3.2).

II.2.1 AMPs: a complex definition

AMPs are a diverse family of small, evolutionary conserved peptides with antimicrobial activity [7]. They are found all throughout the kingdoms of life, including animals, plants and lower life forms such as prokaryotes [75]. AMPs are effector molecules of the innate defense system and are therefore sometimes referred to as '**host defense peptides**' [76]. In higher eukaryotes, the defensive role of AMPs stretches further than their direct microbicidal activity [77]. In humans, for example, AMPs are involved in the inflammatory response of an infection by stimulating phagocytosis and enhancing proliferation of fibroblasts and epithelial cells [78]. The term 'antimicrobial peptide' is ambiguous as, contrary to its name, it is not used to refer to all peptides that have antimicrobial activity. Strictly speaking, only peptides that are ribosomally produced are considered to be "true" AMPs. AMPs are therefore always translated from messenger RNA and encoded

in the organism's genes, in contrast to the peptide secondary metabolites that are synthesized by a multi-enzyme complex [79]. The former peptides are usually linear and cationic and are found in all forms of life, whereas the latter are macrocyclic secondary metabolites unique to microorganisms such as bacteria and fungi [79]. Ribosomally synthesized AMPs can be further divided in those that are unmodified, and those that are post-translationally modified peptides (RiPPs) [80]. All the commercially available peptide antibiotics (e.g. lipopeptides such as daptomycin and glycopeptides such as vancomycin) are non-ribosomal peptides, and hence, not considered to be an AMP. Some sources, however, apply a broader definition when defining an AMP, and consider all antimicrobially active natural peptides as AMPs [7, 81]. The Antimicrobial Peptide Database (APD), for example, focusses on gene-encoded natural peptides but includes non-ribosomally produced peptides as well [82]. For the remainder of this thesis, the narrower definition of an AMP will be applied.

II.2.2 The structure of AMPs

Despite their very divergent distribution in nature, AMPs typically share some characteristic features. Structurally, most AMPs contain 10 to 50 amino acids (AAs) [70, 76]. Shorter AMPs are often preferred in antibiotic development, as they are usually more stable, less cytotoxic, easier to synthesize and less prone to evoke unwanted immune responses [83, 84]. AMPs with minimal AAs on the other hand have a tendency to self-assembly into amphipathic secondary structures, which is related to a decreased antimicrobial activity [84]. When it comes to their primary structure, AMPs are usually abundant in both hydrophobic and positively charged AAs, giving them a positive net charge of +2 to +9 as well as a characteristic hydrophobic region of 40-50% non-polar residues. Structural classification of AMPs is based upon their secondary structure, which in turn is heavily dependent on their AA sequence. Generally, five main groups are distinguished (**Figure II.4**): (**i**) α-helical peptides, (**ii**) AMPs rich in β-sheets or β-hairpins, (**iii**) linear, extended peptides with no α-helices and β-sheets, (**iv**) peptides with a mixed structure, containing both α-helices and β-globular motives, and finally (**v**) AMPs that

harbor a more unconventional structure [85, 86]. AMPs of the α-helical type are the most studied group so far, and some well-known peptides of this type are the human cathelicidin LL-37 and the honeybee venom-derived melittin [73, 84]. AMPs with a β-structure are stabilized by intramolecular disulphide bridges due to the presence of multiple cysteine AA (typically 2 to 10) [79, 84]. Protegrin-1, isolated from porcine leukocytes, is an example of a β-sheet AMP [87]. Extended, linear AMPs with no specific secondary motive are typically characterized by high levels of certain AAs, including proline, glycine, arginine, phenylalanine, and tryptophan [84, 86, 88]. An example of an extended AMP is the honeybee-derived apidaecin [88]. A mixed structure with both α- and β-elements can be seen in the housefly phormicin peptides [89]. In contrast to the first four groups, the AMPs in the last category typically contain more complex structural features, such as a cyclic peptide backbone, thio-ether bridges or post-translational modified AA residues. Although less common, anionic AMPs are described as well, such as the human dermicidin [90]. They are rich in glutamic acid and aspartic acid, and often require the formation of cationic salt bridges to exert their antimicrobial effect [88, 90].

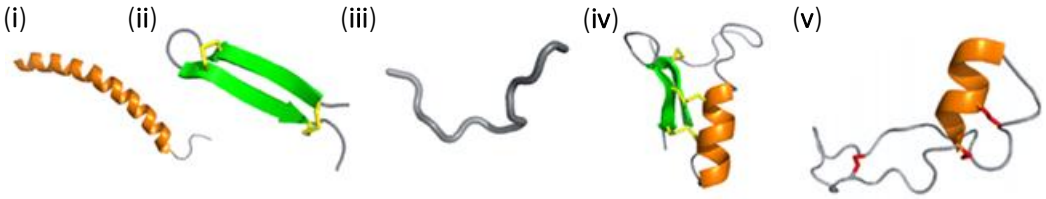


Figure II.4 Five structural categories of antimicrobial peptides. (**i**) Peptides with a uniquely alpha-helical structure. (**ii**) Defensin peptides with beta sheets motives. (**iii**) Linear peptides do not adopt specific structural motives in solution. (**iv**) Peptides with a mixed α-helical and β-sheet structure. (**v**) Antimicrobial peptides with more unconventional motives such as cyclization. Adapted from: Koenbach J. et al., (2019) [73].

II.2.3 Mechanisms of action of antimicrobial peptides

When it comes to the antimicrobial activity of AMPs, a multitude of possible mechanisms of action has been proposed and often, multiple targets are involved. The most widely accepted mode of action is the disruption of the microbial cell envelope by the AMP [78, 84, 91]. By integrating into the lipid bilayers of the microbial membranes, the AMP

reduces their integrity, leading to loss of ions and metabolites and ultimately to the microorganism's death. Both hydrophobic and electrostatic forces are involved in the interaction between AMP and microbial membrane [78]. The amphiphilic nature of the AMP and its interfacial properties are therefore decisive when it comes to their antimicrobial activity [78]. For the remainder of this thesis, the focus will be laid upon the antibacterial activity of AMPs.

II.2.3.1 Initiation of cell wall interaction

Initial attraction to cell wall components is an important step to increase the AMP concentration around the bacterial cell envelope. The outer membrane of Gram-negative bacteria is coated with a layer of protruding LPS, forming salt bridges with cations such as Ca^{2+} and Mg^{2} [92]. This outer electrostatic network prevents most hydrophobic antibiotics from entering the cell. The strong positive charge of AMPs allows them to displace the divalent cations bound to the LPS structures while interacting with the anionic phospholipid groups (**Figure II.5a**) [78, 84]. By disrupting the LPS-cation cross bridges, the outer membrane loses stability, which facilitates uptake of the AMPs through a mechanism called 'self-promoted uptake' [93]. Additionally, the hydrophobic part of the peptides stimulates interaction with the lipid tails of the LPS and insertion into the outer membrane [92]. Then, the peptides can diffuse through the periplasmatic space and adsorb onto the inner, cytoplasmic membrane [92]. For Gram-positive bacteria, the first determinative step is the crossing of the thick peptidoglycan matrix (**Figure II.5b**). Initial adhesion to the peptidoglycan layer is established by interaction with the phosphate groups of teichoic acids [93]. The teichoic acids, anchored to either a glycolipid of the cytoplasmic membrane or to the peptidoglycan layer itself, form a poly-anionic bridge that help the AMPs diffuse to the cytoplasmic membrane [94].

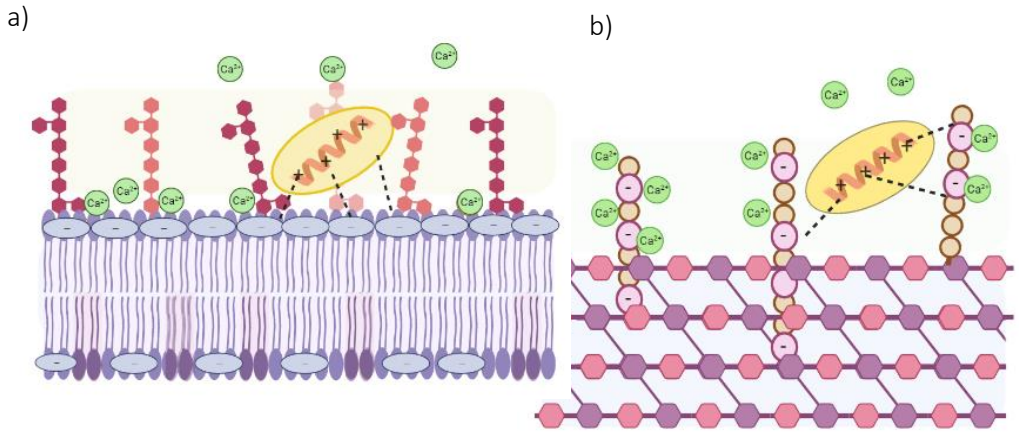


Figure II.5 Initial interaction between AMP and bacterial cell envelope. a) The AMP binds the lipopolysaccharide through electrostatic interactions and disperses the positive ions leading to increased membrane fluidity of Gram-negative bacteria. **b)** The AMP interacts electrostatically with the teichoic acids residues on in the peptidoglycan layer of Gram-positive bacteria. Created using BioRender.

II.2.3.2 Cytoplasmic membrane disruption

After the cell wall has been breached, AMPs can target the inner, cytoplasmic membrane. Initial attraction between AMP and membrane is established by electrostatic forces [95]. The positively charged AA functional groups (e.g. of lysine and arginine) will interact with the anionic lipid head groups on the outer leaflet of the bilayer, such as cardiolipin, phosphatidylglycerol and phosphatidylserine. Upon reaching the microbial membrane, many AMPs undergo conformational changes and switch from a coil-like structure to a helical formation [95]. After initial surface adsorption, the AMPs will weaken the membrane either by complete lysis, pore-formation or other non-pore membrane permeabilization pathways [86, 95]. Three traditional models are often proposed (**Figure II.6**).

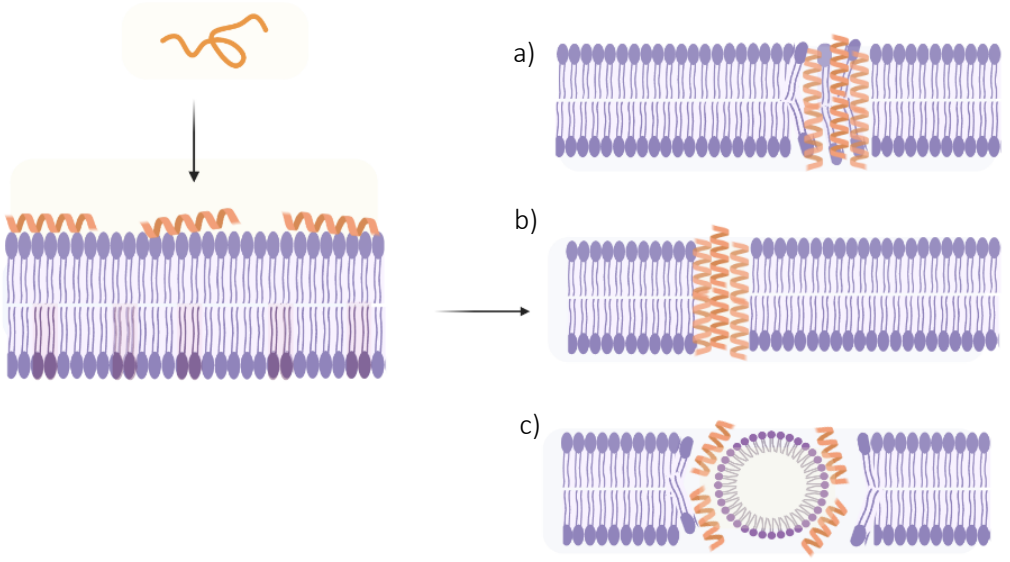


Figure II.6 Membrane-AMP interactions. An α-helical AMP comes in contact with a bacterial membrane and undergoes a conformation change. Three different models for the AMP-induced membrane damage are often proposed: **a)** the toroidal pore model, **b)** the barrel-stave model, and **c)** the carpet model. Created using BioRender.

In the first, called the toroidal pore model, individual AMPs do not self-assembly and are only loosely associated with one another. The created pores are lined with both peptides and interspacing lipid head groups of the membrane (**Figure II.6a**) [86]. In the second, called the barrel-stave model, AMPs will multimerize and interact laterally with each other to form aqueous channels across the membrane (**Figure II.6b**) [86]. A third well-known model is the carpet model, in which the AMPs initially cover the microbial membrane and hereby effectively increase its fluidity [95]. In a final stage of this model, the membrane can be broken up into micelles, ultimately rupturing the structure (**Figure II.6c**) [95].

Apart from these traditionally proposed mechanisms, AMPs can weaken the membranous structure through a plethora of other effects: changing the transmembrane potential, inhibiting ATP-dependent pumps across the membrane, remodeling and weakening the membrane structure by clustering negatively charged

lipids, changing the thickness of the membrane, binding anionic molecules across the membrane and stimulating their efflux,…[86] Both α- and β-type AMPs can act on the microbial membrane. Extended linear peptides that do not adopt secondary structural motives in the biological microenvironment , however, do usually not exhibit antimicrobial activity through membrane interaction [86]. Even though a general, antibacterial mechanism has been described here, AMPs can selectively target either Gram-negative or Gram-positive bacteria, and subtle changes in AA sequence can alter an AMP's spectrum [96].

II.2.3.3 Intracellular modes of action

AMPs can also work through nonmembrane-lytic pathways by directly binding and acting on intracellular targets. In this case, the AMP either enters the cell by endocytosis or passive diffusion [97]. Some intracellular modes of actions of known AMPs include: inhibition of DNA synthesis, inhibition of protein synthesis, metabolism or protein folding, inhibition of proteases, inhibition of cell division, and inhibition of cell wall biosynthesis [97]. Furthermore, it is possible for a single AMP to act on multiple targets simultaneously, which decreases the likelihood of a microbe becoming fully resistant against the peptide. Indolicidin, for example, is a non-lytic AMP that inhibits DNA biosynthesis both by direct DNA binding and by inhibition of topoisomerase enzymes [97].

II.2.3.4 Cell selectivity

Ideally, an antibiotic AMP candidate selectively acts on bacterial cells without disrupting the membranes of human cells. In reality, however, some degree of cell toxicity of AMPs is often reported [98]. Selectivity is mainly based on the lipid composition of the targeted membrane and the acidic residues that contribute to the overall membrane charge [99]. In contrast to bacterial cells, the outer layer of the human cell membrane is low in negatively charged lipids; most of the anionic lipids (e.g. phosphatidylserine) can be found in the inner layer facing the cytoplasm. This neutral net charge of the outer membrane contributes to AMPs' selectivity towards microbes [100]. When performing

early *in vitro* screening for cell toxicity, human erythrocytes are often used, as they contain a higher negative charge on the membrane surface due to the presence of sialylated glycoproteins, and therefore are more likely to attract the positively charged AMPs [99, 101].

II.2.4 AMPs: the road ahead

AMPs are progressively more explored as new anti-infectious treatment strategies since they offer some highly desirable advantages over conventional antibiotics [102]. Two key elements of AMPs are their fast killing time and the ability to work on multiple targets simultaneously [102]. These factors contribute to AMPs' inherent lower susceptibility to resistance development [103]. In contrast to traditional antibiotics, it takes numerous passages on sub-MIC concentration levels to evoke spontaneous resistance formation [102]. In addition, AMPs are often effective against bacterial strains with established drug resistance, and their antimicrobial activity can work synergistically with other peptides as well as conventional antibiotics [102]. Apart from their antimicrobial activity, AMPs often display endotoxin-neutralizing properties, which would make them useful in the treatment of septic shock, as well as immunomodulatory properties, enhancing the immune response [102, 104]. Another advantage of AMPs is the potential for structural simplification with retention of biological activities [98]. In combination with an often broad antimicrobial spectrum and a potent activity at low micromolar concentrations, these characteristics make AMPs suited candidates in the field of antimicrobial development [78, 98]. Nevertheless, AMPs in development struggle to pass the clinical trials and reach marketization phase. The antibiotic drugability of AMPs is often challenged by some unwanted characteristics, including toxic side effects and a low metabolic stability [98].

II.2.4.1 Toxicity of AMPs

Systemic toxicity of AMPs is usually caused by unspecific membrane interactions with the host cells [98, 105]. Often, some degree of hemolytic activity is reported for AMPs. Affinity for erythrocytes is most commonly encountered with highly hydrophobic AMPs

that form stable amphipathic structures and is strongly correlated with the presence of tryptophan, lysine, and arginine AA residues. For example, the indole moiety of tryptophan tends to associate to cholesterol present in the mammalian cell membrane [106]. The occurrence of hemolysis can be a driving factor to opt for a local (often dermal) application of the AMP instead of a systemic route of administration [107]. Another systemic toxic effect that has been encountered for AMPs is nephrotoxicity: the phase III clinical trial for murepavadin, for example, has been terminated prematurely due to acute kidney damage [108]. Another factor to look out for is the potential immunogenicity of AMPs [4]. Although these peptides are usually small with less immunogenic effects than recombinant therapeutic proteins, some degree of immunogenicity cannot be ruled out [107].

II.2.4.2 Challenging *in vivo* conditions

Another important pitfall is the low *in vivo* stability of AMPs leading to a short half-life and a low bioavailable plasma concentration. AMPs are prone to proteolytic degradation, especially upon passing through the small intestines where they encounter both digestive peptides (e.g. chymotrypsin produced by the pancreas), as well as peptidases produced by the mucosal intestinal cells [109, 110]. Many of these proteolytic enzymes have a cleavage site bordered by either a hydrophobic (e.g. tryptophan) or a cationic AA (e.g. lysine), two residues that are very prominent in natural AMPs [109, 110]. Apart from difficulty passing through the gastrointestinal tract, the serum residence time of peptides is generally short and clearance by the kidneys can occur within minutes [111]. This importantly complicates the development and formulation of AMPs and is one of the main reasons why most AMPs currently in clinical trials are dermal formulations or local injections. Often, certain structural modifications are necessary to improve peptide stability, including AA substitution or side chain modification, cyclization and C- or N-terminal modification [112]. Other strategies to improve serum stability include: pegylation to increase serum residence time, or the use of delivery systems such as liposomes or nanoparticles [113] [111]. Another obstacle is that the *in vitro* activity of

AMPs often doesn't correlate predictably with their *in vivo* activity [114]. AMPs can behave differently in serum conditions and their activity can be dependent upon salt concentration and pH levels [114, 115]. Additionally, binding to serum proteins such as albumin can significantly impede AMPs' antimicrobial activity [115]. To successfully develop AMPs as new antibiotics, these challenges will have to be tackled first.

II.2.4.3 AMPs from unconventional places

Ever since the introduction of antibiotics in modern medicine, nature has been a valuable source for the discovery of new antimicrobial biomolecules, and to this day, the vast majority of antibiotic classes have their origin in nature [116]. After the exhaustion of the antibiotic capacity of soil bacteria, researchers turned to medicinal chemistry for the development of semi-synthetic and fully-synthetic antibiotic platforms [21]. Even within the era of synthetic antibiotic design, pharmacophores were often based upon earlier natural antimicrobials, such as the design of fully synthetic penicillin derivatives [21]. Currently, there is growing interest in the revival of nature as a source for novel antibiotics. To maximize the outcome of this more classical approach to antibiotic discovery, scientist are (**i**) using innovative techniques such as the iCHIP to grow microorganisms that were previously deemed uncultivable and (**ii**) turning to underexplored natural sources that have been neglected in earlier antibiotic research [117, 118]. An increasing amount of active molecules with antibiotic potential are being isolated from unconventional sources, such as plants, invertebrate marine animals and insects [119]. Insects in particular are a topic of interest in antibiotic research, and this thesis will dive deeper into the antibiotic potential of insect-derived molecules, in specific AMPs derived from the black soldier fly [120].

II.3 Insects as a source for novel antibiotics

According to an estimation by Stork (2017), there are over 5.5 million different insect species on earth, making the insect class the most diverse amongst all animal classes [121]. On top of the abundance of species, insects are remarkably successful in colonizing a variety of ecological niches [122]. Insects are highly adaptive and are able to mount an

effective, innate immune response when challenged with microbes in their environment [6, 123]. For many species, both feeding and oviposition preferably take place on substrates with a high microbial load such as decomposing plant matter, manure, or animal carcasses. The efficient immune response and the striking diversity in the insect class have made them a research topic of growing interest in recent years [120]. Although insects have been used in medicine for centuries, including the use of *Lucilia sericata* maggots for wound healing, no insect-derived drugs have surfaced on the market [3, 124].

II.3.1 Insect antimicrobial peptides: a survival strategy

The first AMP of insect origin was discovered in 1980 from the pupae of the silk moth *Hyalophora cecropia* and was consequently called cecropin [125]. Since then, research on insect AMPs has come a long way and more are being discovered to this day. Currently (January 2024), the ADP reports on 364 AMPs from insect origin [82, 126]. AMPs are a key component of the humoral defense response against entomopathogens. The humoral immune response is mediated by the secretion of enzymes and effector proteins, which also includes the production of melanin, prophenoloxidase, and lysozyme. In contrast, the cellular immune strategy of insects involves direct cell-mediated responses, including phagocytosis, encapsulation and nodulation. [127]. In holometabolous insects (those that undergo a full metamorphosis from larva to adult insect), AMPs are either produced by epithelial structures such as the trachea or gut to act locally, or by the insect's fat body. After synthesis in the fatty tissues, AMPs are readily secreted in the hemolymph and distributed throughout the whole insect-body [128]. In heterometabolous insects (insects that do not undergo a full metamorphosis), AMPs are produced by the hemocytes in the hemolymph [128]. AMPs can be expressed constitutively, or production can be induced by external factors such as infection. AMPs are thought to be mostly involved in a late-stage immune reaction, eliminating any lingering bacteria hours or days after the initial insect response [129].

The AMP expression pattern can also be dependent on factors such as insect gender and diet, and can be organ-specific [130]. On top of their role in infection, AMPs are also thought to be involved in numerous other insect functions [131]. AMPs are important for maintaining gut homeostasis by managing the endogenous bacterial communities and reducing foreign microbial flora [131, 132]. In *Drosophila melanogaster* AMPs also contribute to the regulation of different nervous system functions, including sleep, memory and learning behavior [131]. Dysregulation of AMP production in *D. melanogaster* has been linked to premature aging [131]. Exposure to pathogens in their environmental niche seems to be the main driving factor behind the evolutionary adaptation of an insect's AMP repertoire. As the (over)expression of AMPs also leads to a significant fitness cost, there is a continuous trade-off between the diversification and expansion of an AMP repertoire and the loss of functional AMP genes [133]. This can partially explain why certain insects have a large amount of AMP encoding genes, whereas others produce very few to no AMPs. The pea aphid *Acyrthosiphon pisum*, for example, feeds on sterile plant phloem and the reduced infection pressure in its environment has led to a complete loss of AMP genes over time [132-136]. The disappearance of entire AMP families has also been described in dipteran lineages feeding on plant substrates [136]. The loss of formerly essential AMP genes can also be facilitated by the acquisition of other survival strategies, such as sanitation of the environment or harboring endosymbionts with antimicrobial activity [132].

II.3.1.1 Insect antimicrobial peptides: structural diversity

Within the large structural groups of AMPs, many subfamilies of insect AMPs are recognized based on specific reoccurring features. Some of these AMP families are large and have been isolated from a wide range of insect orders, such as the insect defensins and cecropins. Other, smaller families have only been found in one order or even in specific insect genera, such as the formicaecins or the apidaecins [125, 134, 137]. Below, some of the most prominent insect AMP families are discussed.

Cecropins

The family of the cecropin AMPs is the largest structural family of insect AMPs and the most heavily studied. Cecropins and cecropin-like AMPs have so far been identified in three different insect orders: Diptera (true flies), Lepidoptera (butterflies and moths) and Coleoptera (beetles) [138]. Cecropins are not unique to insects, however, and some have already been identified in the bacterium *H. pylori*, in some marine invertebrates (tunicates), ascarid nematodes and mammals [139]. Cecropins fall within the large structural group of *α*-helix AMPs and usually contain between 31 and 42 AAs [125, 140]. They often display a broad antimicrobial spectrum, ranging from antibacterial and antifungal activity to antiparasitic activity [125]. Some examples of AMPs that belong to the cecropin superfamily are: papiliocin from the butterfly *Papilio xuthus*, and stomoxyn from the stable fly *Stomoxys calcitrans* [125]. Reoccurring structural elements of the insect cecropins are: a long amphiphilic *α*-helix at the N-terminus, linked to a C-terminal shorter and more hydrophobic helix via a glycine and proline-rich hinge region, an amidated C-terminal residue and a tryptophan AA near the N-terminus [134, 138, 141].

Defensins

Insect defensins are a subfamily of the larger cis-defensin AMP group, characterized by the presence of six cysteine residues and stabilized by three intramolecular disulphide bridges. While defensins are found widespread throughout all the kingdoms of life, the cis-defensin family is exclusively found in plants, fungi and insects. Insect defensins specifically are found in nearly each insect order, including the ancient order of the Odonata (damselflies and dragonflies), hinting at a common ancestor gene and an evolutionary conserved role of the insect defensins [125]. Structurally, insect defensins vary in length between 34-51 AAs and are typically rich in arginine residues [125, 137]. Characteristic for insect defensins is the combination of an N-terminal loop and α-helix and β-sheet structure, linked by two disulfide bonds, also called an “cysteine-stabilized alpha beta motif (CSαβ)” [125, 142]. The spectrum of insect defensins is usually limited to Gram-positive bacteria, although occasionally activity against Gram-negative bacteria

and fungi is seen as well. Gallerimycin, for example, produced by the wax moth *G. mellonella*, only exerts activity against filamentous fungi [142].

Attacins and diptericins

Attacins and diptericins are two closely related families of AMPs. Both comprise glycine-rich peptides of varying length. Attacins were first identified from immunized pupae of *H. cecropiae*, of the Lepidopteran order, but have been identified in Dipteran and Coleopteran insect species as well [143]. In contrast to most other AMP families, anionic peptides rich in aspartic acid are more regularly found among attacins [125]. Attacins contain two glycine-rich domains, can be up to 190 AA long, and adapt a random coil-like structure in solution [144]. Similar to the cecropins, they often adopt helical formations in hydrophobic conditions [125]. Other than the presence of glycine, attacins of different insect taxa have low homology in AA composition [144, 145]. Attacins have predominantly activity against Gram-negative bacteria [125]. Diptericin AMPs share many characteristics with the attacins, such as their antibacterial spectrum and the C-terminal glycine-domain. They do, however, contain an short, proline-rich domain at the N-terminus, in contrast to most attacins [146]. Diptericins were first isolated from the blue-bottle fly *Phormia terranovae* larvae. Similar to attacins, diptericins are usually longer than the average AMP, being up to 82 AA long [137]. More recently, a shorter diptericin-like AMP of 21 AA was isolated from the Hemipteran insect *Rhodnius prolixus* [137].

Knottin-like peptides

Knottin-like peptides, also referred to as 'inhibitor cysteine peptides', belong to the larger group of β-sheet AMPs. They range in length from 30 to 50 AAs and contain a unique structural motif of three or more intertwined disulphide bridges, with two of these bridges forming a ring shape with a third bridge passing through it [147, 148]. This knot-like motif lends the peptides a high stability against high temperatures, enzymatic degradation, and mechanical stress [147]. Knottin-like peptides are commonly found in

various forms of lives [148]. An example of an insect knottin-type peptide is the antifungal Alo-3 AMP from the *Acrocinus longimanus* beetle [149].

Other AMP families

As stated earlier, some AMP families have only been identified in one specific insect order or in a single insect genus. These include **lebocins**, proline-rich AMPs of 32 AA with an O-glycosylated terminus that have so far only been found in Lepidopteran species. The O-glycosylation of lebocins is crucial for the full exertion of their antimicrobial activity, which ranges from activity against Gram-negative and Gram-positive bacteria, to fungi [125]. Next, **moricins** are α-helical AMPs that share some characteristic features with the insect cecropins, but lack the glycine-proline hinge region between the helices [125]. Moricin AMPs have been isolated from a variety of Lepidopteran species, such as *G. mellonella* and *S. litura* [125]. **Gloverins** are another family of Lepidopteran-derived AMPs, first identified in the giant silk moth *Hyalophora gloveri* [150]. They are highly basic and rich in glycine residues (>18 %) [125]. Finally, **coleoptericins** make up a family of glycine and proline-rich AMPs found exclusively in the order of the beetles [134].

II.3.2 Black soldier fly

The BSF, *Hermetia illucens*, (Diptera: Stratiomyidae) is a true fly originating from the neotropical region. At present, the BSF has been introduced to nearly all continents, where it can be found mostly in tropical and temperate regions [151]. They have a wasp-like appearance and mimic some of the wasp's typical behavior, such as flying around agitatedly and pretending to sting [152]. Some external features of the BSF are: 15-20 mm in length, an elongated abdomen with a narrow base, translucent areas on the abdominal segments, a black to brown colored body, wings that are held overlapping, flat on their back, and white tipped legs (**Figure II.7**) [152]. In contrast to the common house fly *Musca domestica*, the BSF does not act as a disease vector, damage farm crops or pollute the environment, and it is non-house invasive [153].

Figure II.7 The black soldier fly and its larvae. The black soldier fly is a true fly belonging to the family of the Stratiomyidae. Its appearance is wasp-like, with an elongated body and white-tipped legs. Images available via a Creative Commons license.

The black soldier fly larvae (BSFL) are saprophagous, feeding preferably on decaying organic matter, including food and agricultural waste, manure, and animal and plant remains [153, 154]. Female flies only mate and oviposit once in their lifetime and lay their eggs (500 to 900) in dry areas near a humid, rotting food source [153, 154]. Eggs hatch after 4 to 14 days, depending on environmental factors such as temperature [153]. The BSF goes through six instar stages during larval development, which usually takes a total of 20 to 25 days [155]. During the last instar, the prepupal phase, the larvae cease feeding and migrate away from the food source to later pupate into adult flies [153, 156]. At this point, the larvae have reached their maximal size, with a large protein (36-48%) and fat content (31-33%), necessary to sustain them through the following metamorphosis at the pupal stage [156]. Adult flies continue to live on for an average of five to eight days [155].

II.3.2.1 Future opportunities of the BSF

To date, the BSFL are mainly used as a sustainable animal feed source. While BSFL as a whole are used as pet feed, BSFL-derived protein and fat are permitted as feed in the farming industry, including the aquaculture, pig, and poultry sector [157]. BSFL as animal feed are specifically desirable for their high nutritional value, their high feed conversion ratio, low space requirement, and their significantly lower environmental impact compared to traditional feed sources [151, 158]. The ability of the BSF to be reared on

waste side streams, such as manure, and to turn low-protein organic matter into high-protein edible biomass, is highly desirable in the face of a circular economy and waste valorization (**Figure II.8**) [151, 158].

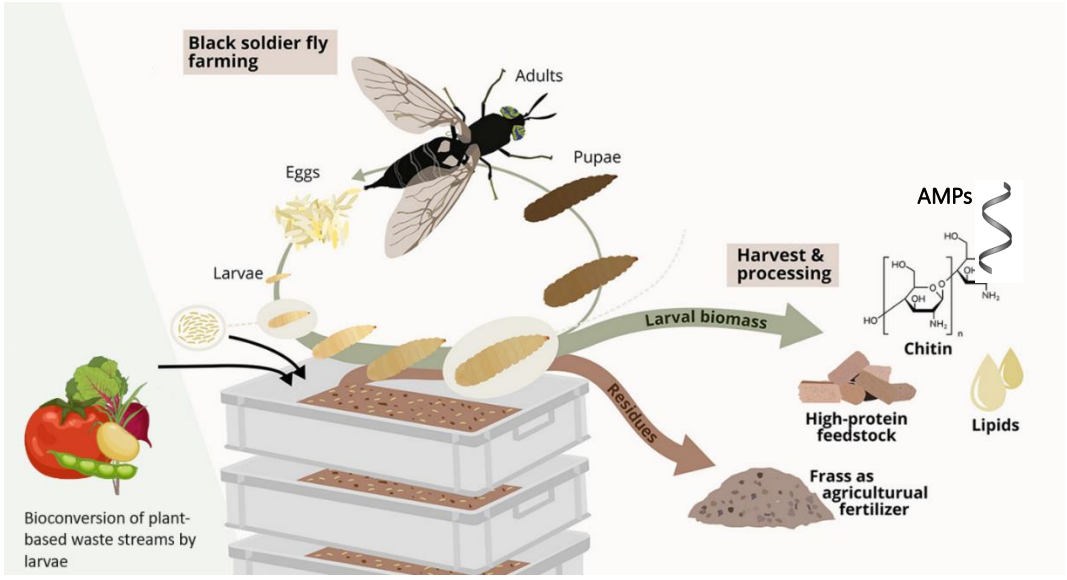


Figure II.8 Life cycle and potential applications of the black soldier fly larvae. The black soldier fly can be reared on a wide array of plant-based side streams, converting waste into valuable larval biomass with a high protein and fat content. Adapted from Liu et al. (2022) [152].

Another use of the BSFL that is currently being explored is the creation of biodiesel out of the larvae's fat content, as alternative for traditional crop oil-based biodiesel [159]. Additionally, BSF and their larvae have been opted as a source for novel bioactive molecules with a multitude of possible applications, including degrading enzymes, chitosan and AMPs [160]. The residue left after BSF farming, frass, can also be used as agricultural fertilizer [158].

II.3.2.2 Antimicrobial activity of the black soldier fly

First mentions of antimicrobial activity of the BSF date back to 2012, when a methanolic larval extract was found to be active against Gram-negative pathogens including *Klebsiella pneumoniae* [161]. Antimicrobial activity of the BSF is usually attributed to the presence of (**i**) AMPs, (**ii**) fatty acids with antimicrobial activity such as lauric acid, and (**iii**)

chitin [162]. Common methods to identify and isolate biologically active compounds from the BSF are the production and characterization of either full body organic extracts of the larvae or hemolymph extracts [162, 163]. Alternatively, researchers have also identified a large number of AMPs through transcriptome analysis [130, 164].

II.3.2.3 Immune response of the black soldier fly

As for other insects, the immune response of the BSF consists of a humoral and a cellular component [165]. The cellular response occurs within five minutes after microbial challenge to rapidly eliminate pathogens from the hemolymph. It involves phagocytosis, encapsulation and nodulation of non-self antigens by the BSF hemocytes [165, 166]. The humoral response kicks in a few hours after the first microbial contact and works synergistically with the cellular response to kill the remaining microorganisms [166]. Humoral immunity components include the production of prophenoloxidase which activates an enzyme cascade leading to melaninization, AMPs, lysozyme, and reactive oxygen species [165, 166]. The transcription of AMPs by the BSFL is strongly induced by infection; the expression of Hidiptericin increases 250-fold 3-6 h post-infection. The BSFL fat body produces AMPs over a prolonged period of time (up to 48 h after infection) [166].

II.3.2.4 Black soldier fly antimicrobial peptides

The first AMP from the BSF was discovered in 2015. A 40 AA-long **defensin**-like peptide (DLP4) with activity against Gram-positive bacteria was isolated from the hemolymph of immunized BSFL. [167]. DLP4 harbors a high sequence homology with other insect defensins such as sapecin from the sarcophagi fly [167]. Further studies on DLP4 and its analogue, DLP2, showed a high thermal stability, low cytotoxicity (RAW264.7) and low hemolytic activity [168, 169]. The high positive net charge (+6) of DLP4 is thought to contribute to its fast antibacterial activity, as its homologue DLP2 with a lower positive charge (+2) needs triple the amount of time to kill 99% of MRSA [169]. Since then, various DLP4-derivatives, including ID13, have been studied as well [170]. The first **cecropin** to be discovered from the BSF, was a 46 AA-long peptide called CLP1. As with DLP4, the

AMP was extracted from the hemolymph of immunized larvae [171]. CLP1 exhibits a 60% sequence homology with cecropin C isolated from *D. melanogaster* and displays activity against Gram-negative bacteria at micromolar concentrations [171]. To date, various other cecropins from BSF origin have been discovered and described, including two stomoxyn-like peptides and four structurally very similar sarcotoxin-like peptides by Elhag et al. [172]. The repertoire of BSF AMPs was expanded due to large scale transcriptomic analyses by Vogel et al. (2018) and later, Moretta et al. (2020) [130, 164]. Currently, over 50 putative AMP sequences of the BSF are known, making it the insect with the second largest amount of AMP encoding genes, only preceded by the ladybird *Harmonia axyridis* [130]. However, before the start of this research, the antimicrobial activity of only 14 BSF AMPs and AMP-derivatives was investigated and verified *in vitro*. No large scale antimicrobial screening of a BSF peptide library had been performed so far, leaving much room for further investigation (see CHAPTER III) [8].

II.3.3 Insect symbionts as a source for novel antibiotics

Apart from the insect itself, microbial symbionts of insects are also a popular research topic in the search for new antibiotics. Insect symbionts are microbes that live in a close, long-term relationship with their insect host [173]. Symbionts can be found in the insect itself, for example in the gut or in specialized cells called bacteriocytes [174]. Some symbionts proliferate on the insect's exterior surface or in their habitat, such as their nest or food provisions [173, 175, 176]. The nature of the symbiosis can be diverse, ranging from a mutual profitable relationship to parasitism [177].

Two arguments support the attention on these symbiotic microorganisms to discover novel drug candidates: first of all, although insects have a well-developed defence system of their own such as an exoskeleton barrier, humoral and cellular immune responses including melanisation, phagocytosis and AMP production [175, 178, 179], many life stages of insects such as the egg and larval stages are still very vulnerable to environmental threats [180]. For various insects it has been shown that defensive symbionts play a crucial role during these developmental stages. Solitary wasps, for

example, use *Streptomyces* bacteria to protect their pupae in brood chambers [180]. Apart from brood protection, these microbes can also help to protect the insect's nutritional resources [175, 181]. Secondly, many defensive symbionts have been characterized for adult insects as well [175, 182-184]. Symbiotic microorganisms play an important role in the insects' defence system against these foreign invaders and help them thrive in challenging environments [180, 185, 186].

Multiple mechanisms that confer this protection against entomopathogens have been identified. The indigenous microbial community can trigger and consecutively increase the insect's own immune response, but they can also reduce the colonization of the insect by entomopathogens, a mechanism called colonization resistance [185]. In this case, the insect microbiota compete with the microbial invaders for nutrients and space, or they can directly take part in the killing of these entomopathogens [185, 187]. The latter phenomenon is especially interesting in the light of the current drug-resistance crisis and could be exploited in the search for new antimicrobials. Indeed, more and more insect symbionts are surfacing in drug research articles and produce metabolites that are being described as "potential antimicrobials". Recent research by Chevrette et al. (2019) for example, highlights the antimicrobial capacity of insect-related *Streptomyces* bacteria, whereas Van Arnam et al. (2018) list molecules that are important in defensive insect-microbe symbiosis [180, 188]. It is clear that there is also increasing interest in the diverse world of insect symbionts to find new, active compounds of interest.

CHAPTER III

In vitro evaluation of a black soldier fly antimicrobial peptide library

This chapter is adapted from:

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III.1 Introduction

As detailed in chapter II, AMPs have gathered a substantial amount of interest as candidates for future antibiotics. Ever since the discovery of cecropin from the pupae of the silk moth *H. cecropia* in 1980 [125], insects have steadily gained attention as AMP producers [134, 137, 189]. Currently, the APD reports on 367 AMPs from insect origin [126]. AMPs are small, evolutionary conserved peptides with antimicrobial activity [7]. In insects, they are the main effector molecules of the innate immune system and increase their resistance to bacterial infections [137]. It is suggested that exposure to pathogens in the insect's environmental niche is a driving factor behind the evolutionary adaptation of the size and diversity of their AMP repertoire [132, 134].

An insect with an AMP repertoire of remarkable size, is the BSF. The BSF (*Hermetia illucens*, Diptera: Stratiomyidae) expresses over 50 genes encoding putative AMPs, a number that so far has only been recorded for the harlequin *H. axyridis* [130, 190]. The larvae of the BSF are saprophagous, feeding preferably on decaying organic matter, including food and agricultural waste, manure, and animal and plant remains [153, 154]. Their expansive AMP gene collection has been linked to their survival in these substrates with a high microbial load [130]. Apart from their role as defenders against infections, AMPs are also involved in maintaining and shaping the bacterial gut community of the BSF [130, 191]. For example, AMP expression in the BSF is diet-dependent and adapts both the feed or substrate microbiota as well as the gut microbiota to allow flexible digestion of the wide range of substrates they encounter in their environment [130, 192]. Overall, the use of BSF AMPs could be exploited beyond antimicrobial drug development to applications in the industrial insect farming sector. For instance, AMP addition could help eliminate food pathogens in the insect rearing cycle or stimulate bioconversion of organic waste by *H. illucens* larvae [130]. This chapter, however, focuses on the potential use of AMPs in the development of novel antimicrobial drugs.

To date, the antimicrobial activity of 14 different BSF AMPs has been confirmed *in vitro*, most of these being defensin AMPs (Table III.1) [164, 169, 171, 172, 193-197]. However,

a detailed characterization of their antimicrobial activity is often missing. So far, no *in vitro* antimicrobial evaluation of a full library of BSF AMPs has been performed, leaving much potential of BSF AMPs to be uncovered. This chapter aims to upgrade the knowledge on the antimicrobial properties of BSF AMPs. This was done in the first place by evaluating a large library of 36 synthetically produced AMPs against a range of pathogenic organisms, and in the second place by carrying out a deeper *in vitro* characterization of the structural, toxicity, and antimicrobial profile of two selected AMPs.

Table III.1 Confirmed *in vitro* antimicrobial activity of antimicrobial peptides from the black soldier fly. Peptides mostly belong to the defensin, cecropin, or attacin class, with activity against both Gram-positive and Gram-negative pathogens.

AMP	AMP family	Study	Activity against	MIC[a] (μM)
DLP4	Defensin	Park et al. (2015)	MRSA[b]	0.59-1.17
			S. aureus KCCM 40881	0.59-1.17
			S. aureus KCCM 12256	1.17-2.34
			S. epidermidis KCCM 35494	0.59-1.17
			B. subtilis KCCM 11316	0.02-0.04
		Li et al. (2017)	*S. aureus* ATCC 25923	0.01
			S. aureus ATCC 43300	0.23
			S. aureus ATCC 6538	0.47
			S. aureus CICC 546	0.47
			S. suis CVCC 606	1.88
			L. ivanovii ATCC 19119	0.12
			S. aureus CVCC 546	3.75
		Li et al. (2020)	*S. epidermis ATCC* 12228	14.99
			S. pneumoniae CVCC 2350	7.50
			S. suis CVCC 3928	3.75
DLP2	Defensin	Li et al. (2017)	*S. aureus* ATCC 25923	0.01
			S. aureus ATCC 43300	0.12
			S. aureus ATCC 6538	0.12
			S. aureus CICC 546	0.23
			S. suis CVCC 606	0.93
			L. ivanovii ATCC 19119	0.12

[a] MIC = minimum inhibitory concentration, [b] MRSA = Multidrug resistant *S. aureus*, [c] Mitochondrial ATPase inhibitory region

Table III.1 Confirmed *in vitro* antimicrobial activity of antimicrobial peptides from the black soldier fly.

AMP	AMP family	Study	Activity against	MIC[a] (µM)
DLP3	Defensin	Park et al. (2017)	MRSA[b]	1.2
			S. aureus KCCM 40881	1.2
			S. aureus KCCM 12256	2.4
			S. epidermis KCCM 25494	2.4
			E. coli KCCM 11234	2.4
			P. aeruginosa KCCM 11328	9.5
ID13	Defensin	Li et al. (2020)	*S. aureus* CVCC 546	0.95
			S. epidermis ATCC 12228	1.91
			S. pneumoniae CVCC 2350	0.95
			S. suis CVCC 3928	0.95
CLP1	Cecropin	Park et al. (2017)	*E. coli* KCCM 11234	0.52 - 1.03
			E. aerogenes KCCM 12177	1.03 - 2.07
			P. aeruginosa KCCM 11328	1.03 - 2.07
Trx-StomoxynZH1a	Cecropin	Elhag et al. 2016)	*E. coli*	-
			S. aureus	-
HI-Attacin	Attacin	Shin et al. (2019)	*E. coli* KCCM 11234	-
			MRSA[b]	-
HiCG13551	IATP[c]	Xu et al. (2020)	*E. coli*	-
			S. aureus	-
			S. pneumoniae	-
Hidefensin-1	Defensin	Xu et al. (2020)	*E. coli*	-
Hidiptericin-1	Diptericin	Xu et al. (2020)	*E. coli*	-
			S. pneumoniae	-
Hill_BB_C6571	Defensin	Moretta et al. (2020)	*E. coli*	-
Hill_ BB_C16634	Defensin	Moretta et al. (2020)	*E. coli*	-
Hill_BB_C46948	Defensin	Moretta et al. (2020)	*E. coli*	-
Hill_BB_C7985	Defensin	Moretta et al.	*E. coli*	-

[a] MIC = minimum inhibitory concentration, [b] MRSA = Multidrug resistant *S. aureus*, [c] Mitochondrial ATPase inhibitory region

III.2 Materials and methods

III.2.1 Antimicrobial peptides

In a previous study, genes were identified in the *H. illucens* transcriptome encoding for putative AMPs [8]. All AMPs of this study that could be produced synthetically were produced by solid-phase peptide synthesis and purified by either COVALAB (Bron, France), Proteogenix (Schiltigheim, France), or Genscript (Leiden, The Netherlands). High-performance liquid chromatography (HPLC)-mass spectrometry was used to determine the peptide purity. Peptide type, AA sequence, purity, C-terminal modifications, isoelectric point and molecular weight are summarized in Table SIII.1. For the experiments, peptides were dissolved in dimethyl sulfoxide (DMSO, Acros Organics) at a concentration of 10 mM and further diluted in sterile demineralized water with DMSO concentrations < 1%.

III.2.2 Bacterial isolates and culture conditions

P. aeruginosa ATCC 9027, *P. aeruginosa* ATCC 15442, *P. aeruginosa* ATCC 15692 (PAO1), *Escherichia coli* ATCC 8739, *S. aureus* ATCC 6538, *K. pneumoniae* ATCC 13883, and *M. tuberculosis* ATCC 25177 (H37Ra) were obtained from the ATCC (American Type Culture Collection, Manassas, USA). *Streptococcus pneumoniae* D39 was obtained via the National Collection of Type Cultures. *Burkholderia cenocepacia* LMG 16656, *P. aeruginosa* LMG 27650, *A. fumigatus* B42928, and *C. albicans* B59630 were obtained from the Belgian Coordinated Collections of Microorganisms. Bacterial strains (except *M. tuberculosis*) were cultured in Mueller-Hinton broth (MHB; Difco) and on Mueller-Hinton agar (MHA; Sigma-Aldrich) or tryptic soy agar (TSA; Sigma-Aldrich). *M. tuberculosis* was grown in complete Middlebrook 7H9 (Sigma-Aldrich) medium. Fungal species were grown in Roswell Park Memorial Institute (RPMI; Gibco) medium.

III.2.3 Antimicrobial activity assay

To detect antimicrobial activity of the BSF AMPs, the peptide library underwent screening against a panel of microorganisms, consisting of *S. aureus* ATCC 6538, *E. coli* ATCC 8739, *P. aeruginosa* ATCC 9027, *C. albicans* B59630 and *A. fumigatus* B42928. Serial dilutions

of the peptides were prepared from the DMSO stock solutions in sterile demineralized water in 96-well plates using an automated liquid-handling workstation (Beckman Coulter® Biomek 3000) in end volumes of 10 µL. The final in-plate concentration of DMSO was <1%. Peptide concentrations ranged from 64 µM to 0.25 µM for the first evaluation, and from 32 µM to 0.016 µM for the independent repeat. As references, doxycycline (Sigma-Aldrich; *S. aureus*, *E. coli*, *P. aeruginosa*), amoxicillin (Sigma-Aldrich; *S. pneumoniae*), flucytosine (Sigma-Aldrich*; C. albicans*) and econazole (Sigma-Aldrich*; A. fumigatus*) were used. Suspensions of an inoculum of 5 x 10^3 CFU/mL (*E. coli*, *S. aureus*, *S. pneumoniae*, *C. albicans*, *A. fumigatus*) and 5 x 10^4 CFU/mL (*P. aeruginosa*) were prepared in MHB (bacterial species) or RPMI medium (fungal species) and 190 µL was added to the 96-well plates. Plates were incubated for 16 h (*E. coli*, *S. aureus, P. aeruginosa*), 24 h (*C. albicans*) or 48 h (*A. fumigatus*) at 37°C. Afterwards, read-out of the antimicrobial activity was performed using a resazurin assay. Resazurin is a commonly used redox indicator that undergoes a fluorescent color change in the presence of viable cells [198]. Twenty µL of a 0.01% (w/v) resazurin (Sigma-Aldrich) solution was added to each well and plates were incubated for 15 min (*S. aureus*), 30 min (*E. coli*), 45 min (*P. aeruginosa*), 4 h (*C. albicans*), or 17 h (*A. fumigatus*) to allow resazurin reduction to take place. Fluorescence was read using a microplate reader (Infinite F Plex, Tecan) at $\lambda_{excitation}$= 550 nm and $\lambda_{emission}$= 590. The results were used to calculate the IC_{50} value, defined as the concentration of peptide causing 50% microbial growth inhibition. MIC values were determined visually. Medium in the wells with no visual growth was plated on MHA to detect the MBC (minimum bactericidal concentration), defined as ≥3 log reduction compared to the growth control.

III.2.4 Cytotoxicity screening of the peptide library

To determine early signs of peptide toxicity, the library was tested against the MRC5-SV2 cell line of human, embryonic lung fibroblasts (Sigma-Aldrich). MRC5-SV2 cells were cultivated in minimum essential medium (MEM, Gibco) supplemented with 20 mM glutamine, 16.5 mM $NaHCO_3$ and 5% inactivated fetal calf serum (iFCS, Gibco). Peptides

were serially diluted in 96-well plates as described earlier at concentrations ranging from 64 µM to 0.25 µM for the first screening, and from 32 µM to 0.016 µM for the independent repeat. Tamoxifen (Sigma-Aldrich) was included as a reference compound. After preparation of the test plates, 190 µL of 1.5 x 10^5 cells/mL was added to the peptides. Plates were incubated for 72 h at 37°C in a 5% CO_2 incubator (Binder). Afterwards, 50 µL of a 0.01% (w/v) resazurin (Sigma-Aldrich) solution was added to the wells to detect cell viability. After 4 h of incubation, fluorescence was read using a microplate reader (Infinite F Plex, Tecan) at $\lambda_{excitation}$ = 550 nm and $\lambda_{emission}$ = 590 and the IC_{50} values were calculated.

III.2.5 *In silico* structural analysis of cecropin family

The amino acid conservation within the cecropin family of AMPs was studies using multiple sequence alignment, using the PRALINE software available at https://www.ibi.vu.nl/programs/pralinewww. Next, a prediction of the 3D conformation of Hill-Cec1 (HC1) and Hill-Cec10 (HC10) was performed using the I-Tasser algorithm via the NovaFold application (DNAStar). Finally, helical wheel projections were constructed of the peptide alpha-helices using the Galaxy GPT software, available at https://cpt.tamu.edu/galaxy-pub.

III.2.6 Circular dichroism spectrometry

Circular dichroism (CD) spectrometry was used to experimentally probe the α-helical character of HC1 and HC10. AMPs were dissolved in potassium phosphate buffer (10 mM, pH 7) with or without the addition of LPS (100 ng/mL) at 32 µM. Spectra were recorded on a Chirascan plus (Applied Photophysics) equipped with a Peltier temperature-controlled (25°C) cell holder in quartz cells with a 0.5 mm pathlength, a step size of 1 nm and bandwidth of 1 nm. Scanned range was 180 nm to 260 nm. Spectra were background-corrected and expressed in molar ellipticity ([θ]), given by m°*M/(10*L*C), where m° is the ellipticity in milli degrees (related to ΔAbsorbance with a factor 32.98), M is the molar mass of the AMPs, L is the pathlength in cm, and C is the concentration of the peptides in g/L.

III.2.7 Evaluation of selected cecropins against an extended bacterial panel

To further investigate the antimicrobial spectrum of the selected AMPs, *in vitro* antimicrobial screening against additional bacteria was performed. The extended panel included *P. aeruginosa* PAO1, *P. aeruginosa* LMG 27650 (a MDR strain), *P. aeruginosa* ATCC 15442, *K. pneumoniae* ATCC 13883, *B. cenocepacia* LMG 16656, *S. pneumoniae* D39, *B. cereus* ATCC 14579, and *M. tuberculosis* H37Ra. *M. tuberculosis* was grown in Middlebrook 7H9 medium, *S. pneumoniae* in MHB + 5% lysed horse blood, and all others in standard MHB. Peptides were serially diluted *in duplo* in sterile demineralized water in 96-well plates using an automated liquid-handling workstation. Bacterial suspensions were prepared at a concentration of 10^4 CFU/mL (*K. pneumoniae*, *B. cereus*, *S. pneumoniae*), 5 x 10^4 CFU/mL (*P. aeruginosa*), 10^5 CFU/mL (*B. cenocepacia*) or 5 x 10^5 CFU/mL (*M. tuberculosis*). 190 µL of this suspension was mixed with the AMPs. For the *M. tuberculosis* screen, the outer wells of the test plates were filled with 200 µL of demineralized water. As references, doxycycline (Sigma-Aldrich; *P. aeruginosa* PAO1, *P. aeruginosa* ATCC 15442, *K. pneumoniae*), polymyxin B sulfate (Sigma-Aldrich; *P. aeruginosa* LMG 27650), moxifloxacin (Sigma-Aldrich; *B. cenocepacia*), vancomycin (*S. pneumoniae*, *B. cereus*), and isoniazid (Sigma-Aldrich; *M. tuberculosis)* were used. Test plates were incubated at 37°C for 16 h (*P. aeruginosa*, *K. pneumoniae*, *B. cereus*, *S. pneumoniae*), 48 h (*B. cenocepacia*) or 7 days (*M. tuberculosis*) and read-out of the antimicrobial activity was performed with a resazurin assay as described earlier (III.2.3). Plates with resazurin were incubated 15 min (*K. pneumoniae*), 30 min (*P. aeruginosa*), 4 h (*B. cenocepacia*), or 1 day (*M. tuberculosis*). The fluorescent signal was read using a microplate reader (Promega) at $\lambda_{excitation}$ = 550 nm and $\lambda_{emission}$ = 590 and the IC_{50} values were calculated. The screening was carried out in biological duplicate.

III.2.8 Antiparasitic screen of selected cecropins

The activity of HC1 and HC10 was additionally tested against a panel of parasites, based on protocols described in literature [199, 200]. AMP dilutions were prepared as described earlier (III.2.3) in a final concentration of 8 µM and a volume of 10 µL. *Trypanosoma brucei*

brucei (*T. b. brucei*, strain Squib 427) and *Trypanosoma brucei rhodesiense* (*T. b. rhodesiense*, strain STIB-900) were maintained in HMI-9 medium + 10% iFCS and added at a density of 7 x 10^4 parasites/mL in 190 µL to the 96 well plates. Plates with *T. b. brucei* and *T. b. rhodesiense* were incubated 72 h (37 °C, 5% CO_2), and activity read-out was performed after 24 h incubation with resazurin (see III.2.3). Next, *Trypanosoma cruzi* (*T. cruzi*, β-galactosidase strain Tulahuen CL2) was cultivated in MRC5-SV2 cells in complete MEM and 190 µL of cell/parasite suspension was added to the test plates (2 x 10^4 cells/mL and 2 x 10^5 parasites/mL), and plates were incubated 168 h (37 °C, 5% CO_2). Antiparasitic activity was determined with a spectrophotometric assay using 50 µL of chlorophenol red β-D-galactopyranoside and 250 µL Nonidet as described elsewhere [199, 200]. *Leishmania infantum* (*L. infantum*, strain MHOM/MA) amastigotes were collected from the spleen of infected hamsters and subsequently maintained in primary mouse macrophages in RPMI medium + 5% iFCS. Then, 190 µL of the cell/parasite inoculum was added to the well plates (3 × 10^5 cells/mL and 3 × 10^6 parasites/well) and the plates were incubated 120 h (37 °C, 5% CO_2). AMP activity against *L. infantis* was assessed microscopically using a standard Giemsa staining protocol [199, 200]. Lastly, *Plasmodium falciparum* (*P. falciparum*, strain Pf-K1) was maintained in human erythrocytes in RPMI medium, supplemented with 0.37 mM hypoxanthine, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, 25 mM $NaHCO_3$ and 10% human serum. Then, 190 µL of *P. falciparum* suspension was added to the AMP test plates (1% parasitaemia, 2% hematocrit), incubated for 72 h in microaerophilic conditions (37 °C, 4% CO_2, 3% O_2 and 93% N_2), and stored at -20 °C until further processing. A Malstat assay was used for activity read-out [199, 200]. As reference compounds suramin (Sigma-Aldrich, *T. b. brucei*, *T. b. rhodesiense*), benznidazole (Sigma-Aldrich, *T. cruzi*), miltefosine (Sigma-Aldrich, *L. infantum*), and chloroquine (Sigma-Aldrich , *P. falciparum*) were used. The screening was carried out in biological duplicate.

III.2.9 Hemolysis analysis

For the analysis of hemolysis, fresh human whole blood was collected in tubes containing 30 units of heparin [201]. Afterwards, the blood was centrifuged (Eppendorf 5427 R,

1000 × g, 5 min) and washed until the supernatant was clear. The erythrocyte pellet was diluted in phosphate buffered saline (PBS; Gibco) to obtain a 2% red blood cell suspension. Next, 150 µL of serially diluted AMPs in PBS were added to microcentrifuge tubes and mixed with 150 µL of the red blood cell suspension. 0.1% Triton-X (Sigma-Aldrich) and PBS were used as positive and negative controls respectively. The samples were incubated at 37°C for 1 h. Afterwards, the tubes were centrifuged at 1000 × g for 5 min and 200 µL of the supernatant was transferred to a 96-well plate. Absorbance was measured with a microplate reader (Promega) at λ = 570 nm to detect hemoglobin release. Results were used to calculate the percentage of hemolysis. The hemolysis analysis was carried out in biological duplicate. In addition, hemolysis was predicted using the online software HemoPred [202].

III.2.10 Time kill curves

To investigate the bactericidal kinetics of the selected AMPs, a time kill analysis was performed as described previously by Mascio et al. [203]. Briefly, peptide serial dilutions were prepared in 96-well plates (10µL) and mixed with 190 µL of a *P. aeruginosa* ATCC 9027 culture of an optical density measured at a wavelength of 600 nm (OD_{600}) of 0.1, which corresponds to a concentration of approximately 10^8 CFU/mL. Plates were incubated at 37°C and at selected timepoints (0, 0.5, 1, 2, 4, and 5 h), aliquots were removed and serially diluted in PBS. The dilutions were plated on TSA and incubated overnight at 37°C. Afterwards, bacterial viability was assessed by performing a standard plate count. Bactericidal activity was defined as a ≥3-log reduction compared to the untreated bacterial control. To avoid carry-over effect of the peptides, the undiluted sample was not plated, leading to a quantification limit of 10^3 CFU/mL [203]. Five biological repeats were performed.

III.2.11 Inner membrane permeabilization by the propidium iodide uptake assay

To investigate the inner membrane (IM) damage induced by the selected AMPs, a propidium iodide (PI; Sigma-Aldrich) uptake assay was performed, adapted from Dassanayake et al. [204]. *P. aeruginosa* ATCC 9027 was grown until the mid-log phase in

MHB. The bacteria were centrifuged (Eppendorf, 3000 × g, 15 min) and resuspended in 5 mM HEPES (Sigma-Aldrich) buffer (pH 7.4) to an OD_{600} of 0.5. Next, 50 µL of serially diluted peptides were added *in duplo* to a 96-well plate and mixed with a 150 µL bacterial suspension containing 4 µM PI (final in-plate concentration of 3 µM). Peptide test concentrations ranged from 32 µM to 0.25 µM. A high concentration of polymyxin B sulfate (16 µM) was implemented as a positive control. Fluorescence was measured every 5 min during 1 h using a microplate reader (Promega) at $\lambda_{excitation}$ = 530 nm and $\lambda_{emission}$ = 620. Data were normalized based on the fluorescent signal in the presence of 50 µL HEPES buffer and 150 µL PI bacterial suspension. The assay was carried out in biological triplicate.

III.2.12 Outer membrane permeabilization with n-phenyl-naphthylamine

N-phenyl-naphthylamine (NPN; TCI) was used to study the permeabilization of the outer membrane (OM) as described by Helander et al [205]. Briefly, *P. aeruginosa* ATCC 9027 was grown until the mid-log phase in MHB. Afterwards, the cells were centrifuged (Eppendorf, 3000 × g, 15 min) and resuspended in 5 mM HEPES buffer (pH 7.4) to an OD_{600} of 0.5. Next, 50 µL of peptides were added *in duplo* to a 96-well plate (final concentrations ranging from 32 µM to 0.25 µM). As a positive control, 16 µM of polymyxin B was included. The peptides were mixed with 50 µL of a 40 µM NPN solution and 100 µL of *P. aeruginosa*. Fluorescence was measured every 5 min during 1 h using a microplate reader (Promega) with $\lambda_{excitation}$ = 350 nm and $\lambda_{emission}$ = 420. Data were normalized based on the fluorescent signal in the presence of 50 µL HEPES buffer, 50 µL NPN and 100 µL bacteria. Experiments were carried out in biological triplicate.

III.2.13 Cytoplasmic membrane depolarization assay

To study the effects of the peptides on the membrane potential, 3,3′-dipropylthiadicarbocyanine iodide (diSC3(5); TCI) was used as described by Kwon et al [206]. *P. aeruginosa* ATCC 9027 was grown until the mid-log phase in MHB. Afterwards, the bacteria were centrifuged (Eppendorf, 3000 × g, 15 min), washed and resuspended in 5 mM HEPES buffer (pH 7.4) supplemented with 20 mM glucose and 100 mM KCl to

an OD_{600} of 0.2. DiSC3(5) was added to the *P. aeruginosa* suspension at a concentration of 1.33 µM (final in-plate concentration of 1 µM), and the mixture was left to stand for 1.5 h to stabilize the fluorescent signal. Serially diluted peptides (50 µL) were added *in duplo* to 96-well plates (final concentrations ranging from 32 µM to 0.25 µM) and mixed with 150 µL of bacteria with diSC3(5). The fluorescent signal was measured at $\lambda_{excitation}$ = 620 nm and $\lambda_{emission}$ = 670 with a microplate reader (Promega) every 5 min during 1 h. 0.1% Triton-X was used as a positive control. Data were normalized based on the fluorescence of 50 µL HEPES buffer and 150 µL diSC3(5)-bacterial suspension. The assay was carried out in biological triplicate.

III.3 Results

III.3.1 Antimicrobial activity of the BSF peptide library

To identify antimicrobial activity of the AMPs, a screening against one Gram-positive, two Gram-negative and two fungal species was performed (**Table III.2, SIII.2**). One peptide (Hill-Stom2) was not included in the screening due to poor DMSO solubility. Among the tested peptides, the most promising activity was found within the cecropin family of AMPs. Aside from one peptide (Hill-Cec6), all cecropins showed activity against *E. coli* and *P. aeruginosa* at low micromolar concentrations, with MIC values ranging from 0.50 µM to 2 µM. Cecropins are α-helical AMPs without cysteine residues and β-sheet motifs [137, 189]. Potent activity of insect cecropins with a strict Gram-negative spectrum has been reported for other insect species earlier, such as for *Lucilia sericata* [207, 208]. One of the earlier described BSF cecropins (CLP1) has a 95% sequence homology with the closest related AMP of our library (Hill-Cec2), and exhibits antibacterial activity in the same concentration range [171]. Next, one diptericin (Hill-Dip6) also showed activity against *E. coli* (MIC of 2 µM), but activity against *P. aeruginosa* was not recorded. Antibacterial activity against *S. aureus* was reported for three defensins (Hill-Def2a, Hill-Def2b, and Hill-Def4) but was absent for all other AMPs. Interestingly, the free cysteine residues of Hill-Def2a seem crucial for its potent antibacterial activity, as its counterpart with disulfide bridges (Hill-Def2b) needed much higher concentrations (32 µM and 64 µM) for

90% growth inhibition of *S. aureus*. Finally, no peptide showed signs of activity against *A. fumigatus* and *C. albicans* within the tested concentration ranges.

III.3.2 Bactericidal activity of BSF peptide library

All cecropins exhibited some degree of bactericidal activity against *E. coli* and *P. aeruginosa*. The MBC of the peptides was either equal to their MIC or higher (up to 64 times) and generally, a higher concentration of AMP was required to kill *P. aeruginosa* compared to *E. coli* (Table III.2). MBC values were notably variable between the two independent repeat screenings, while for the MIC values only a maximum of a factor two difference was noted between experiments. Thus, the reference strains seem to show a variable susceptibility to the bactericidal mechanism of the cecropins. Other active AMPs, such as the defensins Hill-Def2 and Hill-Def4, did not exhibit clear bactericidal activity, but a bacteriostatic mechanism of action.

Table III.2 Results of antimicrobial and cytotoxicity screening of peptide library. IC_{50} values (concentrations causing 50% growth inhibition), MIC values (minimum inhibitory concentrations needed for visual absence of bacterial growth), and MBC (minimum bactericidal concentration) values obtained in two individual screenings: the first starting at 64 µM, the second at 32 µM. Peptides with activity < 32 µM against either the cell line (MRC5-SV2) or the microbial reference strains are shown. S = bacteriostatic activity.

		IC_{50} (µM)						MIC (µM)			MBC (µM)		
AMP	Repeat	MRC5-SV2	*S. aureus*	*E. coli*	*P. aeruginosa*	*C. albicans*	*A. fumigatus*	*S. aureus*	*E. coli*	*P. aeruginosa*	*S. aureus*	*E. coli*	*P. aeruginosa*
Hill-Knot2	1	**9.73**	> 64.00	> 64.0	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	-	-	-
	2	**3.76**	> 32.00	> 32.0	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	-	-	-
Hill-Knot3	1	> 64.00	> 64.00	> 64.0	**22.63**	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	-	-	-
	2	> 32.00	> 32.00	> 32.0	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	-	-	-
Hill-Def2a	1	> 64.00	< **0.25**	**35.3**	**32.86**	> 64.00	> 64.00	**0.25**	> 64.00	> 64.00	S	-	-
	2	> 32.00	**0.19**	> 32.0	> 32.00	> 32.00	> 32.00	**2.00**	> 32.00	> 32.00	S	-	-
Hill-Def4	1	> 64.00	**0.50**	> 64.0	**32.00**	> 64.00	> 64.00	**1.00**	> 64.00	> 64.00	S	-	-
	2	> 32.00	**0.70**	> 32.0	> 32.00	> 32.00	> 32.00	**8.00**	> 32.00	> 32.00	S	-	-
Hill-Def2b	1	> 64.00	**8.50**	> 64.0	> 64.00	> 64.00	> 64.00	**64.00**	32.00	> 64.00	S	-	-
	2	> 32.00	**13.45**	> 32.0	> 32.00	> 32.00	> 32.00	**32.00**	64.00	> 32.00	S	-	-

Table III.2 Results of antimicrobial and cytotoxicity screening of peptide library. S = bacteriostatic activity.

		IC_{50} (µM)						MIC (µM)			MBC (µM)		
AMP	Repeat	MRC5-SV2	*S. aureus*	*E. coli*	*P. aeruginosa*	*C. albicans*	*A. fumigatus*	*S. aureus*	*E. coli*	*P. aeruginosa*	*S. aureus*	*E. coli*	*P. aeruginosa*
Hill-Cec1	1	> 64.00	> 64.00	< 0.25	< 0.25	> 64.00	> 64.00	> 64.00	0.25	1.00	-	1.00	4.00
	2	> 32.00	> 32.00	0.21	0.20	> 32.00	> 32.00	> 32.00	0.50	0.50	-	0.50	0.50
Hill-Cec2	1	> 64.00	> 64.00	0.50	0.93	> 64.00	> 64.00	> 64.00	1.00	4.00	-	1.00	16.00
	2	> 32.00	4.36	0.31	0.76	> 32.00	> 32.00	> 32.00	2.00	2.00	-	2.00	2.00
Hill-Cec3	1	> 64.00	> 64.00	0.49	0.40	> 64.00	> 64.00	> 64.00	1.00	4.00	-	1.00	16.00
	2	> 32.00	> 32.00	0.22	0.79	> 32.00	> 32.00	> 32.00	0.50	2.00	-	32.00	2.00
Hill-Cec4	1	37.37	> 64.00	0.50	< 0.25	> 64.00	> 64.00	> 64.00	1.00	1.00	-	S	16.00
	2	> 32.00	> 32.00	0.32	0.22	> 32.00	> 32.00	> 32.00	2.00	2.00	-	2.00	2.00
Hill-Cec5	1	> 64.00	> 64.00	0.50	2.61	> 64.00	> 64.00	> 64.00	1.00	16.00	-	S	S
	2	> 32.00	> 32.00	0.23	3.60	> 32.00	> 32.00	> 32.00	0.50	32.00	-	0.50	32.00
Hill-Cec7	1	> 64.00	> 64.00	0.38	0.40	> 64.00	> 64.00	> 64.00	1.00	4.00	-	1.00	16.00
	2	> 32.00	> 32.00	0.32	0.78	> 32.00	> 32.00	> 32.00	2.00	2.00	-	2.00	2.00
Hill-Cec8	1	> 64.00	> 64.00	0.45	1.29	> 64.00	> 64.00	> 64.00	1.00	4.00	-	1.00	64.00
	2	> 32.00	> 32.00	0.68	1.73	> 32.00	> 32.00	> 32.00	2.00	8.00	-	8.00	8.00

Table III.2 Results of antimicrobial and cytotoxicity screening of peptide library. S = bacteriostatic activity.

		IC_{50} (µM)						MIC (µM)			MBC (µM)		
AMP	Repeat	MRC5-SV2	*S. aureus*	*E. coli*	*P. aeruginosa*	*C. albicans*	*A. fumigatus*	*S. aureus*	*E. coli*	*P. aeruginosa*	*S. aureus*	*E. coli*	*P. aeruginosa*
Hill-Cec9	1	> 64.00	> 64.00	**0.43**	**0.38**	> 64.00	> 64.00	> 64.00	**1.00**	**1.00**	-	1.00	S
	2	> 32.00	> 32.00	**0.21**	**0.88**	> 32.00	> 32.00	> 32.00	**0.50**	**2.00**	-	0.50	2.00
Hill-Cec10	1	> 64.00	32.74	< **0.25**	< **0.25**	> 64.00	> 64.00	> 64.00	**0.25**	**1.00**	-	0.25	16.00
	2	> 32.00	> 32.00	**0.22**	**0.22**	> 32.00	> 32.00	> 32.00	**0.50**	**2.00**	-	32.00	2.00
Hill-Cec11	1	> 64.00	> 64.00	< **0.25**	< **0.25**	> 64.00	> 64.00	> 64.00	**1.00**	**1.00**	-	1.00	4.00
	2	> 32.00	> 32.00	**0.22**	**0.24**	> 32.00	> 32.00	> 32.00	**0.50**	**2.00**	-	32.00	8.00
Hill-Cec12	1	> 64.00	> 64.00	< **0.25**	< **0.25**	> 64.00	> 64.00	> 64.00	**0.25**	**1.00**	-	1.00	S
	2	19.45	> 32.00	**0.32**	**0.23**	> 32.00	> 32.00	> 32.00	**2.00**	**2.00**	-	8.00	8.00
Hill-Cec13	1	> 64.00	> 64.00	< **0.25**	< **0.25**	> 64.00	> 64.00	> 64.00	**1.00**	**1.00**	-	1.00	64.00
	2	> 32.00	> 32.00	**0.23**	**0.25**	> 32.00	> 32.00	> 32.00	**0.50**	**2.00**	-	0.50	2.00
Hill-Stom1	1	**11.65**	**58.58**	> 64.0	**16.00**	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	-	-	-
	2	**24.42**	> 32.00	> 32.0	**17.67**	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	-	-	-
Hill-Dip6	1	**4.54**	> 32.00	**0.85**	> 32.00	> 64.00	> 64.00	> 64.00	**2.00**	> 32.00	-	S	-
	2	**7.73**	> 32.00	**1.00**	> 32.00	> 32.00	> 32.00	> 32.00	**2.00**	> 64.00	-	8.00	-

III.3.3 Primary toxicity of BSF peptide library

Primary peptide toxicity was evaluated using a cell line of human lung fibroblasts (MRC5-SV2) (**Table III.2**). The majority of the tested peptides had no effect on cell viability within the tested concentration ranges. For three AMPs, IC_{50} values below 32 µM were recorded for cell toxicity: Hill-Knot2, a knottin-like peptide, Hill-Stom1, a stomoxyn-like peptide, and Hill-Dip6, a diptericin.

III.3.4 Structural analysis of the cecropin family

To study amino acid conservation among the cecropin family and to link possible conserved sequence features to the *in vitro* antimicrobial activity, multiple sequence alignment was performed (**Figure III.1**) [209]. Sequence analysis showed strong conservation of most amino acid residues in both the hydrophobic and the polar regions. The absence of a tryptophan residue at the N-terminal region of Hill-Cec6, a reoccurring feature for insect cecropins, could play a role in its lack of antibacterial activity (**Tables SIII.1, SIII.2**) [134]. Hill-Cec6 also differs from most other cecropins at the C-terminal region, having no proline residues. It contains more acidic AA as well, giving it a considerably lower net charge (+2) than the other cecropins (+4 to +7).

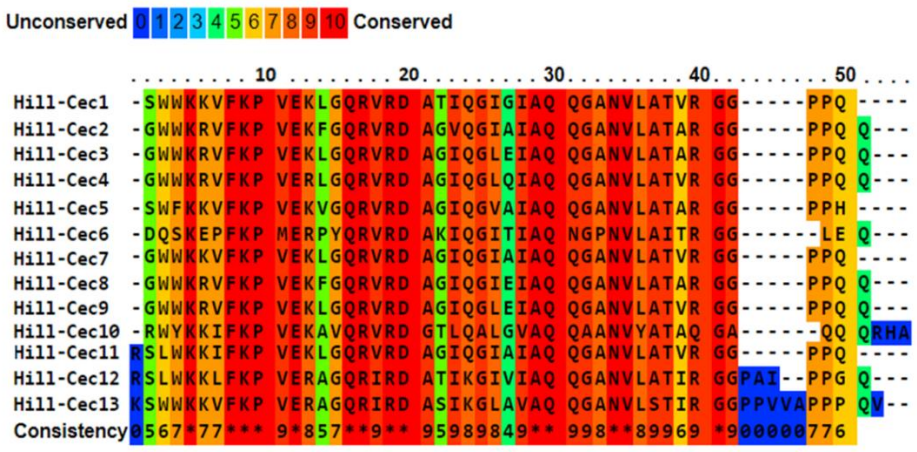


Figure III.1 Primary sequence alignment of the BSF AMPs from the cecropin family. The colour of the amino acids indicates the extent of conservation among the different peptides. The figure was constructed using the PRALINE software available at https://www.ibi.vu.nl/programs/pralinewww.

Two peptides, HC1 and HC10, from the cecropin family were selected for further *in vitro* characterization. As all cecropins presented comparable activity parameters, HC1 and HC10 were chosen based on their structural divergence and lower amount of sequence overlap. HC1 has a charge of +5 and is 44 AA long, whereas HC10 has a stronger positive charge of +7 and a length of 47 AA. Both have potent antibacterial activity against the Gram-negative test strains. A 3D-structure was predicted for HC1 and HC10 using the NovaFold application, which uses the I-Tasser algorithm (**Figure III.2**). For both AMPs, the software predicted a protein structure with a double alpha helix, linked by a short hinge region, a structure typically found in cecropins [141, 210].

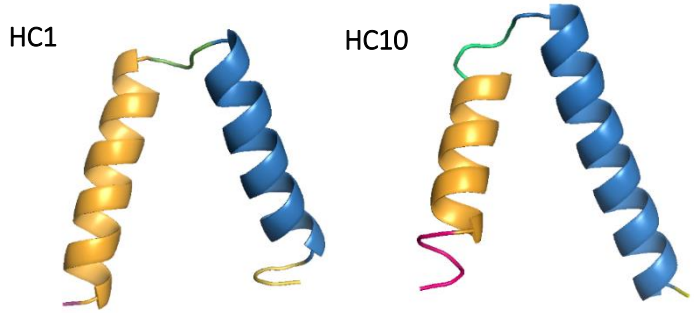


Figure III.2 *In silico* predicted 3D-models for HC1 (left) and HC10 (right). Both peptides contain features typically found in cecropin AMPs, including a double alpha helix and a short hinge region in between. Models were predicted with the I-Tasser software, available true the NovaFold software (DNAstar). The predicted models have a TM-score of 0.53 ± 0.15 (HC1), and 0.52 ± 0.15 (HC10).

A helical wheel projection was constructed to visualize the distribution of hydrophobic and hydrophilic amino acids among the helical axis of the helixes of the peptides (**Figure III.3**) [211]. The N-terminal helix of HC1 and HC10 has a high density of charged, mainly cationic, amino acid residues, whereas the C-terminal helix is rich in polar, but uncharged amino acids. Both helices have an amphipathic character, with the hydrophobic amino acids oriented opposite the hydrophilic helical face.

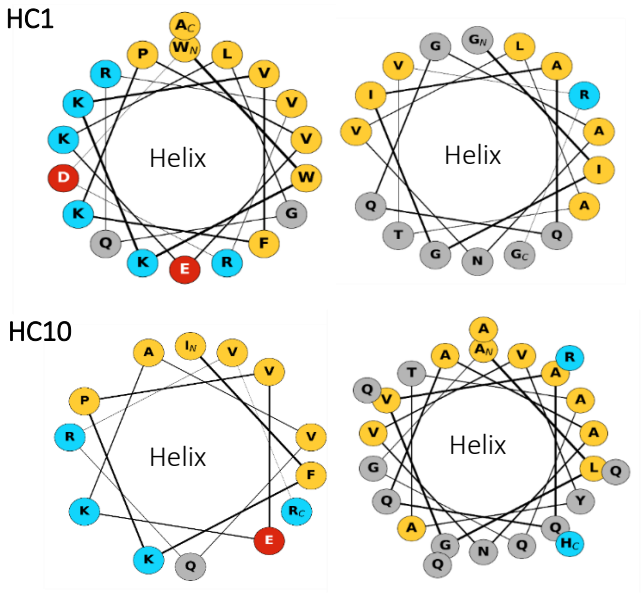


Figure III.3 Helical wheel projection HC1 and HC10. Projections were constructed using the online Galaxy CPT software available at https://cpt.tamu.edu/galaxy-pub. Polar residues with a positive charge are indicated in blue, negatively charged polar residues are red. Uncharged polar amino acids are indicated in grey, and hydrophobic residues have a yellow colour.

In addition to *in silico* analysis, CD spectroscopy was performed to test the helical character of HC1 and HC10. CD spectroscopy is a technique that can give structural information about optically active molecules based on differential absorption of left and right circularly polarized light [212]. For peptides, it can distinguish motives such as α-helices, β-sheets, and random coil formations [212]. As biological material such as LPS can induce conformational changes, spectra were recorded with or without the addition of LPS [213]. The spectrum of HC1 showed two bands around 208 nm and 225 nm (**Figure III.4**), characteristic for α-helices, indicating that at least part of the AMP in solution adapts a helix structure prior to microbial contact. For HC10, however, a strong band around 200 was seen, indicative for a random coil formation (**Figure III.4**). Addition of LPS did not induce significant confirmation changes (data not shown). Potentially, the dose of LPS (100 ng/mL) was too low for significant confirmational changes at a dose of 32 µM.

Experiments should ideally be repeated with increasing concentrations of LPS. In addition, as the bands seen for HC1 are fairly weak, settings and AMPs could be optimized as well.

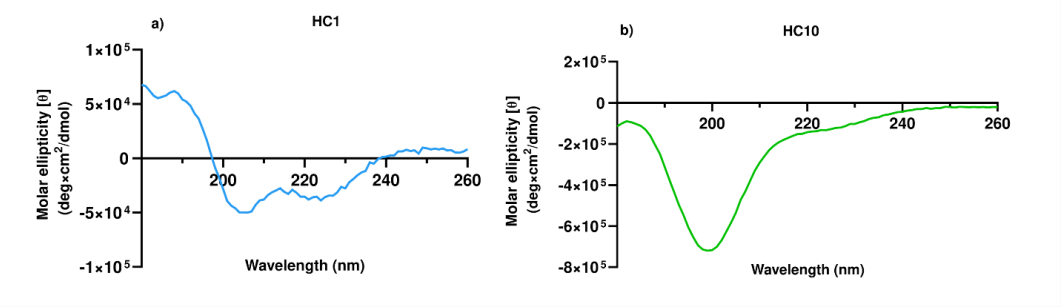


Figure III.4 Circular dichroism spectra for HC1 and HC10. a) The HC1 AMP shows bands at 208 and 225 nm, typically seen for α-helical peptides. **b**) HC10 adopts a random coil formation, as indicated by the 200 nm band.

III.3.5 Screening of selected cecropins against an extended microbial panel

The antibacterial activity of HC1 and HC10 was further explored against an extended panel of microorganisms (**Table III.3, Table III.4**). *P. aeruginosa* PAO1, a moderately virulent clinical isolate [214], *P. aeruginosa* LMG 27650, a multi-drug resistant clinical strain [215], and *P. aeruginosa* ATCC 15442, an environmental strain [216], were susceptible to the cecropins. The activity of HC1 was comparable with its activity against the non-virulent test strain *P. aeruginosa* ATCC 9027 [217]. The MIC of HC10, however, increased by a factor 2 to 4. HC10 also required notably higher concentrations for bactericidal activity. Additionally, HC1 and HC10 were both highly active against *K. pneumoniae*, another species known to cause critical lung infections [218], with MIC values between 0.25 and 0.5 µM for HC1 and between 0.5 and 1 µM for HC10. No activity against *B. cenocepacia*, *S. pneumoniae*, *B. cereus*, or *M. tuberculosis* was found within the tested concentration range.

Table III.3 Extended antimicrobial screening of HC1 and HC10. IC50 values (concentrations leading to 50% growth inhibition of the pathogen), MIC values (minimum inhibitory concentrations needed for visual absence of bacterial growth), and MBC (minimum bactericidal concentration) values obtained in two individual screenings of the selected cecropins HC1 and HC10 from concentrations starting at 32 µM. MDR = multi-drug-resistant.

		IC_{50} (µM)		MIC (µM)		MBC (µM)	
Strain	Repeat	HC1	HC10	HC1	HC10	HC1	HC10
P. aeruginosa ATCC 15692 (PAO1)	1	0.77	2.48	1	8	4	16
	2	0.39	0.76	0.50	2	0.50	16
P. aeruginosa LMG 27650 (MDR)	1	1.34	2.96	2	4	4	32
	2	0.66	3.35	1	8	16	16
P. aeruginosa ATCC 15442	1	0.73	2.82	1	4	4	16
	2	0.38	2.71	1	4	8	32
K. pneumoniae ATCC 13883	1	0.36	0.75	0.50	1	2	4
	2	0.18	0.31	0.25	0.50	4	8
B. cenocepacia LMG 16656	1	>32	>32	>32	>32	-	-
	2	>32	>32	>32	>32	-	-
S. pneumoniae D39	1	>32	>32	>32	>32	-	-
	2	>32	>32	>32	>32	-	-
B. cereus ATCC 14579	1	>32	>32	>32	>32	-	-
	2	>32	>32	>32	>32	-	-
M. tuberculosis ATCC 25177 (H37Ra)	1	>32	>32	>32	>32	-	-
	2	>32	>32	>32	>32	-	-

Next, HC1 and HC10 were also tested against a panel of parasites, including *T. b. brucei* and *T. b. rhodesiense*, both causative agents of African sleeping sickness, *T. cruzi*, which causes Chagas disease, *L. infantum*, involved in visceral leishmaniasis, and a malaria parasite (*P. falciparum*) [199]. Interestingly, both cecropins showed moderate activity

against the African trypanosome species, while activity against the other parasites was not noted at concentrations up until 8 µM (Table III.4).

Table III.4 Antiparasitic screening of HC1 and HC10. IC50 values (concentrations leading to 50% growth inhibition of the pathogen) and MIC values (minimum inhibitory concentrations needed for visual absence of bacterial growth) obtained in two individual screenings of the selected cecropins HC1 and HC10 against parasitic species performed at concentrations starting at 8 µM.

		IC_{50} (µM)		MIC (µM)	
Strain	Repeat	HC1	HC10	HC1	HC10
T. b. brucei (Squib 427)	1	5.20	1.19	8	> 8
	2	6.28	1.48	8	> 8
T. b. rhodesiense (STIB-900)	1	1.19	6.89	2	> 8
	2	1.48	> 8	2	> 8
T. cruzi (Tulahuen CL2)	1	> 8	> 8	> 8	> 8
	2	> 8	> 8	> 8	> 8
L. infantum (MHOM/MA)	1	> 8	> 8	> 8	> 8
	2	> 8	> 8	> 8	> 8
P. falciparum (Pf-K1)	1	> 8	> 8	> 8	> 8
	2	> 8	> 8	> 8	> 8

III.3.6 Hemolysis analysis

In addition to the primary toxicity screening against human fibroblasts, a hemolytic assay was performed using human red blood cells. The OM leaflets of red blood cells are rich in sialic acid residues, which gives them a lower negative charge than other mammalian cell types [101, 219]. This negative charge makes them prone to interactions with the positively charged AMPs. Hence, a hemolysis analysis is often routinely included in AMP research. Apart from cationicity, hydrophobicity is also positively correlated with the hemolytic capacity of AMPs [220]. Both cecropins showed hemolysis of less than 10% at the highest concentration tested of 64 µM. These results predict low hemolysis *in vivo*.

However, results will need to be confirmed later on *in vivo*, as the experimental conditions differ from the protein-rich environment of whole blood [98]. Results of the hemolysis analysis were in line with the bio-informatical predictions of HemoPred which identified the two cecropins as 'non-hemolytic' [202].

III.3.7 Time kill analysis

To investigate the onset of action and the bactericidal activity of the peptides, a time kill analysis was performed. Both cecropins have a rapid onset of action and cause growth inhibition of *P. aeruginosa* within 30 min (**Figure III.5**). To obtain bactericidal activity (≥3-log10 reduction compared to the growth control), both AMPs require at least a concentration of four times their MIC values. At 4 µM (4 x MIC), HC1 achieved rapid bactericidal activity, leading to a log10 reduction of 4.74 ± 0.55 after 1 h. However, afterwards there was a clear increase in *P. aeruginosa* growth, and at 5 h, HC1 had variable activity throughout the different experiments, causing on average a 3.32 ± 1.40 log10 reduction (**Table SIII.3**). In contrast, a higher HC1 concentration of 8 µM (8 X MIC) consistently achieved bactericidal activity at all time points tested, leading to a log10 reduction in bacteria of 5.50 ± 0.44 after 5 h. For HC10, a similar pattern was observed. The highest concentration of 16 µM (8 X MIC), always caused at least a 3-log10 reduction of bacteria, although the exact amount of bacterial killing varied throughout the independent experiments, leading to a log10 reduction of 4.60 ± 0.94 after 5 h (**Table SIII.4**). At 8 µM (4 x MIC), the AMP was bactericidal up until 1 h, but was not able to sustain this killing effect for the next time points tested (log10 reduction of 2.44 ± 0.46 after 5 h). Important to mention is the variability in bactericidal activity noticed in the time kill experiments. This variability was also seen in our earlier MBC experiments, and is suspected to be caused by natural variation in the peptides' MIC and MBC values in-between experiments.

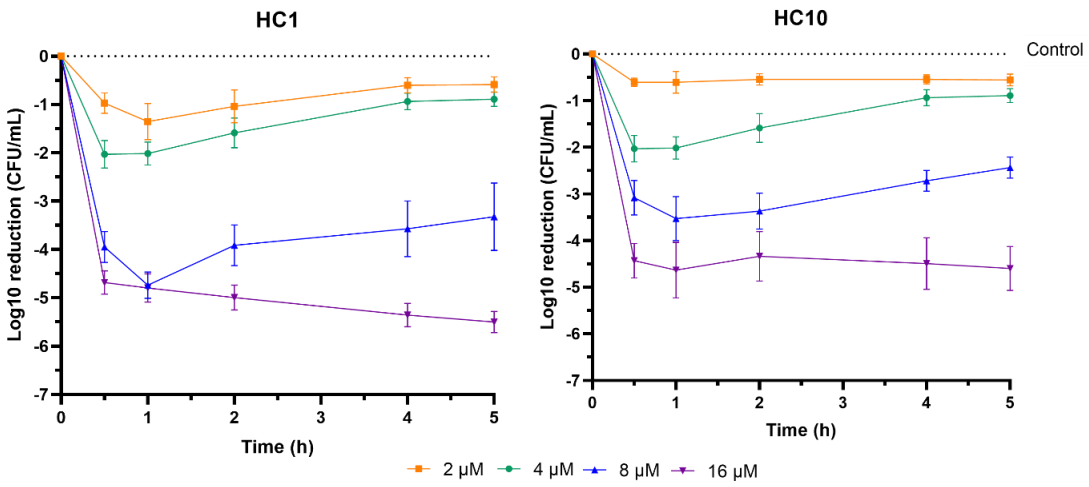


Figure III.5 Time kill curves showing the log10 reductions in *P. aeruginosa* ATCC 9027 caused by HC1 and HC10
Concentrations of 2 µM, 4 µM, 8 µM, and 16 µM were tested. The dashed line indicates the bacterial growth control, to which the data has been normalized. Graphs represent the mean ± standard deviation of five independent experiments.

III.3.8 Membrane permeabilization and disruption

Both cecropins are able to permeabilize the cell membranes of *P. aeruginosa*, as confirmed by the NPN and PI uptake assays (**Figure III.6**). NPN, a hydrophobic probe, is normally excluded from bacterial membranes. It will, however, accumulate in the OM of Gram-negative bacteria when the barrier is compromised, leading to an increase in fluorescence [221, 222]. NPN uptake in the OM occurs within the first 5 min, after which the fluorescent signal stabilizes (**Figure III.6a, III.6b**). The NPN uptake is concentration-dependent and reaches a maximum of 100% (as compared to 16 µM of polymyxin B) at 32 µM for HC1, and a maximum of 75% at 32 µM for HC10. Supra-MIC concentrations are needed to reach 50% or more NPN uptake. Upon treatment with PI, a sharp increase in fluorescence is also seen immediately after exposure, after which the signal quickly stabilizes (**Figure III.6c, III.6d**). PI is a DNA intercalating dye, which is not able to traverse intact bacterial membranes [223]. When the membranes are permeabilized, PI can reach the cytoplasm where it binds to nucleic acids, which increases its fluorescent signal [221]. PI fluorescence is therefore an indicator of IM permeabilization. The PI uptake is

concentration-dependent for both cecropins. At high concentrations (32 µM) the membrane permeabilization exceeds that of a high dose of polymyxin B (16 µM). The NPN and PI uptake assays show that HC1 and HC10 can permeabilize both the OM and IM of *P. aeruginosa*. The majority of AMPs with a characterized mechanism of action work by decreasing the integrity of the bacterial membranes through, for example, pore-formation or complete membrane lysis [138]. As the increase in fluorescence for both the NPN and PI uptake occurs rapidly (within 5 min) and is present at non-lethal concentrations, the membrane permeabilization is likely directly linked to the mechanism of action of the BSF AMPs, and not a secondary effect of the bacteria dying through other, non-related intracellular mechanisms [224].

III.3.9 Cytoplasmic membrane depolarization

DiSC3(5), a voltage-sensitive cationic dye, was used to study cytoplasmic membrane depolarization. Under normal conditions, the dye is concentrated in the (hyper)polarized cytoplasmic membrane, leading to self-quenching of its fluorescence. Upon depolarization, however, diSC3(5) is released into the cytoplasm causing an increase in fluorescence [225]. Both HC1 and HC10 cause cytoplasmic membrane depolarization of *P. aeruginosa*, as indicated by the increase in diSC3(5) fluorescence (Figure III.6e, III.6f). The depolarization is largely concentration-dependent, although more outspoken for HC10. At high concentrations, cytoplasmic depolarization exceeds that of polymyxin B (16 µM), especially for HC1. As the PI uptake assay showed that the cecropins permeabilize the IM, it is possible that the membrane depolarization is largely caused by membrane damage.

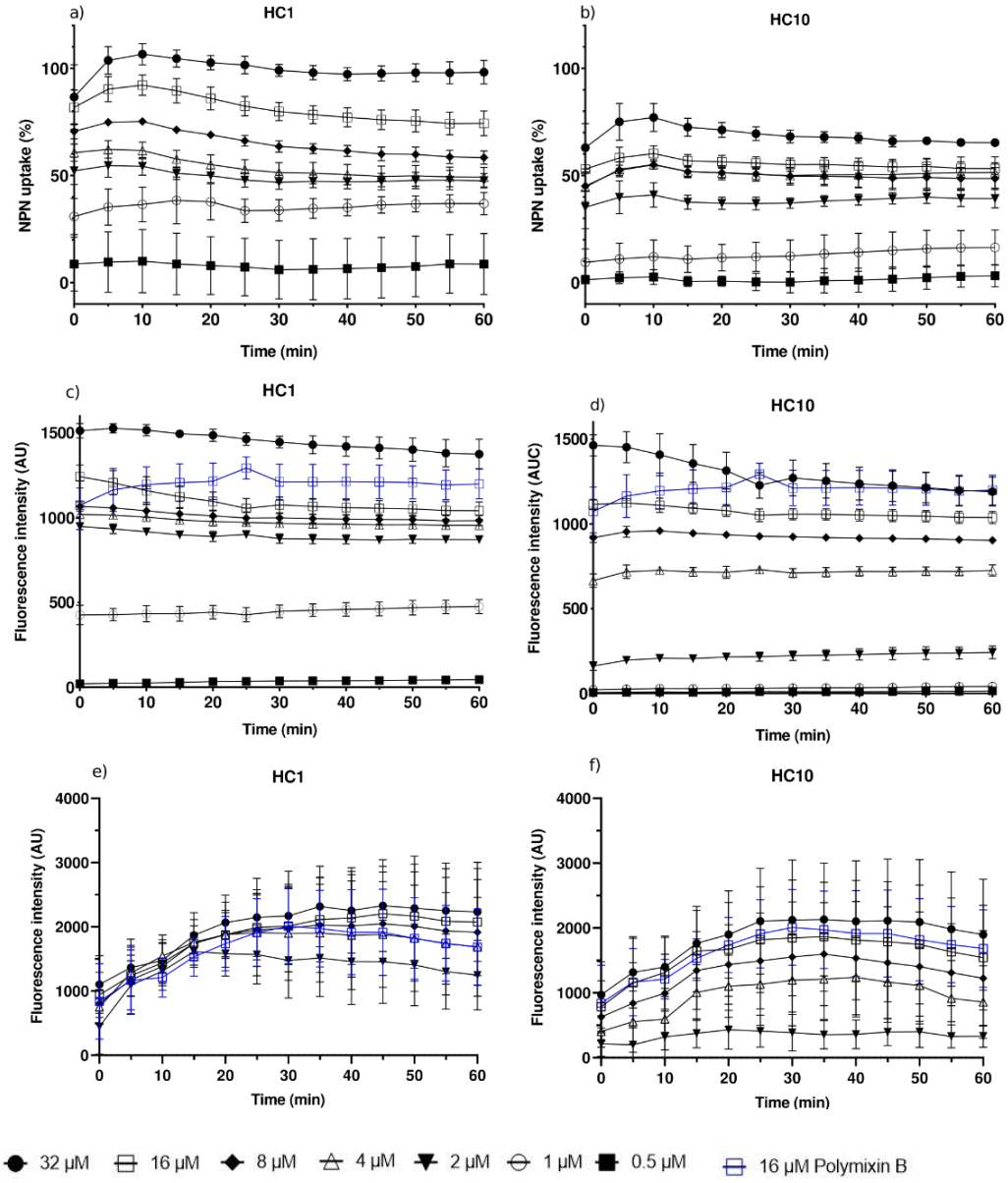


Figure III.6 Membrane activity of HC1 and HC10. **a,b**) Outer membrane permeabilization of *P. aeruginosa* caused by HC1 (a) and HC10 (b) using N-phenyl-naphthylamine (NPN). NPN uptake is expressed as a percentage of the maximal uptake recorded with 16 µM of polymyxin B. **c,d**) Fluorescence caused by propidium iodide (PI) uptake in *P. aeruginosa* after addition of HC1 (c) or HC10 (d). Values were normalized with the negative control. AU: arbitrary units. **e,f**) Fluorescent signal of 3,3′-dipropylthiadicarbocyanine iodide (diSC3(5)) as an indicator of cytoplasmic membrane depolarization of *P. aeruginosa* caused by HC1 (e) or HC10 (f). Concentrations < 2 µM had no measurable effects. Fluorescent signals were normalized with the negative control. The assay was carried out in biological triplicate. The mean ± standard deviation is given.

III. 4 Discussion

III.4.1 Antimicrobial evaluation of black soldier fly peptide library

As a decomposer of biowaste, the BSF larva lives in close contact with potentially hazardous microorganisms [172]. The evolutionary expansion and diversification of the BSF AMP repertoire could explain their successful colonization of these microbe-rich substrates [130]. Here, a library of BSF peptides was evaluated for its antimicrobial activity. Potent activity against Gram-negative bacteria was mostly recorded for the cecropin AMP family, while some defensin peptides showed antibacterial activity in the Gram-positive spectrum. No antifungal activity was recorded. Remarkably, for a significant part of the AMP library including the majority of the defensins, antimicrobial activity was undetected. While possible that these peptides have functionally diversified to fulfil other biological functions in the BSF, the chosen experimental procedures might also have interfered with their *in vitro* activity. For example, even low amounts of DMSO can influence protein folding, denaturation, aggregation and target-binding. In addition, the AMPS produced via solid-phase synthesis might differ from their natural counterparts in terms of protein folding or aggregation, side chain integrity and post-translational modifications.

III.4.2 Biological function of cecropins in the black soldier fly

Apart from Hill-Cec6 all cecropins showed strong activity against the Gram-negative test strains at low micromolar concentrations. Cecropin AMPs are widely distributed among the insect orders, and have so far been identified in the Coleoptera, Diptera and Lepidoptera. It is suggested, however, that they are produced by all holometabolous insects with the exception of Hymenopteran species [134]. The amount and diversity of cecropins found in insects hints at more preserved functions of these AMPs. In the BSF, AMPs are involved in maintaining and shaping the bacterial gut communities and maintaining eubiosis in the gut [130, 191], but cecropins could have additional biological roles as well. For example, the *Aedes aegypti* cecropin B is involved in the formation of the cuticle of adult mosquitoes [226]. Knockdown of the cecropin B gene in the pupae

led to high mortality, deformed adults, and impaired cuticle lamellae with disorganized chitin fibrils [226]. It is suggested that cecropin B works through upregulation of the expression of prophenoloxidases, which are involved in cuticle formation. Prophenoloxidases also play a crucial role in insect immunity. The enzymes are, for example, involved in the induction of the melanisation process, which leads to encapsulation of invading pathogens [227, 228].

III.4.3 Potential of black soldier fly cecropins in drug development

The activity against Gram-negative pathogens of cecropins has made them compounds of interest for new antimicrobial drug development [138]. In this study, we selected two cecropin AMPs, HC1 and HC10, for *in vitro* characterization. As all antibacterial cecropins presented comparable activity parameters, HC1 and HC10 were chosen based on their structural divergence and lower amount of sequence overlap. In line with other cecropins, they have an alpha-helical, amphipathic character and a strong positive net charge. As AMPs are known to be toxic to human cells due to unspecific membrane interactions, we studied their effect on lung fibroblasts and erythrocytes [98]. Both peptides showed no signs of *in vitro* hemolysis or cytotoxicity at the tested concentration levels. The antimicrobial activity of the cecropins was further explored, and apart from the *E. coli* and *P. aeruginosa* lab strains, the cecropins were also active against other *P. aeruginosa* species, including a multi-drug resistant strain, and *K. pneumoniae*, *T. b. brucei*, and *T. b. rhodesiense*.

Next, we characterized the antimicrobial profile of HC1 and HC10 against *P. aeruginosa* more in-depth. *P. aeruginosa* is an opportunistic pathogen that is a major cause of nosocomial infections [229]. Vulnerable patients, such as cystic fibrosis (CF) and burn wound patients, are especially at risk of *Pseudomona*s infections [229, 230]. Eradication of *P. aeruginosa* has become increasingly difficult due to the rise of multi-drug resistant strains [231]. AMPs with activity against these drug-resistant strains could be valuable in the treatment of these critical infections [138]. Both cecropins are able to kill *P. aeruginosa* bacteria within the first 30 min of exposure. However, to maintain

bactericidal activity, supra-MIC concentrations are needed (4 to 8 µM for HC1, 16 µM or higher for HC10). Bactericidal activity has been linked to membrane permeabilization for other cecropins [232, 233]. Indeed, the NPN uptake assay confirmed that HC1 and HC10 can permeabilize the OM of *P. aeruginosa*. In line with the killing kinetics, the peptides are able to disrupt the OM fast (within 5 min), but need supra-MIC concentrations (2 µM for HC1, 4µM for HC10) to achieve at least 50% NPN uptake. The PI and $diSC_3(5)$ assays show that both cecropins are also able to target the inner, cytoplasmic membrane. Membrane disruption by AMPs is one of the main mechanisms that prevents pathogens to develop resistance [234, 235]. The combination of their activity against multi-drug resistant *Pseudomonas*, their low cytotoxicity, their fast killing time and the membrane disruptive mechanism of action, make HC1 and HC10 candidates for new anti-pseudomonal drug leads to treat acute infections, such as skin or lung infections [236, 237].

III.4.4 Importance in industrial black soldier fly larvae production

Apart from antimicrobial drug development, there is substantial interest in exploiting active AMPs towards other applications and industries. AMPs could, for example, be used as additives in the agriculture, food, and feed industries [238-240]. BSFL are being studied in waste management and recycling in livestock farming, for example in the hygienization of manure [241-245]. The reduction of pathogens in these highly contaminated substrates by the BSFL is partially attributed to the larvae's production of AMPs [245]. Furthermore, AMPs could also be of use in the insect industry itself. Insect farming is a flourishing sector [246], and the application of AMPs in BSF farming could help to lower the bioburden, including the number of human pathogens, present in the substrate during rearing, the insect biomass and the leftover frass [247, 248]. However, to develop these AMPs into usable additives, more research needs to be done on the stability of these peptides, for example in the substrate, and possible AMP formulations.

III.4.5 Concluding remarks

Overall, our selected cecropin peptides have shown promising activity against Gram-negative pathogens, such as *P. aeruginosa*. This leaves potential for various industrial applications including antimicrobial drug development. As insect AMPs are studied as alternative treatments for infections, such as skin, eye, and lung infections, these BSF cecropins expand the library of peptide templates usable for antimicrobial drug development [236]. However, many more aspects, such as synergy with conventional antibiotics, of these cecropin AMPs remain to be characterized (see CHAPTER IV) [249, 250]. Further development will also have to address obstacles commonly associated with peptide-based drugs, including poor metabolic stability [107, 134].

Table SIII.1 Primary structure of black soldier fly peptides. Their name, sequence, isoelectric point (pI), molar mass, purity and structural modification are mentioned below.

Peptide	Sequence	Purity (%)	Modification	pI	Molar Mass (g/mol)
Alo1-like					
Hill-Alo1	CINNGDGCQPDGRQGNCCSGYCHKEPGWVTGYCR	90.94	-	6.67	3679.04
Hill-Alo2	CIANGNGCQPDGRQGNCCSGFCYKQRGWVAGYCRRR	95.69	-	8.77	3985.5
Diptericin fragment					
Hill-Dip1	ASVPEPADLREAFETEDAIYIPISIEEANRLRLPR	92.77	-	4.04	3982.41
Hill-Dip2	SAPKLEEESALSYIPDAAEAEIAEPASSHGRVRR	96.53	-	3.37	3637.92
Hill-Dip3	DVEQVLETEDAYYVPVSDEEAEVLRLPR	93.08	-	3.37	3264.5
Hill-Dip4	SIDDLTLSEDGEDHVEIITDDEVQRAKR	93.25	-	3.7	3199.35
Diptericin					
Hill-Dip5	QLNIQGGGSPHSGFDLSVQGRAKIWESDNGRNTLYGTGQY-GQHLGGPYGNSEPSFGGGLMFSHRF	Crude	-	7.72	6928.42
Hill-Dip6	QIFAQGGGSPGKGYDIYAQGRAKLWESQNQRNSLHGTASY-SQHLGGPYGNSRPNVGGGLTFTHRF	90.00	-	10.28	6981.51
Knottin-like					
Hill-Knot1	IKCTASICTQICRILKYKCGYCASASRCVCLK	91.56	-	8.79	3531.37
Hill-Knot2	IKCVPSQCNQICRVLGKKCGYCKNASTCVCL	96.43	C-terminal amidation	8.81	3363.14
Hill-Knot3	RKCTASQCTRVCKKLGYKRGYCQSSTKCVC	90.22	-	9.8	3390.06
Defensin without SS[b]					
Hill-Def1	VTCDLLEPFLGPAPCMIHCIVRFRKRTGYCNSQNVCVCR	94.26	C-terminal amidation	8.41	4443.34
Hill-Def2a	ATCDLLSPFKVGHAACAAHCIARGKRGGWCDKRAVCNCRK	93.75	-	9.26	4275.04
Hill-Def3	ATCTNWNCRTQCIARGKRGGYCVERNICKCTS	93.97	-	8.76	3597.16

Table SIII.1 Primary structure of black soldier fly peptides

Peptide	Sequence	Purity (%)	Modification	pI	Molar Mass (g/mol)
Hill-Def4	ATCDLLSPFKVGHAACALHCIALGRRGGWCDGRAVCNCRR	92.58	-	8.43	4259
Hill-Def5	AMCDLLSGLNMGRSVCAMRCILKGHRGGWCDDQGVCNCRV	91.95	-	7.84	4330.15
Hill-Def6	LSCLFENQAVSAIACGASCITRKGKRGGWCSNGVCRCTPN	90.56	-	8.42	4162.82
Hill-Def7	TTCDLISGTKIENIACAAHCIAMGHKGGYCNSNLICICR	93.94	-	7.46	4099.82
Hill-Def8	QLPCDYLSGLGFGEDACNTDCIAKGHKSGFCTGLVCRCRTL	92.20	-	6.66	4353.99
Defensin with SS[b]					
Hill-Def2b	ATCDLLSPFKVGHAACAAHCIARGKRGGWCDKRAVCNCRK	99.10	SS-Bridges: 3-36; 16-30; 20-38	9.26	4275.04
Linear peptide					
Hill-Lin1	AIYHRSIRSPQFSGSSASANAQSSSGGRGGFGGSSSSSSANAETNSFGG	92.77	C-terminal amidation	10.92	4712.8
Hill-Lin2	AIYHRSIRSPQFSGSSASANAQSSSGGRGGFGGSSSSSSANAETNSFGG	Crude	C-terminal amidation	11.82	4712.8
Cecropin					
Hill-Cec1	SWWKKVFKPVEKLGQRVRDATIQGIGIAQQGANVLATVRGGPPQ	96.40	C-terminal amidation	11.93	4785.52
Hill-Cec2	GWWKRVFKPVEKFGQRVRDAGVQGIAIAQQGANVLATARGGPPQQ	90.49	C-terminal amidation	12.14	4873.54
Hill-Cec3	GWWKRVFKPVEKLGQRVRDAGIQGLEIAQQGANVLATARGGPPQQ	90.85	C-terminal amidation	11.75	4911.59
Hill-Cec4	GWWKRVFKPVERLGQRVRDAGIQGLQIAQQGANVLATVRGGPPQQ	90.12	C-terminal amidation	12.29	4966.67
Hill-Cec5	SWFKKVFKPVEKVGQRVRDAGIQGVAIAQQGANVLATARGGPPH	91.89	C-terminal amidation	11.93	4669.36

Table SIII.1 Primary structure of black soldier fly peptides

Peptide	Sequence	Purity (%)	Modification	pI	Molar Mass (g/mol)
Hill-Cec6	DQSKEPFKPMERPYQRVRDAKIQGITIAQNGPNVLAITRGGLEQ	90.87	C-terminal amidation	10.54	4950.6
Hill-Cec7	GWWKKVFKPVEKLGQRVRDAGIQGIAIAQQGANVLATVRGGPPQ	92.21	C-terminal amidation	11.93	4725.46
Hill-Cec8	GWWKRVFKPVEKFGQRVRDAGIQGIEIAQQGANVLATARGGPPQQ	94.74	C-terminal amidation	11.75	4945.6
Hill-Cec9	GWWKRVFKPVEKLGQRVRDAGIQGLEIAQQGANVLATVRGGPPQQ	91.69	C-terminal amidation	11.75	4939.64
Hill-Cec10	RWYKKIFKPVEKAVQRVRDGTLQALGVAQQAANVYATAQGAQQQRHA	92.13	C-terminal amidation	11.42	5279.98
Hill-Cec11	RSLWKKIFKPVEKLGQRVRDAGIQGIAIAQQGANVLATVRGGPPQ	94.37	C-terminal amidation	12.16	4852.65
Hill-Cec12	RSLWKKLFKPVERAGQRIRDATIKGIVIAQQGANVLATIRGGPAIPPGQ	91.34	C-terminal amidation	12.31	5277.19
Hill-Cec13	KSWWKKVFKPVERAGQRIRDASIKGLAVAQQGANVLSTIRGGPPVVAPPPQV	91.28	C-terminal amidation	12.17	5589.51
Stomoxyn-like					
Hill-Stom1	FNNLPICVEGLAGDIGSILLGVESDIGALAGAIANLALIAGECAAQGEAGAAICA	Crude	C-terminal amidation	3.27	5181.92
Hill-Stom2	TKAGPIVGNVLSIVGDAVAAIGIAIARAPAIVADVEYCATNVVATTVATLSKFGAAVKKCA	55.10	C-terminal amidation	9.4	5911.94

[a] The molecular weight (MW) and isoelectric point (pI) of the peptides were calculated using the online software at http://pepcalc.com/

[b] SS = disulfide bridges

Table SIII.2 Antimicrobial activity of black soldier fly peptide library. IC50 values, MIC values (minimum inhibitory concentration), and MBC values (minimum bactericidal concentrations) obtained in two individual screenings: the first screening starting at 64µM, the second screening starting at 32 µM. S = bacteriostatic activity.

		IC50 (µM)						MIC (µM)			MBC (µM)		
AMP	Repeat	MRC-5 SV2	*S. aureus*	*E. coli*	*P. aeruginosa*	*C. albicans*	*A. fumigatus*	*S. aureus*	*E. coli*	*P. aeruginosa*	*S. aureus*	*E. coli*	*P. aeruginosa*
Hill-Alo1	1	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	-	-	-
	2	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	-	-	-
Hill-Alo2	1	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	-	-	-
	2	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	-	-	-
Hill-Dip1	1	**32.00**	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	-	-	-
	2	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	-	-	-
Hill-Dip2	1	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	-	-	-
	2	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	-	-	-
Hill-Dip3	1	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	-	-	-
	2	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	-	-	-
Hill-Dip4	1	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	-	-	-
	2	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	-	-	-
Hill-Dip5	1	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 32.00	> 32.00	> 32.00	-	-	-
	2	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 64.00	> 64.00	> 64.00	-	-	-
Hill-Dip6	1	**4.54**	> 32.00	**0.85**	> 32.00	> 64.00	> 64.00	> 32.00	**2.00**	> 3200	-	S	-
	2	**7.73**	> 32.00	**1.00**	> 32.00	> 32.00	> 32.00	> 64.00	**2.00**	> 64.00	-	8,00	-

Table SIII.2 Antimicrobial activity of black soldier fly peptide library

		IC50 (µM)						MIC (µM)			MBC (µM)		
AMP	Repeat	MRC-5 SV2	*S. aureus*	*E. coli*	*P. aeruginosa*	*C. albicans*	*A. fumigatus*	*S. aureus*	*E. coli*	*P. aeruginosa*	*S. aureus*	*E. coli*	*P. aeruginosa*
Hill-Knot1	1	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	-	-	-
	2	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	-	-	-
Hill-Knot2	1	9.73	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	-	-	-
	2	3.76	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	-	-	-
Hill-Knot3	1	> 64.00	> 64.00	> 64.00	22.63	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	-	-	-
	2	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	-	-	-
Hill-Def1	1	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	-	-	-
	2	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	-	-	-
Hill-Def2a	1	> 64.00	< 0.25	35.33	32.86	> 64.00	> 64.00	0.25	> 64.00	> 64.00	S	-	-
	2	> 32.00	0.19	> 32.00	> 32.00	> 32.00	> 32.00	2.00	> 32.00	> 32.00	S	-	-
Hill-Def3	1	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	-	-	-
	2	> 32.00	> 32.00	> 32.00	> 3.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	-	-	-
Hill-Def4	1	> 64.00	0.50	> 64.00	32.00	> 64.00	> 64.00	1.00	> 64.00	> 64.00	S	-	-
	2	> 32.00	0.70	> 32.00	> 32.00	> 32.00	> 32.00	8.00	> 32.00	> 32.00	S	-	-
Hill-Def5	1	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	-	-	-
	2	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	-	-	-
Hill-Def6	1	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	-	-	-
	2	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	-	-	-

Table SIII.2 Antimicrobial activity of black soldier fly peptide library

		IC50 (µM)						MIC (µM)			MBC (µM)		
AMP	Repeat	MRC-5 SV2	*S. aureus*	*E. coli*	*P. aeruginosa*	*C. albicans*	*A. fumigatus*	*S. aureus*	*E. coli*	*P. aeruginosa*	*S. aureus*	*E. coli*	*P. aeruginosa*
Hill-Cec1	1	> 64.00	> 64.00	< 0.25	< 0.25	> 64.00	> 64.00	> 64.00	0.25	1.00	-	1.00	4.00
	2	> 32.00	> 32.00	0.21	0.20	> 32.00	> 32.00	> 32.00	0.50	0.50	-	0.50	0.50
Hill-Cec2	1	> 64.00	> 64.00	**0.50**	**0.93**	> 64.00	> 64.00	> 64.00	**1.00**	**4.00**	-	1.00	16.00
	2	> 32.00	**4.36**	**0.31**	**0.76**	> 32.00	> 32.00	> 32.00	**2.00**	**2.00**	-	2.00	2.00
Hill-Cec3	1	> 64.00	> 64.00	**0.49**	**0.40**	> 64.00	> 64.00	> 64.00	**1.00**	**4.00**	-	1.00	16.00
	2	> 32.00	> 32.00	**0.22**	**0.79**	> 32.00	> 32.00	> 32.00	**0.50**	**2.00**	-	32.00	2.00
Hill-Cec4	1	37.37	> 64.00	**0.50**	**<** **0.25**	> 64.00	> 64.00	> 64.00	**1.00**	**1.00**	-	S	16.00
	2	> 32.00	> 32.00	**0.32**	**0.22**	> 32.00	> 32.00	> 32.00	**2.00**	**2.00**	-	2.00	2.00
Hill-Cec5	1	> 64.00	> 64.00	**0.50**	**2.61**	> 64.00	> 64.00	> 64.00	**1.00**	**16.00**	-	S	S
	2	> 32.00	> 32.00	**0.23**	**3.60**	> 32.00	> 32.00	> 32.00	**0.50**	**32.00**	-	0.50	32.00
Hill-Cec6	1	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 32.00	> 32.00	-	-	-
	2	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 32.00	> 64.00	> 64.00	-	-	-
Hill-Cec7	1	> 64.00	> 64.00	**0.38**	**0.40**	> 64.00	> 64.00	> 64.00	**1.00**	**4.00**	-	1.00	16.00
	2	> 32.00	> 32.00	**0.32**	**0.78**	> 32.00	> 32.00	> 32.00	**2.00**	**2.00**	-	2.00	2.00
Hill-Cec8	1	> 64.00	> 64.00	**0.45**	**1.29**	> 64.00	> 64.00	> 64.00	**1.00**	**4.00**	-	1.00	64.00
	2	> 32.00	> 32.00	**0.68**	**1.73**	> 32.00	> 32.00	> 32.00	**2.00**	**8.00**	-	8.00	8.00

Table SIII.2 Antimicrobial activity of black soldier fly peptide library

		IC50 (μM)						MIC (μM)			MBC (μM)		
AMP	Repeat	MRC-5 SV2	*S. aureus*	*E. coli*	*P. aeruginosa*	*C. albicans*	*A. fumigatus*	*S. aureus*	*E. coli*	*P. aeruginosa*	*S. aureus*	*E. coli*	*P. aeruginosa*
Hill-Cec9	1	> 64.00	> 64.00	**0.43**	**0.38**	> 64.00	> 64.00	> 64.00	**1.00**	**1.00**	-	1.00	S
	2	> 32.00	> 32.00	**0.21**	**0.88**	> 32.00	> 32.00	> 32.00	**0.50**	**2.00**	-	0.50	2.00
Hill-Cec10	1	> 64.00	32.74	**< 0.25**	**< 0.25**	> 64.00	> 64.00	> 64.00	**0.25**	**1.00**	-	0.25	16.00
	2	> 32.00	> 32.00	**0.22**	**0.22**	> 32.00	> 32.00	> 32.00	**0.50**	**2.00**	-	32.00	2.00
Hill-Cec11	1	> 64.00	> 64.00	**< 0.25**	**< 0.25**	> 64.00	> 64.00	> 64.00	**1.00**	**1.00**	-	1.00	4.00
	2	> 32.00	> 32.00	**0.22**	**0.24**	> 32.00	> 32.00	> 32.00	**0.50**	**2.00**	-	32.00	8.00
Hill-Cec12	1	> 64.00	> 64.00	< 0.25	< 0.25	> 64.00	> 64.00	> 64.00	0.25	1.00	-	1.00	static
	2	19.45	> 32.00	0.32	0.23	> 32.00	> 32.00	> 32.00	2.00	2.00	-	8.00	8.00
Hill-Cec13	1	> 64.00	> 64.00	< 0.25	< 0.25	> 64.00	> 64.00	> 64.00	1.00	1.00	-	1.00	64.00
	2	> 32.00	> 32.00	0.23	0.25	> 32.00	> 32.00	> 32.00	0.50	2.00	-	0.50	2.00
Hill-Stom1	1	11.65	58.58	> 64.00	16.00	> 64.00	> 64.00	> 64.00	> 32.00	> 32.00	-	1.00	4.00
	2	24.42	> 32.00	> 32.00	17.67	> 32.00	> 32.00	> 32.00	> 64.00	> 64.00	-	0.50	0.50

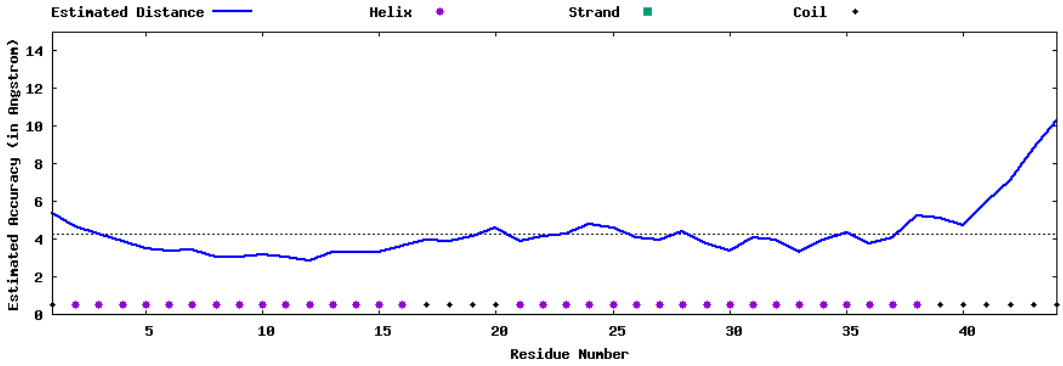
Table SIII.3 Time kill analysis results for HC1. Average log CFU/mL reductions caused by HC1 during the time kill experiments with standard deviations (SD). Samples were taken at 5 different time points at different peptide concentration levels.

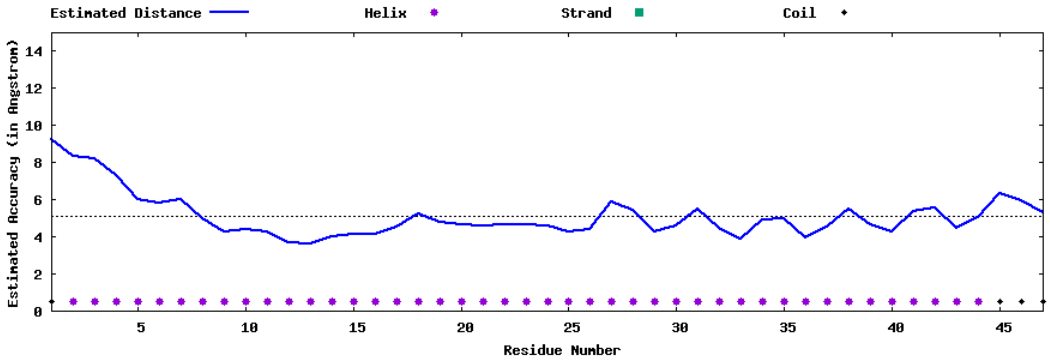
	Average log reduction ± SD compared to growth control (CFU/mL)							
Time (h)	1 µM		2 µM		4 µM		8 µM	
0.5	0.94 ±	0.54	2.37 ±	0.48	3.84 ±	0.75	4.30 ±	0.12
1	1.48 ±	0.93	2.38 ±	0.09	4.36 ±	0.23	4.36 ±	0.23
2	1.06 ±	0.87	1.92 ±	0.58	3.44 ±	0.78	4.61 ±	0.17
4	0.36 ±	0.12	0.82 ±	0.40	3.30 ±	1.43	5.00 ±	0.20
5	0.34 ±	0.16	0.75 ±	0.31	2.74 ±	1.94	5.24 ±	0.18

Table SIII.4 Time kill analysis results for HC10. Average log CFU/mL reductions caused by H10 during the time kill experiments with standard deviations (SD). Samples were taken at 5 different time points at different peptide concentration levels.

	Average log reduction ± SD compared to growth control (CFU/mL)							
Time (h)	1 µM		2 µM		4 µM		8 µM	
0.5	0.82 ±	0.48	1.78 ±	0.72	2.88 ±	1.11	3.90 ±	0.45
1	1.18 ±	1.11	1.16 ±	0.96	2.38 ±	1.25	3.41 ±	0.47
2	0.95 ±	0.94	1.01 ±	0.78	2.27 ±	1.56	3.27 ±	0.82
4	0.35 ±	0.14	0.57 ±	0.17	2.09 ±	1.90	3.21 ±	1.12
5	0.36 ±	0.22	0.56 ±	0.34	2.29 ±	2.24	2.71 ±	1.96

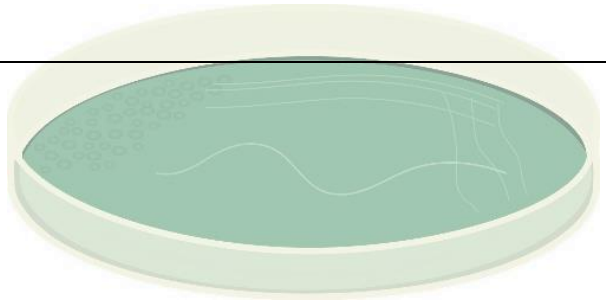
Figure SIII.1 Local accuracy estimation for the I-tasser prediction models for HC1 (top) and HC10 (bottom)





CHAPTER IV

In vitro anti-pseudomonal potential of HC1 and HC10



Biofilm experiments were published in:

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IV.1 Introduction

As the cecropins HC1 and HC10 showed good activity against *P. aeruginosa*, this chapter will first introduce this pathogen and its current treatment options more in detail. *P. aeruginosa* is a Gram-negative, non-fermenting, facultative aerobic and rod-shaped bacterium [251, 252]. It was first isolated in 1882 from a soldier's wound from which the bandage showed strong green-blue discoloration [251, 252]. *P. aeruginosa* is pervasive in various environmental niches such as water and soil, but also has high clinical relevance [253]. In 2019*, P. aeruginosa* was estimated to be responsible for 559.000 deaths globally, and related to 18.9 million lost life years [254]. As an opportunistic pathogen, *P. aeruginosa* mostly causes infection in immunocompromised patients, including burn wound patients, hospitalized elderly people, cancer patients, and those suffering from chronic lung diseases such as CF. [255]. *P. aeruginosa* can affect virtually all organs, but cystitis, skin infections, pneumonia, keratitis, and otitis media are most seen [253, 255]. In addition, *P. aeruginosa* is adept at colonizing abiotic surfaces, which can lead to ventilator-associated pneumonia (VAP) in intubated patients and catheter-related bloodstream infections [253]. *P. aeruginosa* is the pathogen most frequently associated with VAP infections, and higher VAP-related mortality is noted than for other causative bacteria [256]. Transmission of *P. aeruginosa* occurs mostly due to contact with an environmental or hospital reservoir, or contaminated material [251]. There is, however, increasing evidence of the importance of patient-to-patient transmission in CF specialized centers via infected cough droplets [257]. Most CF patients acquire the bacterium in childhood, and although *P. aeruginosa* prevalence in CF patients has been steadily decreasing the past decade, the bacterium is still associated with the majority of respiratory failure and mortality cases in CF [251, 258].

P. aeruginosa is a highly versatile bacterium with remarkable genetic plasticity, allowing the bacterium to easily adapt its metabolism when colonizing a new environment [259, 260]. Its accessory genes can make up to 20% of the total bacterial genome [260]. *P. aeruginosa* also contains a wide array of virulence factors and a striking intrinsic antibiotic

resistance [260, 261]. Mechanisms such as the inactivation of antibiotics, modification of drug targets, expression of efflux pumps, decrease of membrane permeability, biofilm formation and quorum-sensing, render the bacterium remarkably resilient to antibiotics [260, 261]. Biofilm formation is a hallmark of chronic *P. aeruginosa* infections, leading to long treatments and difficult pathogen eradication [253, 261]. Biofilms are clusters of bacterial cells encapsulated in a self-produced extracellular matrix [253]. Their formation follows a continuous cycle; after bacterial surface attachment, the biofilm proliferates and matures, after which certain cells disperse in order to colonize elsewhere [262]. Biofilm growth is tightly regulated by inter-cell communication quorum sensing systems [262]. This survival strategy allows *P. aeruginosa* to withstand external stress factors, including nutrient depletion and low oxygen levels [253]. Biofilms can grow in the human body (e.g. the lungs) during infection, or they can form on abiotic surfaces such as medical devices [262]. Antibiotic eradication of biofilms is challenging due to (**i**) decreased penetration of antimicrobials into the biofilm layers, and (**ii**) decreased antibiotic susceptibility in subpopulations of biofilm bacteria [263]. In the inner core of a biofilm, many bacteria will have a low metabolic rate, leaving multiple targets of antibiotics inactivated [263]. These bacteria are often said to be in an 'antibiotic persistent' state. Antibiotic persistence refers to the ability of a subpopulation of a bacterial cells to survive treatment with bactericidal concentrations of antibiotics [264]. Persistence results from a transient phenotypic switch of the bacterium, and not from the acquisition of AMR genes. Hence, when the antibiotic pressure is removed, the persistent fraction will give rise to a daughter population that is equally antibiotic susceptible as the original population (MIC remains the same) [264]. Bacterial persisters are heterogeneous in nature and can form spontaneously or be induced by external stress factors such as antibiotic pressure or nutrient starvation [13-14]. Persisters also occur in non-biofilm *P. aeruginosa* populations, and are thought to be an important cause of treatment failure and relapse [265]. Apart from biofilm formation and quorum sensing, other important virulence factors include the type II and III secretion systems, flagellins,

pyoverdine production, outer membrane proteins, and lipopolysaccharide (LPS) (see CHAPTER V) [255, 266].

For the first-line treatment of *P. aeruginosa* infections, one of the following antibiotics is often preferred: ciprofloxacin (a fluoroquinolone, Figure IV.1a), meropenem (a carbapenem, Figure IV.1b), aztreonam (a monobactam, Figure IV.1c), ceftazidime or cefepime (cephalosporins, Figure IV.1d), or piperacillin-tazobactam (a penicillin with a β-lactamase inhibitor, Figure IV.1e) [267]. Aminoglycosides (Figure IV.1f) are mostly used in combination therapy, while peptide antibiotics such as polymyxin B and colistin (Figure IV.1g) are reserved for multi-drug resistant isolates [267].

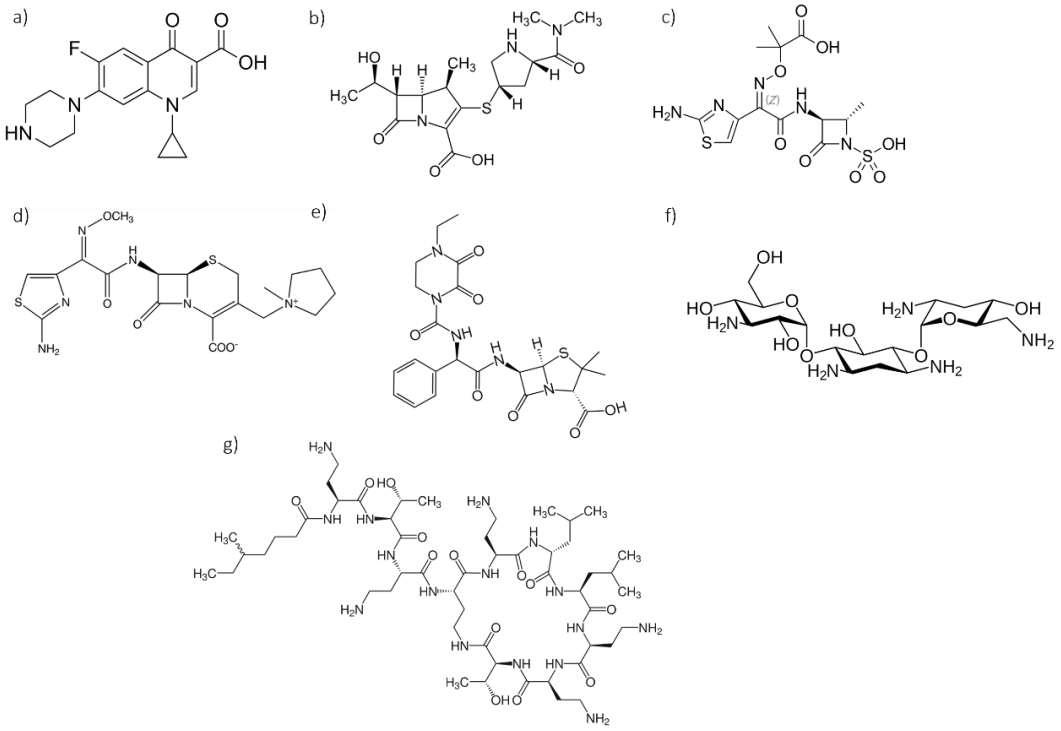


Figure IV.1 Molecular structures of antibiotics used in the treatment of *P. aeruginosa.* **a**) ciprofloxacin, **b**) meropenem, **c**) aztreonam, **d**) cefepime, **e**) piperacillin, **f**) tobramycin, and **g**) colistin.

Despite available antibiotics, the health care burden of *P. aeruginosa* remains high and new, innovative treatments or prevention measures are urgently needed [268]. The most recently approved antibiotic for *P. aeruginosa* is cefideracol, a cephalosporin with an iron-binding siderophore side chain [268, 269]. Currently, the clinical pipeline of anti-pseudomonal drugs is a mix of traditional and non-traditional antibiotics. For instance, many new combinations of β-lactam antibiotics and β-lactamase inhibitors are being investigated [268]. As for the non-traditional therapeutics, various bacteriolytic phage cocktails are currently in phase I clinical trials [268]. Anti-pseudomonal vaccines and antibodies, however, have mostly failed gaining approval due to not meeting the trial endpoints [268]. Within anti-virulence therapies, ftortiazinon, a type III secretion system inhibitor, in combination with cefepime is currently tested in phase II trials [268, 270]. Peptide antibiotics remain well represented in the clinical trials, including polymyxin-derivatives and AMPs. Although the AMP murepavadin was recently withdrawn from clinical trials due to nephrotoxicity, the WLBU2 AMP is under trial as anti-biofilm therapy [268, 271]. AMPs are often favoured for (i) lower emergence of resistance due to fast, membranolytic action, (ii) possible synergy with traditional antibiotics, (iii) their immunomodulatory activity, and (iv) activity against multi-drug resistant bacteria [272-274]. Of additional interest is the ability of some AMPs to eradicate biofilms or disrupt the biofilm formation process, and to kill persistent bacteria [275-277].

In this chapter, the anti-pseudomonal activity of HC1 and HC10 is further investigated. The focus is first laid on anti-biofilm activity (prevention and eradication). Then, anti-persister activity of HC1 and HC10 is studied. Specifically, the antimicrobial activity against *P. aeruginosa* bacteria that have survived treatment with a high dose of a bactericidal antibiotic (ciprofloxacin) is studied. Lastly, synergy of the cecropins with conventional antibiotics and resistance development in *P. aeruginosa* after AMP exposure are studied.

IV.2 Materials and methods

IV.2.1 Bacterial strains and culture conditions

P. aeruginosa 15442 and *P. aeruginosa* 15692 (PAO1) were obtained from the ATCC. *P. aeruginosa* LMG 27650 was obtained from the BCCM. Bacteria were cultured in MHB and plated on TSA.

IV.2.2 Inhibition of biofilm formation

To investigate the effect of the AMPs on biofilm formation, serially diluted peptides were added *in duplo* to 96-well plates, with final concentrations ranging from 64 to 0.5 µM. Next, a *P. aeruginosa* ATCC 15442 culture of approximately 10^6 CFU/mL was added. Plates were incubated for 16 h at 37 °C and shaking at 110 rpm (New Brunswick Innova 4300). Afterwards, viability of the bacterial cells in the biofilm was determined following an assay described previously by Gilbert-Girard et al. (2020) [278]. Briefly, the growth medium was removed from the well plates and the bacterial biofilms were washed once with PBS to remove remaining planktonic cells. Afterwards, 200 µL of a 20 µM resazurin solution was added to each well and the plates were incubated 4 h at 37 °C with agitation at 110 rpm (New Brunswick Innova 4300). The fluorescence of the wells was measured at $\lambda_{excitation}$ = 550 nm and $\lambda_{emission}$ = 590 using a microplate reader (Promega). Afterwards, a crystal violet staining was applied to determine the biomass of the biofilms [278]. The resazurin solution was removed from the well plates and 200 µL of 100% ethanol (VWR Chemicals) was added to fix the biofilms. After 15 min of incubation, the ethanol was discarded from the wells and the plates were left to air-dry for 30 min. Afterwards, 200 µL of a 0.023% (w/V) crystal violet (Merck) solution was added to the biofilms. The staining solution was removed after 5 min and the biofilms were washed with PBS. After air-drying for 10 min, the crystal violet stain was solubilized in 200 µL of 100% ethanol. Absorbance was measured at λ = 595 nm with a microplate reader (Promega) and, where possible, IC_{50} values were calculated. The experiment was carried out in biological triplicate. Polymyxin B sulfate (Sigma-Aldrich) was included as a reference peptide antibiotic.

IV.2.3 Biofilm eradication

In addition to biofilm formation, the effect of the AMPs on preformed biofilms was investigated to measure the amount of biofilm eradication. *P. aeruginosa* ATCC 15442 culture in MHB was added to 96-well plates to a concentration of 10^6 CFU/mL. After 16 h incubation shaking at 110 rpm, the medium was removed and 10 µL of serially diluted peptides were added *in duplo* to the plates at concentrations ranging from 128 µM to 0.5 µM (final concentration). Fresh MHB was added and the well plates were incubated again for 16 h at 37 °C and shaking at 110 rpm (New Brunswick Innova 4300). The next day, bacterial viability and biomass were determined with respectively a resazurin assay and crystal violet assay as described earlier. The experiment was carried out in biological triplicate. Polymyxin B sulfate was included as a reference peptide antibiotic.

IV.2.4 Isolation of *P. aeruginosa* antibiotic persisters

To validate the antibiotic dose and exposure time needed to obtain persistent *P. aeruginosa* POA1 bacteria, killing curve experiments were performed. An overnight stationary phase culture was treated with a high dose (200 µg/mL) of the bactericidal antibiotic ciprofloxacin HCl (= 100 x MIC; Sigma) as described elsewhere [279, 280]. Antibiotic-treated bacteria and a non-treated control were incubated at 37°C for 5 h. A killing curve was constructed by sampling the bacteria at regular time intervals and performing a viable plate count. Briefly, samples were taken at 30 min, 1 h, 2 h, 3.5 h, and 5 h post-treatment, centrifuged (4000 rpm), washed thrice in PBS, and enumerated on TSA. After overnight (16h) incubation (37 °C), colonies were counted and the fraction of surviving bacteria was determined for each timepoint. The killing dynamics (monophasic versus biphasic killing pattern) were analysed using goodness-of-fit statistics. For the 5 h timepoint, three colonies were randomly selected and regrown in fresh MHB overnight. To determine whether the antibiotic survival was indeed due to the presence of persistent bacteria and not due to antibiotic resistance, the ciprofloxacin treatment was repeated and killing patterns and survival fractions were compared to those obtained earlier. The experiment was carried out in biological triplicate.

IV.2.5 Antimicrobial activity against *P. aeruginosa* persisters

To obtain persistent *P. aeruginosa* bacteria, an overnight culture was treated with a high dose (200 µg/mL) of ciprofloxacin HCl for 3.5 h as described earlier (IV.2.4). To remove the antibiotic, the bacterial culture was centrifuged (Beckman Coulter Allegra X-12R, 4000 rpm) and washed thrice with PBS. Next, the bacterial culture was divided over Eppendorf tubes and treated with high, bactericidal concentrations of HC1 and HC10 (32 µM, 16 µM, and 8 µM). Bacteria were sampled at regular intervals (30 m, 1 h, 2 h, and 3.5 h), washed and enumerated on TSA to determine the viable plate count. A control of non AMP-treated persisters was include as well. The experiment was carried out in biological triplicate.

IV.2.6 Synergy of AMPs with conventional antibiotics

To test for synergy between HC1 and HC10 and other antimicrobials, a panel of eight commercially available antibiotics was chosen. Seven of these antibiotics are clinically used in the treatment of *P. aeruginosa*; these include polymyxin B (Sigma-Aldrich), ciprofloxacin (Sigma-Aldrich), tobramycin (TCI), doxycycline (Sigma-Aldrich), meropenem (tebu-bio), ceftazidime (tebu-bio), and cefepime (tebu-bio). Erythromycin (Sigma-Aldrich), a macrolide that has no activity against *P. aeruginosa*, was included in the test panel as well [281]. Synergy was tested for a sensitive laboratory strain (PAO1), and a multi-drug resistant isolate (LMG 27650). In a first step, the MIC values of the chosen antibiotics were determined against both *P. aeruginosa* strains, using a resazurin assay as described earlier in III.2.3. Next, synergy was tested using a modified checkerboard assay as described by He et al. (2015) [282]. In a 96-well plate, 2/3 serial dilutions of AMP (column 1-4) and antibiotic (column 5-8) were prepared in MHB from top to bottom, with an end volume of 100 µL (**Figure IV.4**). Final in-plate starting concentrations were equal to three times the earlier determined MIC. In the last four columns, a 2/3 serial dilution of the AMP and antibiotic combination was made, starting from 1.5 times their individual MIC values. Next, 100 µL of bacterial suspension in MHB was added to a final inoculum of 5 x 10^4 CFU/mL and plates were incubated overnight at 37 °C. After incubation, the

presence of clear and opaque wells was noted. Compound concentrations corresponding to the clear wells without bacterial growth were used for the calculation of the fractional inhibitory concentration (FIC). The FIC value is an indication of synergy and is given by the formula $FIC_{AB} = \frac{\mathbf{MIC_A^{Combo}}}{\mathbf{MIC_A}} + \frac{\mathbf{MIC_B^{Combo}}}{\mathbf{MIC_B}}$, where A is the AMP and B is the antibiotic, MIC_A and MIC_B are their individual minimum inhibitory concentrations, MIC_A^{Combo} refers to the MIC value of the AMP when added in combination with the antibiotic, and MIC_B^{Combo} is the MIC value of the antibiotic when acting in combination with the AMP [282]. Two biological replicates of the experiment were performed. FIC values are represented as the mean ± standard deviation of all technical and biological measurements. The combinatory activity of AMP and antibiotic was considered to be synergistic when FIC ≤ 0.5, additive when FIC was > 0.5 and ≤ 1, indifferent when FIC > 1 and ≤ 2, and antagonistic when FIC was > 2 [249, 283].

IV.2.7 Serial passage mutagenesis

A serial passage mutagenesis assay was used to study spontaneous induction of resistance in *P. aeruginosa* to HC1 and HC10 after daily passage under AMP pressure as described by Elliott et al. [284]. 1/2 serial dilutions of HC1, HC10, and ciprofloxacin HCl were prepared in triplicate in individual 96-well plates starting from 32 µM (AMPs) or 4 µM (ciprofloxacin HCl) in an end volume of 100 µL. Ciprofloxacin was included as a reference, as it is known to induce spontaneous AMR in *P. aeruginosa* within days [285]. Bacterial suspension of *P. aeruginosa* PAO1 in MHB was added to the well plates at a final volume of 200 µL and concentration of 5×10^4 CFU/mL. After overnight incubation (37°C), the IC_{50} value was determined using the earlier described resazurin protocol (III.2.3). Before addition of resazurin, 2 µL of the first non-inhibited well was inoculated into fresh MHB medium for each plate. This *P. aeruginosa* culture exposed to sub-MIC doses of either HC1, HC0 or ciprofloxacin was used as the inoculum for the next screening passage. For each compound and each cycle, a control screening with a standard PAO1 culture was performed as well to obtain reference MIC values (as described in III.2.3). The experiment was repeated for 20 cycles in total. Back-up cryo stocks were made for

each cycle, and bacterial inoculi were regularly plated on TSA to check for contamination. Each day, the average IC_{50} value was calculated, and the fold increase compared to the control MIC was calculated (IC_{50} day_b/IC_{50} day_a).

IV.2.8 Statistical analysis

For the biofilm experiments, a non-parametric Kruskal Wallis test with Dunnett's T3 multiple comparison's was used to compare the means of the treated groups to the mean of the untreated control (data had equal variances but were not normally distributed). For the antibiotic persister experiments, the goodness of fit of a monophasic (one killing rate) and biphasic (two killing rates) curve were compared to determine which is most probable to describe the experimental dataset, using the Aikake information criterion. The biphasic model is described by the equation $\log(Y) = \log[(N - P_0)^{(-kn \times t)} + P_0^{(-kp \times t)}]$ and the monophasic model by $\log(Y) = \log([(N)^{(-kn \times t)}]$ where Y is the surviving fraction of bacteria, t is the treatment duration (in h), P_0 describes persister fraction at timepoint 0, and kn and kp define the the killing rates of normal and persistent bacteria [286]. A parametric one-way ANOVA test was used to test for statistical difference in the anti-persister assay (both between different concentrations of the same AMP, as well as between AMPs at equal concentrations and timepoints). For the analysis of synergy, obtained FIC values were compared with a non-parametric Kruskal Wallis test (equal variances, but no normal distribution of data). All analysis were made in Graphpad 9.5.1.

IV.3 Results

IV.3.1 Anti-biofilm activity of HC1 and HC10

As biofilm formation of *P. aeruginosa* strains contributes to their tolerance to antibiotics, the effect of the cecropin AMPs on **biofilm formation** was investigated [287]. *P. aeruginosa* ATCC 15442 was used since it showed less variability in biofilm formation in our assay compared to *P. aeruginosa* PAO1, as observed in preliminary experiments (data not shown). Both peptides exhibited a concentration-dependent inhibition of biofilm

formation. For HC1, a statistically significant decrease in biofilm mass ($p < 0.05$) and viability ($p < 0.001$) at sub-MIC concentrations (0.5 µM) was noted compared to the untreated control. HC1 achieved 50% reduction in biofilm mass at a concentration close to its MIC (IC_{50} of 1.3 ± 0.57 µM), and 50% reduction of biofilm viability at supra-MIC concentrations (IC_{50} of 2.1 ± 0.52 µM) (**Figure IV.2a, 2b**). For HC10, however, higher concentrations were needed to obtain 50% reduction or more of biofilm mass (IC_{50} of 7.5 ± 3.5 µM) or biofilm viability (IC_{50} of 11 ± 1.7 µM) (**Figure IV.2a, 2b**). The difference in bactericidal concentrations between both cecropins could explain their difference in inhibition of biofilm formation. As for **eradication** of preformed biofilms, both HC1 and HC10 were not successful at the tested concentrations. Only marginal reductions of biofilm mass were seen for HC1 (between 9% and 23%) and HC10 (between 10% and 23%), and the decrease was not concentration-dependent (**Figure IV.2c**). Additionally, no significant effect on biofilm viability compared to the untreated control was detected (**Figure IV.2d**). In comparison, the reference polymyxin B showed over 70% reduction in viability at concentrations of 64 µM and 128 µM.

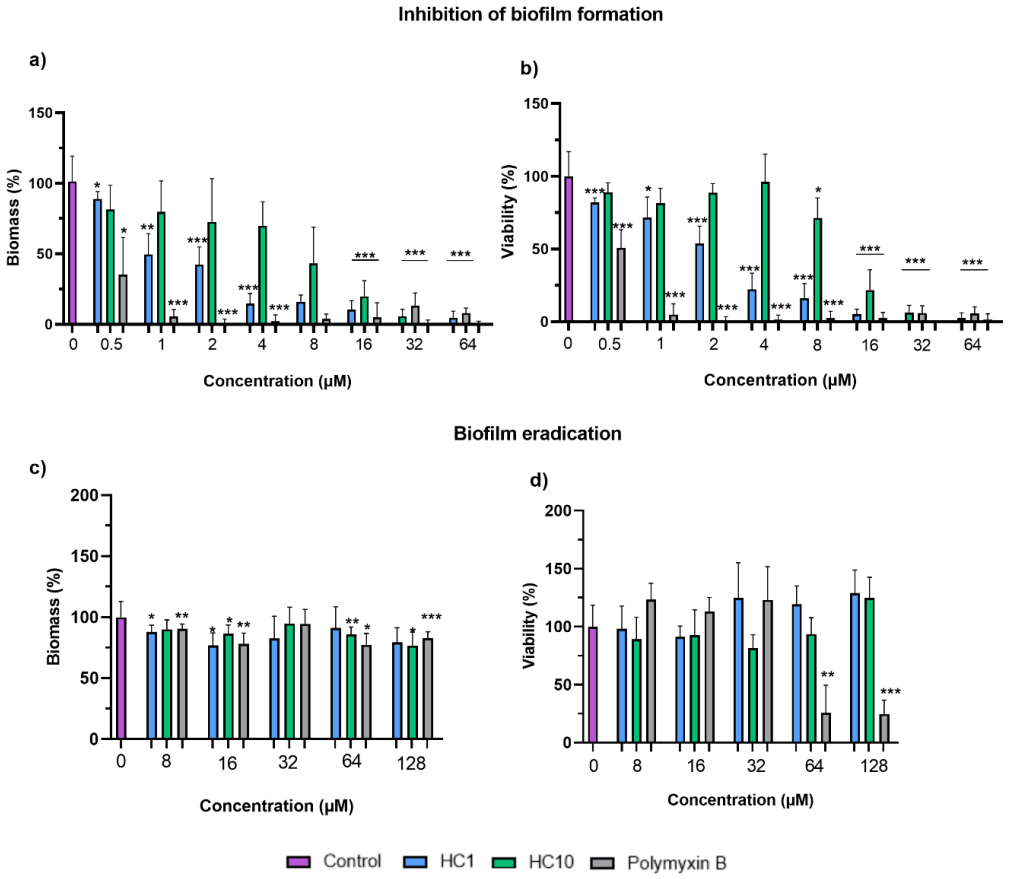


Figure IV.2 Anti-biofilm activity of cecropins HC1 and HC10 against *P. aeruginosa* ATCC 15442. a) Effect of HC1 and HC10 on *P. aeruginosa* biofilm formation as measured by biofilm mass. **b**) Effect of HC1 and HC10 on *P. aeruginosa* biofilm formation as measured by biofilm viability. **c)** Effect of HC1 and HC10 on the eradication of a preformed *P. aeruginosa* biofilm as measured by biofilm mass. **d)** Effect of HC1 and HC10 on the eradication of a preformed *P. aeruginosa* biofilm as measured by biofilm viability. Bars represent the mean ± standard deviation of three independent experiments. Treated groups were compared with the non-treated control with a Kruskal Wallis test .* = $p < 0.5$, ** = $p < 0.1$, and *** = $p < 0.001$.

IV.3.2 *P. aeruginosa* antibiotic persistence

As stated earlier, antibiotic persistence refers to the ability of a subpopulation of bacteria to survive lethal concentrations of otherwise bactericidal antibiotics, without being genetically resistant [264, 288]. Two important hallmarks of antibiotic persistence include: **i**) a biphasic killing pattern when exposed to a bactericidal antibiotic, as the persistent population is killed at a much slower rate than the antibiotic sensitive cells. **ii**)

When regrowing the antibiotic persistent bacteria, they give rise to a culture that is as susceptible to the antibiotic as the parent population [264]. Analysis of the time-kill curve of stationary *P. aeruginosa* PAO1 bacteria after exposure to a high dose of ciprofloxacin showed a high probability (> 99.99%) of a biphasic killing curve compared to monophasic killing (Figure IV.3). At 5 h after treatment, the persistent fraction accounted for 0.019 ± 0.005 % of the original bacterial count. Although persister fractions of stationary phase cultures can be as high as 1% of the total population, these levels are within a normal range of typical persister counts and are in accordance with other studies on *P. aeruginosa* persisters [280, 289, 290]. After repetition of the experiment with antibiotic-treated bacteria, the same killing pattern was observed (data not shown), indicative of antibiotic persistence. For the following experiments, the timepoint 3.5 h was chosen as all sensitive bacteria were killed at this moment.

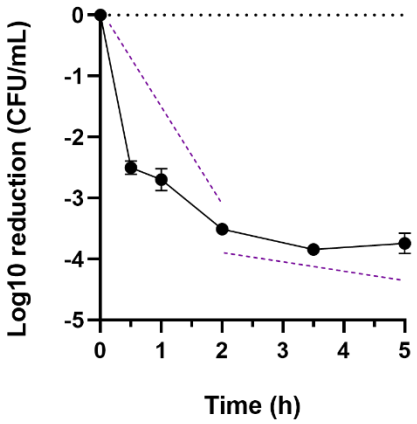


Figure IV.3 Killing curve of *P. aeruginosa* PAO1 exposed to a high dose (200 µg/mL) of ciprofloxacin. The log reduction in bacteria was examined over a period of 5 h. After 2 h, the killing rate slowed and the remaining fraction of bacteria is presumed to be antibiotic persistent. The overall killing curve is biphasic, indicative of the presence of antibiotic persistent bacteria.

IV.3.3 Anti-persister activity of HC1 and HC10

After treating a stationary phase *P. aeruginosa* culture with a high dose of ciprofloxacin to kill all antibiotic sensitive cells, the remaining persistent bacteria were additionally

treated with bactericidal concentrations of AMPs (8, 16, and 32 µM). Both HC1 and HC10 are able to reduce the bacterial count with an extra 2-3 log after a treatment of 3.5 h, indicating activity against antibiotic persistent bacteria (**Figure IV.4**). No statistical difference between the different AMP concentrations was noted ($p > 0.5$, One-way ANOVA), suggesting that a high, bactericidal dose of AMP is sufficient to obtain the anti-persister killing activity in this experimental set-up. Additionally, no difference between the activity of HC1 and HC10 was found ($p > 0.5$, One-way ANOVA). Although HC1 and HC10 show no synergy with ciprofloxacin (see IV.3.4), it cannot be ruled out that an additive effect of the AMPs and the fluoroquinolone is at play, as even washing the culture thrice may not have removed all ciprofloxacin present. After 3.5 h, about 10^2 CFU of bacteria remain, which indicates that the applied conditions (time and AMP dose) did not manage to eradicate the bacterial culture completely. Important to note, however, is that dead bacteria were not removed from the ciprofloxacin-treated culture. As the AMPs are likely to bind to membrane material, including that of dead bacteria, this might lead to confounding effects, complicating the estimation of anti-persister activity at a given AMP concentration.

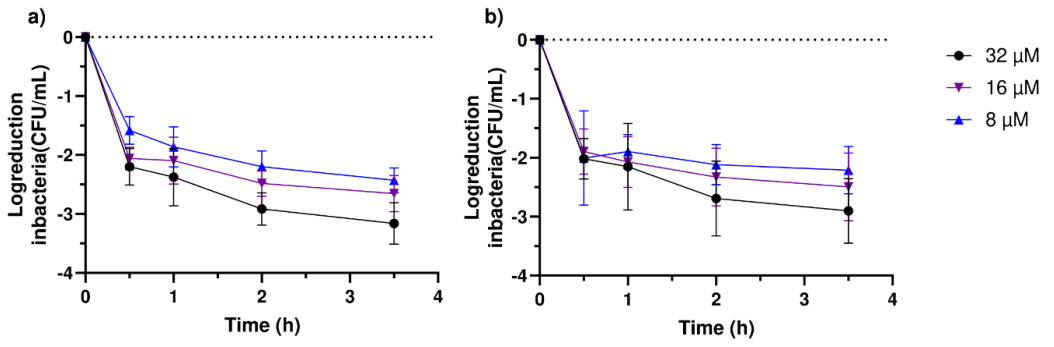


Figure IV.4 Activity of HC1 (a) and HC10 (b) against *P. aeruginosa* PAO1 antibiotic persisters. After selection of persistent bacteria with a treatment of a high dose of ciprofloxacin, the bacterial cultures were treated with 8 µM, 16 µM or 32 µM of the AMPs. Bacterial killing was followed for an additional 3.5 h and expressed as a log reduction in viable count compared to the ciprofloxacin-treated culture. No statistical difference ($p > 0.05$) was found between HC1 and HC10 (at equal concentrations and timepoints) as well as between different concentrations (8, 16, and 32 µM) of the same peptide (One-way ANOVA).

IV.3.4 Activity of antibiotic panel against selected *P. aeruginosa* strains

Before investigating the presence of synergy, the activity of the antibiotic panel was tested against a normal, sensitive lab strain (PAO1) and a multi-drug resistant clinical isolate (LMG 27650). The range of MIC values obtained from two biological independent screening assays, both with two technical replicates, are presented in **Table IV.1**. As expected, *P. aeruginosa* PAO1 was susceptible to all antibiotics except erythromycin. *P. aeruginosa* has an intrinsic resistance towards macrolide antibiotics due to the presence of multi-drug efflux pumps [291]. The LMG 27650 strain showed resistance towards all antibiotics for which the European Committee for Antimicrobial Susceptibility Testing (EUCAST) has reported on a breakpoint MIC value [292].

Table IV.1 Antimicrobial activity of a panel of commercially available antibiotics against two strains of *P. aeruginosa*. The minimum inhibitory concentration (MIC) represents the lowest tested concentration at which no bacterial growth was noted. The MIC range of two biological repeats, each with two technical repeats, is given. The MIC breakpoint indicates the minimum inhibitory concentration at which the strain is resistant towards the antibiotic according to the EUCAST guidelines. PAO1 is a sensitive laboratory strain, while LMG 27650 is a multidrug resistant *P. aeruginosa* strain.

Antibiotic	Class	MIC (range, µM)		MIC breakpoint (µM)
		PAO1	LMG 27650	
Polymyxin B	Peptide antibiotic	0.5 - 1	0.5	3.5*
Ciprofloxacin	Fluoroquinolone	0.0625 – 0.125	32	> 1.5
Tobramycin	Aminoglycoside	0.5	128	> 4.3
Doxycycline	Tetracycline	4	8	-
Meropenem	Carbapenem	2	64	> 21
Ceftazidime	Cephalosporin	2 - 8	128	> 15
Cefepime	Cephalosporin	1	32	> 17
Erythromycin	Macrolide	256 – 512	256	-

*MIC breakpoint for colistin (polymyxin E) is given

IV.3.5 Synergy of HC1 and HC10 with conventional antibiotics

Synergistic action between AMPs and conventional antibiotics has been reported multiple times in recent literature [293-296]. The checkerboard assay remains a hallmark of synergy testing and is still routinely used in research on AMP-antibiotic interactions (Figure IV.5a). However, in 2015 He and colleagues proposed a different screening set-up to obtain a more robust quantitation of synergy [282]. The traditional checkerboard assay has several drawbacks. For example, the standard set-up does not include in-plate

MIC measurement. For the FIC calculations, the checkerboard assay relies on MIC values measured independently in another assay. Experimental variation of the activity is especially common for AMPs and makes the FIC calculation for the checkerboard assay less reliable [282]. Moreover, in a checkerboard assay each well contains a unique combination of compound concentrations. The lack of technical replicates leads to statistical weak data that are highly sensitive to errors and difficult to interpret [282]. Hence, to determine the synergy of the cecropin AMPs with the panel of antibiotics, the modified version of the assay proposed by He et al. was used (Figure IV.5b). In the modified version, in-plate MIC measurement largely cancels out variation in FIC calculations due to experimental variation in antimicrobial activity. Moreover, four technical replicates for synergy measurement are present per plate, increasing statistical power and robustness. To do so, the Interaction type of AMP and antibiotic are only investigated at fixed, equal MIC ratios, where synergistic action is most likely to occur [282]. In addition, 2/3 serial dilutions were performed instead of the standard ½ dilutions, for more accurate MIC and FIC measurement.

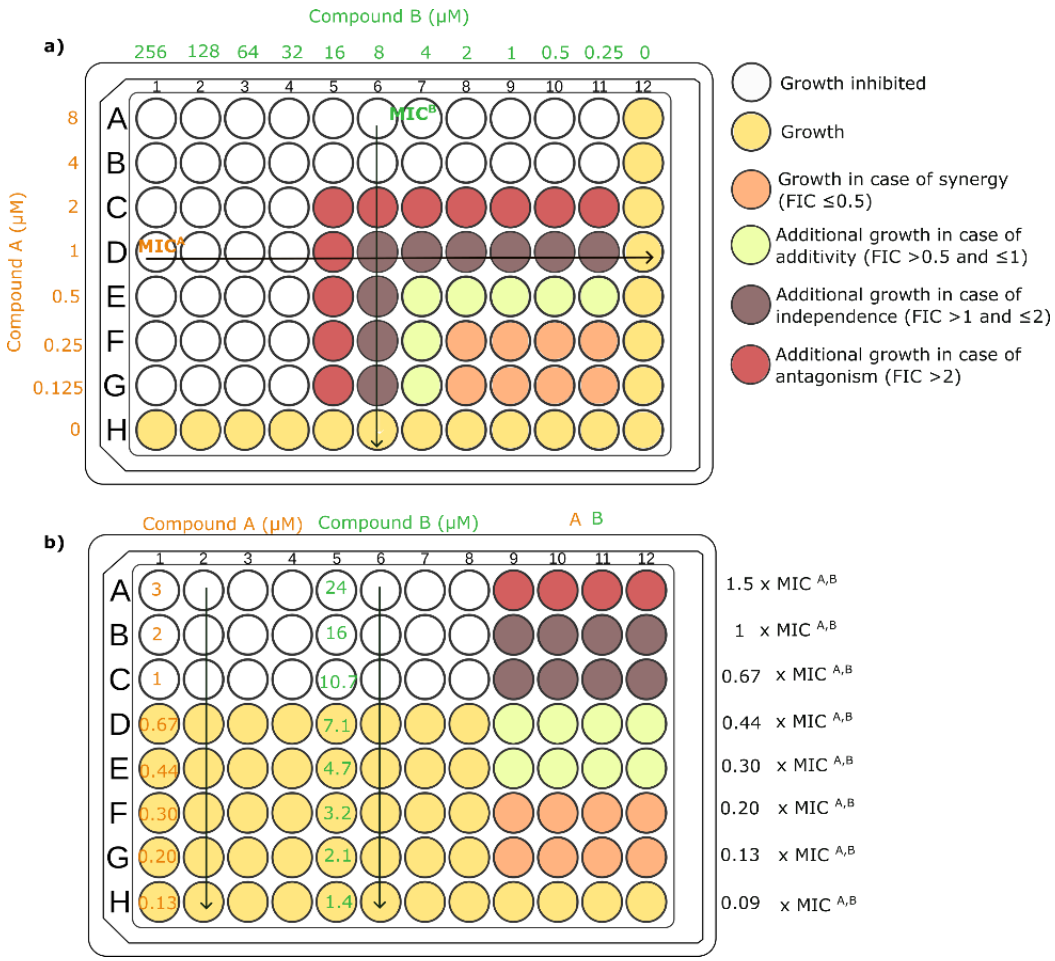


Figure IV.5 Visual comparison of two synergy assays. a) The checkerboard assay remains the golden standard for synergy testing. Two compounds are serially diluted horizontally (compound A) or vertically (compound B) in a 96-well plate. Each well represents a unique combination of the MIC ratios of both compounds. Experimental variation in a checkerboard assay often leads to difficulties in interpretation of synergy results. **b**) The modified synergy assay as proposed by He et al. that is used in this thesis. Synergy is only tested at compound concentrations at equal MIC ratios. Eight columns are reserved for in-plate MIC measurement, and a 2/3 serial dilution is used for more accurate MIC and FIC measurement.

When adhering to the EUCAST guidelines for synergy (FIC ≤ 0.5), no combination of AMP and antibiotic shows synergistic action on any of the two tested strains (**Table IV.2**). However, the authors of the applied modified synergy assay propose a less conservative cut off (FIC ≤1) [282]. According to these less stringent guidelines, still only mild synergy

at best is noted for the AMPs with erythromycin and doxycycline. Both antibiotics inhibit protein synthesis by binding to ribosomal subunits and are targeted by multidrug efflux pumps produced by *P. aeruginosa* [291, 297]. As these efflux pumps significantly decrease the cell entry of these antibiotics, the membrane permeabilizing action of the AMPs could possibly increase the antibiotic's cell uptake [298, 299]. While erythromycin is not used in clinical practice due to its low anti-pseudomonal activity, doxycycline is used occasionally for the treatment of non-systemic cystitis [300]. For all other antibiotics, combinatory action with AMPs is mostly either additive or independent. No antagonism was seen for any of the tested combinations. Additionally, no statistical difference ($p > 0.5$) was seen between the FIC values obtained for PAO1 and those noted for LMG 27650, or between the FIC values recorded for HC1 and HC10.

Table IV.2 Synergy of HC1 and HC10 with a panel of commercially available antibiotics. The fractional inhibitory concentration (FIC) is an indication of the type of interaction between AMP and antibiotic. Synergy is given by a FIC ≤ 0.5, additive interaction by a FIC > 0.5 and ≤ 1, indifferent action when FIC > 1 and ≤2, and antagonism when FIC is> 2. The mean FIC ± standard deviation of all biological and technical repeats is given. PAO1 is a sensitive laboratory strain, while LMG 27650 is a multi-drug resistant *P. aeruginosa* strain. SD = standard deviation

	PAO1		LMG 27650	
	Average FIC ± SD		Average FIC ± SD	
	HC1	HC10	HC1	HC10
Polymyxin B	1.44 ± 0.45	1.01 ± 0.18	0.83 ± 0.17	0.77 ± 0.19
Ciprofloxacin	1.07 ± 0.15	1.18 ± 0.075	1.05 ± 0.050	0.97 ± 0.00
Tobramycine	1.39 ± 0.26	1.23 ± 0.21	1.80 ± 0.25	2.25 ± 0.30
Doxycycline	0.75 ± 0.12	0.92 ± 0.22	0.57 ± 0.13	0.70 ± 0.10
Meropenem	1.16 ± 0.16	1.18 ± 0.22	0.91 ± 0.14	0.94 ± 0.16
Ceftazidime	1.05 ± 0.11	1.23 ± 0.30	0.87 ± 0.070	1.10 ± 0.16
Cefepime	1.37 ± 0.34	1.09 ± 0.23	0.94 ± 0.18	0.95 ± 0.17
Erythromycin	0.75 ± 0.12	0.80 ± 0.15	0.76 ± 0.015	0.66 ± 0.14

IV.3.6 Resistance development

Apart from the acquisition of resistance genes through contact with other bacteria (horizontal gene transfer), bacteria can gain increased AMR through spontaneous gene mutations [301]. The latter can be investigated by exposing *P. aeruginosa* continuously to sub-MIC doses of antimicrobial compounds and tracking changes in MIC values over time. For ciprofloxacin HCl, first decrease in susceptibility of PAO1 was noted at day 4

(5.9 ± 0.6 fold increase in IC_{50}). At day 14, the IC_{50} value increased sharply again with a factor of 13.1 ± 4.4 (**Figure IV.6**). At this point, the EUCAST MIC resistance breakpoint was also crossed. Similar findings were reported earlier in literature [285]. For HC1 and HC10, however, the IC_{50} stayed close to their baseline values during the course of the experiment, indicating absence of spontaneous resistance induction for at least 20 days of exposure (**Figure IV.6**).

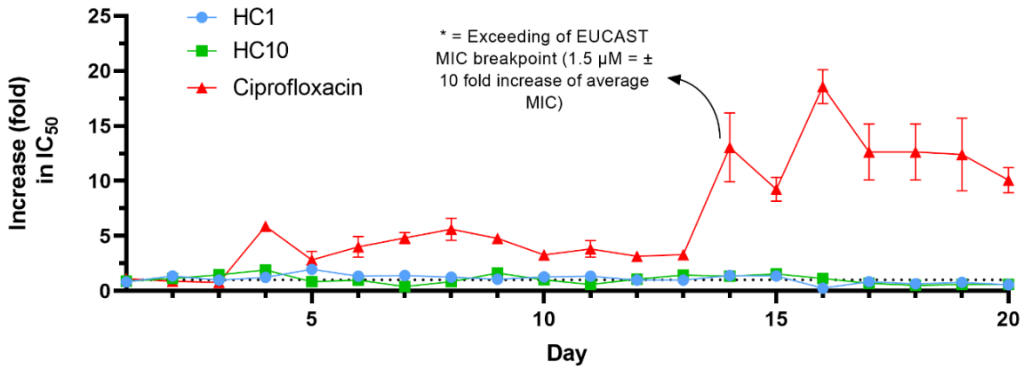


Figure IV.6 Induction of antimicrobial resistance in *P. aeruginosa* PAO1. *P. aeruginosa* PAO1 was continuously exposed to sub-inhibitory doses of HC1, HC10 and ciprofloxacin (reference) for 20 cycles. For ciprofloxacin, notable increases in IC_{50} value were seen at day 4 and day 14. For the cecropin AMPs HC1 and HC10, susceptibility of *P. aeruginosa* remained the same throughout the 20 day period.

IV.4 Discussion

P. aeruginosa continues to be a scourge for immunocompromised people, ranging from burn wound patients to CF patients and hospitalized people [229, 230]. To decrease its impact on global mortality and morbidity, finding new efficient treatments remains a priority, especially due to rising circulation of drug-resistant strains. In this chapter, the anti-pseudomonal activity of the BSF AMPs HC1 and HC10 was investigated. The peptide characteristics and activities that are often reported as therapeutic advantages were of specific interest, including anti-biofilm and anti-persister activity, synergy with existing antibiotics, and lack of resistance development.

IV.4.1 AMPs to combat biofilm and persistent infections

Firstly, anti-biofilm activity of the cecropins was studied. Biofilm formation of *P. aeruginosa* can lead to persistent infections that respond poorly to antibiotic treatment. The biofilm matrix can slow antibiotic penetration, while sessile bacteria enter into a lower metabolic state characterized by a decreased susceptibility to antimicrobials [287]. Two strategies to combat biofilms are (**i**) the prevention of biofilm formation by either inhibiting bacterial adhesion, preventing inter-cell communication, or by reducing initial bacterial growth, and (**ii**) the eradication of mature biofilms [302, 303]. Both strategies were investigated within this study. HC1 and HC10 showed a significant decrease in biofilm mass and viability when they were added immediately to planktonic *P. aeruginosa* bacteria. Bactericidal concentrations of the cecropins were able to obtain 75% or more reduction of biofilm formation, indicating that most of the inhibitory action of the peptides is directly linked to their activity on the planktonic bacteria. Cecropins with inhibitory activity can be useful to prevent biofilms after surgery or as a coating on medical devices [304-306]. When the peptides were added to 16-h mature biofilms, no significant effect on biofilm viability was observed at concentrations up to 128 µM. Possibly, their high positive charge leads to electrostatic interactions with anionic matrix components such as alginate, causing them to be trapped [303, 307]. Thus, HC1 and HC10 do not seem to exhibit the same promising biofilm eradication activities as reported

for some other cecropins [308, 309]. A possible strategy to circumvent matrix interactions is the encapsulation of AMPs in nanocarriers [303]. In addition, the combinatorial activity of AMPs and compounds that destabilize or breakdown the biofilm matrix (e.g. EDTA, DNase, alginate lyase,…) could be studied [310].

Related to anti-biofilm activity, anti-persister activity was investigated. Antibiotic persistence refers to the ability of a subpopulation of bacteria to survive lethal concentrations of bactericidal antibiotics [264, 288]. In contrast to resistance, antibiotic persistence is not directly inheritable to daughter populations of a persistent cell, but rather results from a transient phenotypic switch. Although the nature of persistent bacteria is heterogeneous and persister cells can show varying degrees of metabolic activity, metabolic inactivity and a non-dividing state are often seen [289]. Antibiotic persistence can occur stochastically in a bacterial population as a surviving strategy, but can also be induced by external stress factors such as antibiotic pressure or nutrient starvation [289, 311]. Hence, bacterial populations at the core of a biofilm, where nutrients and oxygen are low, are often deemed to be in a persistent state. For *P. aeruginosa*, antibiotic persistence has previously been linked to chronic infections and biofilm-mediated recurrent infections [312]. As AMPs mostly work independently from bacterial metabolism through membrane disruption, they are often regarded as a potential anti-persister strategy [275, 277]. HC1 and HC10 both showed clear antimicrobial activity against *P. aeruginosa* persister cells, which were selected by first treating a culture with a high (100 x MIC) dose of the bactericidal antibiotic ciprofloxacin. The killing dynamics of HC1 and HC10 on persistent *P. aeruginosa* were characterized by a fast, sharp decrease (± 2 $\log_{10}$ reduction) in bacteria within the first 30 min, followed by a slowed killing phase the following hours of exposure. This biphasic killing pattern is indicative of antibiotic persistence itself, suggesting the existence of a small (< 0.00001 % of starting count) 'super-persistent' subpopulation that also withstands fast killing by AMPs. Overall, the bacterial count of the original *P. aeruginosa* population (± 1.8 x 10^9 CFU/mL) was first reduced by ciprofloxacin to a concentration of approximately 2.1 x 10^5

CFU/mL, and then lowered to ± 1.4 x 10^2 CFU/mL (HC1) or ± 3.7 x 10^2 CFU/mL (HC10) by the addition of 32 µM of AMP. To investigate the anti-persister activity in more detail, experiments could be repeated at higher AMP concentrations and longer treatment times. In addition persistent bacteria could be removed first to avoid any confounding effects by dead bacteria present in the culture (e.g. by bead purification) [313]. Nevertheless, it is clear that HC1 and HC10 do exhibit significant activity against persistent *P. aeruginosa* bacteria, which could be an asset when treating chronic, recurrent infections. Other studies have also highlighted anti-persister activity of AMPs, including activity of cecropin-4 fragments against MRSA persisters [314]. To the best of our knowledge, however, this is the first study detailing the anti-persister activity of cecropin AMPs for a Gram-negative bacterium. In addition, these results confirm that matrix interactions are probably at play in the absence of biofilm eradication, instead of a lack of activity against the biofilm bacteria themselves.

IV.4.2 Lack of synergy for HC1 and HC10

Antimicrobial synergy of HC1 and HC10 was tested for a panel of conventional antibiotics used in clinical treatment of *P. aeruginosa*. Contrary to what is reported for many other α-helical peptides, no clear synergy was reported for the BSF AMPs. Only the combinatorial activity of HC1 and HC10 with erythromycin and doxycycline was (depending on the chosen cut-off values) mildly synergistic. Both antibiotics are targeted by common drug efflux pumps on the bacterium's membrane, and increased activity could be explained by the heightened membrane permeability upon activity of the peptides [298, 299]. A possible reason why these findings are not in line with the general expectations of synergy for AMPs, is the lack of a standardized, robust assay for AMP synergy. A thorough comparative analysis of He et al. (2015) exposed various issues related to the commonly used checkerboard assay [282]. This assay specifically lacks internal MIC controls and technical replicates. As activity of AMPs specifically is prone to inter-experimental variability, this significantly complicates the analysis of the checkerboard results. Moreover, there is no uniform consensus on how the average FIC

value should be calculated for checkerboard assays [282, 315]. He et al. concluded that synergy is often overestimated when performing checkerboard assays, proposing instead a modified alternative. In addition, membrane permeabilization of AMPs does not immediately lead to synergistic action [282]. Nevertheless, the applied testing method has only investigated synergy at a fixed ratio of HC1/HC10 and antibiotic. For a more in-depth examination of synergy, preliminary checkerboard assays could be used to establish the optimum concentration ratios of the antimicrobials, before performing the modified synergy assay, as advised recently by Howell et al. (2022) [315]. Apart from synergy in regular antimicrobial activity assays, anti-biofilm synergy could be studied as well. Polymyxin B, for example, was found to be synergistic with doxycycline in the eradication of *P. aeruginosa* biofilms [316] .

IV.4.3 Free from resistance development?

Lastly, spontaneous induction of resistance by the AMPs in *P. aeruginosa* was investigated by a serial passage mutagenesis assay. Apart from horizontal gene transfer, spontaneous mutagenesis due to antibiotic exposure is an import driving factor for development of resistance [317]. Continuous exposure of bacteria to sub-MIC concentrations is often seen in chronic infections. For ciprofloxacin, it has been well studied that serial passage under sub-MIC concentrations leads to fast acquisition of mutations conferring fluoroquinolone resistance, including genes encoding the antibiotic target *gyrA* and *gyrB*, and genes involved in efflux pump control *mexZ*, *mexR*, *mexS* and *nalC* [317, 318]. AMPs are often associated with a reduced risk of resistance emergence, mostly due to their fast membranolytic action, leaving only a small time window for the bacteria to acquire resistance strategies. In addition, their ability to work on multiple targets and their (often) aspecific membrane-binding makes it challenging for bacteria to become fully resistant [319, 320]. Indeed, HC1 and HC10 did not induce spontaneous resistance after 20 day passage at sub-MIC concentrations, in contrast to ciprofloxacin. Nevertheless, resistance of *P. aeruginosa* against cationic peptides has been reported, mostly by mutations in the arnBCADTEF (LPS modification) operon, which in turn is regulated by several two-

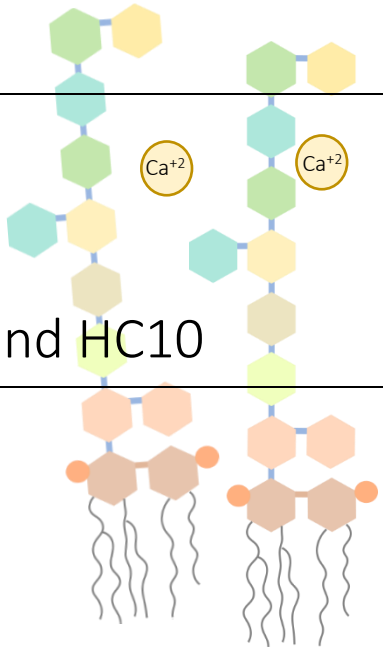
component systems (e.g. PmrB-PmrA) [321, 322]. The addition of 4-amino-l-arabinose to LPS, for example, decreases its affinity for AMPs (CHAPTER V) [321]. Apart from cell wall and membrane modifications, bacteria can acquire AMP resistance through stimulated protease secretion, efflux pump upregulation, and AMP trapping through increased outer membrane vesicle production [323]. Even though AMPs are not completely safe from resistance development over time, the absence of fast spontaneous mutation after HC1 and HC10 exposure is an asset when developing new anti-pseudomonal compounds.

IV.4.4 Concluding remarks

In this chapter, the specific anti-pseudomonal characteristics of HC1 and HC10 were investigated, with a focus on anti-biofilm and anti-persister activity, synergy, and resistance development. The BSF AMPs showed mixed results in their potential as anti-pseudomonal drugs; while anti-persister activity and biofilm prevention was noted, biofilm eradication was not, presumably due to matrix interactions. In addition, synergy with conventional antibiotics was not strongly present. Although development of resistance was not noted in *P. aeruginosa*, it is generally expected that changes in LPS structure can still confer decreased susceptibility over time. In CHAPTER V, the LPS interactions of HC1 and HC10 will be studied more in depth.

CHAPTER v

LPS-binding effects of HC1 and HC10



This chapter is adapted from:

Van Moll, L., Wouters, M., De Smet, J., Delputte, P., Van Der Borght, M., Cos, P., In-depth biological characterization of two black soldier fly anti-Pseudomonas peptides reveals LPS-binding and immunomodulating effects. mSphere, 2023. 8(5): p. e00454-23.

V.1 Introduction

As detailed in the previous chapter, the opportunistic pathogen *P. aeruginosa* remains a serious threat to hospitalized patients and immunocompromised people. The increasing acquisition of multi-drug resistance, including carbapenem resistance, severely limits treatment options and has made *P. aeruginosa* both part of the ESKAPE pathogens as well a WHO priority pathogen [324-326]. *P. aeruginosa* also has a remarkable intrinsic resilience against antibiotics [327, 328]. The bacterium secretes a diverse array of virulence factors, contributing to the bacterium's ability to adapt to challenging conditions such as the host's immune response [255, 329]. As for other Gram-negative bacteria, one of *P. aeruginosa's* important virulence factors is LPS.

LPS is an important structural component of the outer membrane of Gram-negative bacteria [330]. The glycolipid has a three part structure: (**i**) a hydrophobic, lipid A anchor, (**ii**) an oligosaccharide core, and (**iii**) the protruding O-antigen, consisting of repeating oligosaccharide units [330, 331]. The LPS layer has an overall negative charge and is stabilized by the presence of divalent cations. Hence, LPS contributes to the permeability barrier of *P. aeruginosa* and its intrinsic antibiotic resistance [330]. The O-antigen and lipid A of are known to be prone to structural modifications [330, 332]. The lipid A part consists of acylated fatty acid chains linked to a glucosamine backbone [330]. By decreasing the negative charge on lipid A, *P. aeruginosa* can decrease its immunogenicity, sometimes seen in chronic infections [330, 332, 333]. LPS is often also referred to as endotoxin. It can activate an immune cascade after recognition by the Toll-like receptor 4 and co-receptor myeloid differentiation factor 2, present on monocytes, macrophages and dendritic cells [330]. When present in the bloodstream, LPS can drive the development of sepsis [334].

LPS neutralization can be an interesting asset of new antimicrobial compounds when combatting Gram-negative infections. Many AMPs do not only have strong bactericidal activity, but immunomodulating activity as well [335]. By regulating gene expression in immune cells, modulating chemotaxis of leukocytes, activating the complement system

and stimulating angiogenesis, AMPs are able to directly influence the course of the immune response [7, 335, 336]. Some AMPs, including the human cathelicidin LL-37, are also able to bind and neutralize LPS [334, 336, 337]. LPS-AMP binding is mediated by both electrostatic and hydrophobic interactions, and can be an important first step in the AMPs' membrane insertion [338].

Despite their many promising characteristics, the development of AMPs into successful antibiotics has been hampered by intrinsic peptide limitations, such as poor metabolic stability, low bioavailability, and decreased activity in physiological conditions due to salt interactions [339, 340]. Moreover, many AMPs show some degree of unselective cell binding, leading to unwanted side effects such as hemolysis [98]. In the previous chapters III and IV, a biological characterization of HC1 and HC10 was performed, both showing strong activity against *P. aeruginosa* and absence of hemolysis at active concentrations [8]. Nothing yet, however, is known about their LPS-binding effects and the effect of physiological salt concentrations on their antimicrobial activity. Hence, this chapter aims to further characterize the *in vitro* activity profile of HC1 and HC10, with a focus on their salt sensitivity and LPS-neutralizing activity.

V.2 Materials and methods

V.2.1 Materials and peptide synthesis

HC1 and HC10 were synthesized using conventional solid-phase peptide synthesis using a 9-fluorenylmethoxycarbonyl protection strategy by Proteogenix (France). Piperidine (20% in dimethyl formamide) was used as deprotection agent and trifluoroacetic acid (TFA) was used for resin cleavage. Both peptides were purified using reverse phase liquid chromatography with a purity of > 90 %. Data of peptide characterization including HPLC and Mass Spectrometry were enclosed by the manufacturer. HC1 is a 44 amino acid long peptide, while HC10 has 47 amino acids. Both peptides are amidated at the C-terminus [8]. For the experiments, AMP stock solutions of 10 mM were prepared in DMSO (Acros Organics) and further diluted in sterile demineralized water. The LPS from *P. aeruginosa*

used in the experiments was purified by phenol extraction and bought from Sigma-Aldrich.

V.2.2 Cell culture and viability

The murine macrophage cell line RAW264.7 (ATCC® TIB-71™) was cultivated in DMEM, supplemented with 10 % iFCS and 2 mM L-glutamine, at 37 °C and 5 % CO_2. For the cell viability assay, HC1 and HC10 (32 – 2 µM) with or without the addition of LPS (100 ng/mL), LPS alone (100 ng/mL), and tamoxifen (128 – 1µM, Sigma) were prepared in DMEM and added to 96-well plates. Tamoxifen was included as a cytotoxic reference compound. As a negative control, wells with untreated cells were included. Next, RAW264.7 cell suspension was added to the plates at a concentration of 1.5 x 10^5 cells/mL. After 48 h of incubation, 50 µL of a 0.01 % resazurin solution (Sigma-Aldrich) was added to each well. After 4 h, the fluorescent signal was read using a plate reader (GloMax, Promega) at $\lambda_{excitation}$= 550 nm and $\lambda_{emission}$= 590. The fluorescent data were used to calculate the cell viability percentage. The viability assay was performed in biological triplicate.

IV.2.3 Antimicrobial activity screening

The activity of HC1 and HC10 against a broad range of clinical and non-clinical *P. aeruginosa* isolates was investigated using an antimicrobial screening assay with resazurin as viability indicator. The panel consisted of *P. aeruginosa* PAO1, *P. aeruginosa* PA14, *P. aeruginosa* LMG 27650, *P. aeruginosa* AA2, *P. aeruginosa* RP73, *P. aeruginosa* NH57388A, *P. aeruginosa* AMT0023-34, *P. aeruginosa* LMG 14084, and *P. aeruginosa* PR355. For the screening, serial dilutions of the peptides were prepared in technical triplicate in MHB (Difco) in 96-well plates in a final volume of 100 µL. Test concentrations started at 32 µM. Polymyxin B, a clinically used peptide antibiotic with a Gram-negative spectrum, was included as a reference compound [341]. Afterwards, 100 µL of bacterial *P. aeruginosa* suspension was added to the test plates, to a final inoculum concentration of 5 x 10^4 CFU/mL. Plates were incubated overnight at 37°C. The next day, 20 µL of a 0.01% resazurin solution was added to each well. After reduction of resazurin to resorufin (4 h for *P. aeruginosa* RP73 and NH57388A, 15 min for all other isolates), the fluorescent

signal was read using a plate reader (Glomax, Promega) at $\lambda_{excitation}$= 550 nm and $\lambda_{emission}$= 590. The fluorescent data were used to calculate the concentration of the peptides leading to 50% growth inhibition of the bacteria (IC50). The MIC was determined visually as the lowest concentration where no bacterial growth occurred, according to the EUCAST (2022) guidelines [342]. At least two biological repeats of the screening assay were performed. The IC_{50} values are reported as the mean ± standard deviations of all technical and biological repeats. For the MIC values, a concentration range was reported, as these concentrations represent discrete instead of continuous values.

V.2.4 Salt sensitivity of HC1 and HC10

To probe the sensitivity of HC1 and HC10 to salts, their activity was first tested in a physiological salt medium representative of the human lung condition. Briefly, an antimicrobial screening experiment was carried out in a modified Gamble's solution with resazurin as viability indicator. The Gamble's solution was prepared as described by Calas et al., but components were dissolved in Müeller Hinton bacterial medium (pH 7.4) (Table SV.1) [343]. The antimicrobial activity against *P. aeruginosa* PAO1 was determined similar to the protocol described earlier in the text. The fold increase in activity (IC_{50} value) compared to non-supplemented bacterial broth was calculated. Secondly, the determine whether the salt sensitivity was concentration-dependent, the activity of HC1 and HC10 was tested in the presence of increasing concentrations of monovalent salt (NaCl) and divalent salt ($CaCl_2$). Briefly, the MHB was supplemented with either 10, 25, 50, 75, 100, 150, or 300 mM of NaCl, and 0.05, 0.1, 0.25, 0.5, 1, or 2.5 mM of $CaCl_2$. The antimicrobial activity was determined similar to the protocol earlier described in the text. The activity in salt-supplemented conditions was compared with the activity in the normal, non-supplemented medium control by calculating the fold change in IC50 value. The experiment was carried out in biological triplicate. The experiments were performed in biological triplicate.

V.2.5 Endotoxin neutralization using the limulus amebocyte lysate assay

LPS neutralization was investigated using a commercially available chromogenic limulus amebocyte (LAL) assay (Toxinsensor^{TM}, Genscript). Briefly, free endotoxins can activate an enzyme cascade, leading to the activation of the clotting factor C in the LAL extract. The activated clotting factor will trigger the release of a chromogenic substrate, which can be quantified spectrophotometrically. Peptides were prepared in endotoxin-free water at final concentrations ranging from 32 to 1 µM and mixed with 0.5 endotoxin units (EU)/mL of standard *E. coli* endotoxin. After 30 min of incubation at 37 °C, the amount of free endotoxin was determined according to the manufacturer's instructions. Absorbance was read at 560 nm on a plate reader (GloMax, Promega). A standard curve was included to calculate the amount of free endotoxins present in the samples. The LAL assay was performed in biological triplicate.

V.2.6 LPS-binding using Bodipy TR Cadaverine displacement assay

To determine the binding of HC1 and HC10 to the lipid A part of LPS, a Bodipy TR Cadaverine (BC; Invitrogen) displacement assay was carried out. Serial dilutions of HC1 and HC10 (32 – 2 µM) were prepared in technical triplicate in 50 mM Tris buffer (pH 7.4) in black 96-well plates. LPS and BC solutions in Tris buffer were added at final concentrations of 100 ng/mL and 2.5 µM respectively. Fluorescence was read using a plate reader (Infinite F Plex, Tecan) at $\lambda_{excitation}$= 580 nm and $\lambda_{emission}$= 620 during a 1 h cycle. Additionally, the effect of divalent cations on the AMP-LPS binding was studied by repeating the assay in 2.5 mM $CaCl_2$ supplemented Tris buffer. Both BC displacement assays were performed in biological triplicate.

V.2.7 Nitrite detection using the Griess reaction

To determine the amount of nitrite produced by RAW264.7 macrophages after LPS and/or AMP treatment, a Griess reaction (Invitrogen) was performed. Cells were seeded in 96-well plates at a density of 5 x 10^5 cells/mL. After 24 h of incubation, the cell medium was discarded and replaced by AMP serial dilutions in DMEM in technical duplicate (32 – 2 µM) with or without the addition of LPS (100 ng/mL). Controls of LPS without peptide

(100 ng/mL) and DMEM medium were included in the test as well. After 48 h of incubation, the nitrite content in the cell supernatant was determined following the kit manufacturer's instructions. Briefly, 20 µL of a mixture of equal volumes of N-(1-naphthyl)ethylenediamine and sulfanilic acid were added to 150 µL of cell supernatant. After 30 min, the absorbance was read at 560 nm using a plate reader (GloMax, Promega). A nitrite standard curve was included to calculate the amount of nitrite present in the supernatant. The Griess reaction was performed in biological triplicate.

V.2.8 Detection of TNFα and IL-6 using ELISA

Enzyme-linked immunosorbent assays (ELISA, Invitrogen) were used to determine the amount of TNFα and IL-6 present in the supernatant of LPS and/or AMP treated RAW264.7 cells. Macrophages were seeded and treated similarly as described for the nitrite detection. After 24 h of treatment, the TNFα and IL-6 levels were determined following the manufacturer's instructions. After addition of the stop solution (1M phosphoric acid), the absorbance of the micro wells was read using a plate reader (GloMax, Promega) at 450 nm. TNF-α and IL-6 levels were calculated using the absorbance data of the standard curves. The ELISA experiments were carried out in biological triplicate.

V.2.9 Detection of TNFα, IL-1β, IL-6, and IL-12β using qPCR

RAW264.7 cells were seeded in 24-well plates at a density of 5 x 10^5 cells/mL and treated similarly as described earlier for the nitrite detection. After 4 h of LPS and/or peptide treatment, total RNA was extracted from the cells using TRIzol reagent (ambion), according to the manufacturer's instructions. The resulting RNA was treated with ezDNAse (Invitrogen), and cDNA was prepared using the Superscript™ IV reverse transcriptase (Invitrogen). The SensiFAST SYBR® No-ROX One-Step kit (Bioline) was used to amplify the genes of interest from the cDNA. Mouse-specific primers for *TNFα* (forward: CATCTTCTCAAAATTCGAGTGACAA, reverse: TGGGAGTAGACAAGGTACAACCC), *IL-1*β (forward: CAACCAACAAGTGATATTCTCCATG, reverse: GATCCACACTCTCCAGCTGCA), *IL-6* (forward: GAGGATACCAC-TCCCAACAGACC, reverse:

AAGTGCATCATCGTTGTTCATACA), and *IL-12*β (forward: GGAAGCACGGCAGCAGAATA, reverse: AACTTGAGGGAGAAGTAGGAATGG) were used. *GAPDH* (forward: TCACCACCATGGAGAAGGC, reverse: GCTAAGCAGTTGGTGGTGCA) was used as the house-keeping gene control. The Lightcycler 480 II system and corresponding software (Roche Diagnostics) were used to perform the qPCR experiments. The polymerase was activated at 95 °C for 2 min, followed by 40 cycles of 2-step amplification (denaturation at 95 °C for 5 s, followed by an annealing step at 60 °C for 20 s). Relative gene expression, normalized to *GAPDH*, was calculated using the Pfaffle equation, given by $\frac{(E_{GOI})^{\Delta Ct_{GOI}}}{(E_{GAPDH})^{\Delta Ct_{GAPDH}}}$ where GOI refers to the gene of interest, E the primer efficiency, and ΔCt the difference in cycle thresholds [344]. Three biological repeats of the qPCR experiments were carried out.

V.2.10 Statistical analysis

Results are reported as mean ± standard deviation of all technical and biological replicates. For the salt sensitivity assay, a one sample t-test was used to determine if the fold increase in IC50 was significantly different from 1. For the BC displacement assay, a one-way ANOVA test was used to determine significant difference between groups after 1 h of treatment. As not all data sets were normally distributed but most had equal variances for the ELISA and qPCR experiments, a non-parametric Kruskal-Wallis test was used to compare the treated samples with the controls. Graphpad Prism 9.5 was used for all data visualization and statistical analysis.

V.3 Results

V.3.1 Antimicrobial activity of HC1 and HC10 against a panel of *P. aeruginosa* isolates

The activity of HC1 and HC10 against a panel of eight different *P. aeruginosa* isolates, chosen to represent a broad variety in origin (clinical or environmental) and phenotype, was investigated [215, 333, 345]. Both peptides showed activity against all strains tested, including intermediate and multi-drug resistant strains. IC50 values for all isolates are in the low micromolar range (Table V.1). Activity of the clinically used polymyxin B is higher than that of HC1 and HC1, but in the same concentration range. *P. aeruginosa* PA14, a highly virulent strain causing acute infections, was noticeably more susceptible to both HC1, HC10, and polymyxin B [346]. In contrast, HC1, HC10, and polymyxin B were less active against the low-inflammatory *P. aeruginosa* RP73 isolate from a patient with cystic fibrosis, showing a threefold increase in IC50 value compared to PAO1 [333].

Table V.1: **Antimicrobial activity of HC1 and HC10 against a panel of *P. aeruginosa* isolates.** IC50 values (concentration of peptide leading to 50 % of growth inhibition), and the MIC value (minimum inhibitory concentration) in the low micromolar range, show the high activity of HC1 and HC10 against all strains tested. Polymyxin B was included as a reference antibiotic. Data are represented as the mean ± the standard deviation of all technical and biological repeats.

Strain	Origin	Resistant?	Average IC_{50} (μM)			Range MIC (μM)	
			HC1	HC10	P[c]	HC1	HC10
PAO1	Non-CF[a], wound [347]	No	1.38 ± 0.37	1.15 ± 0.36	0.53 ± 0.15	2	2
PA14	Non-CF[a], burn wound [346, 347]	No	0.47 ± 0.16	0.63 ± 0.17	0.16 ± 0.04	1-2	1-2
LMG 27650	Non-CF[a], clinical [348]	Yes (MDR[b])	1.39 ± 0.37	0.99 ± 0.40	0.42 ± 0.18	2-4	2
AA2	CF[a] (acute) [349]	Cefoperazone, piperacillin	1.31 ± 0.35	1.11 ± 0.31	0.51 ± 0.25	2	2
RP73	CF[a] (chronic) [333]	Meropenem, ceftazidime, gentamicin	3.91 ± 1.27	3.73 ± 1.47	1.00 ± 0.22	4-8	4-8
NH573-88A	CF[a] (chronic) [215]	Quinolones, β-lactam antibiotics	1.39 ± 0.18	1.28 ± 0.12	0.30 ± 0.06	2	2
AMT0023-34	CF[a] (acute) [215]	Quinolones, amino-glycosides	0.89 ± 0.63	0.95 ± 0.37	0.37 ± 0.05	1-2	1-2
LMG 14084	Non-clinical, environment [348]	No	1.48 ± 0.26	1.00 ± 0.47	0.52 ± 0.06	2	2
PR355	Non-clinical, hospital [348]	No	1.19 ± 0.43	1.17 ± 0.46	0.35 ± 0.09	1-2	1-2

[a] CF = cystic fibrosis, [b] MDR = multi-drug resistant, [c] P = polymyxin B

V.3.2 Salt sensitivity of HC1 and HC10

As *P. aeruginosa* is a pathogen that commonly affects the lungs, HC1 and HC10 were screened in a simulated lung fluid medium (Gamble's solution). Lung fluid is rich in various salts, which are known to interact with AMPs [350]. Both HC1 and HC10 indeed showed a strong increase in IC_{50} values, indicating a decreased anti-pseudomonal activity in the Gamble's medium (**Figure V.1a**). To study whether the decrease in antimicrobial activity of HC1 and HC10 occurred due to the presence of monovalent or divalent salts, the peptides were screened against *P. aeruginosa* in increasing concentrations of NaCl and $CaCl_2$. The addition of NaCl did not have a significant effect on the antimicrobial activity of HC1 and HC10 up until concentrations of 75 mM (**Figure V.1b, V.1c**).

At 100 mM NaCl, the concentration of salt present in lung fluid, the activity only minorly decreased compared to the activity in non-supplemented medium, with an 1.3-fold increase in IC_{50} for HC1, and 1.2-fold increase for HC10 [343]. In 150 mM NaCl, approximately the concentration found in human blood, the IC_{50} increased by a factor of 1.8 for HC1, and 1.5 for HC10 [351]. The effect of $CaCl_2$ on the AMP antimicrobial activity was more outspoken than that of NaCl. For both peptides, a significant decrease in activity was found from 0.25 mM $CaCl_2$ onwards (**Figure V.1d, V.1e**). At 2.5 mM $CaCl_2$, the antimicrobial activity strongly decreased, with an average 17-fold increase in IC_{50} for both HC1 and HC10. As this is also the concentration found in human lung fluid, the presence of divalent salts presumably causes the significant decrease in activity in lung conditions [343].

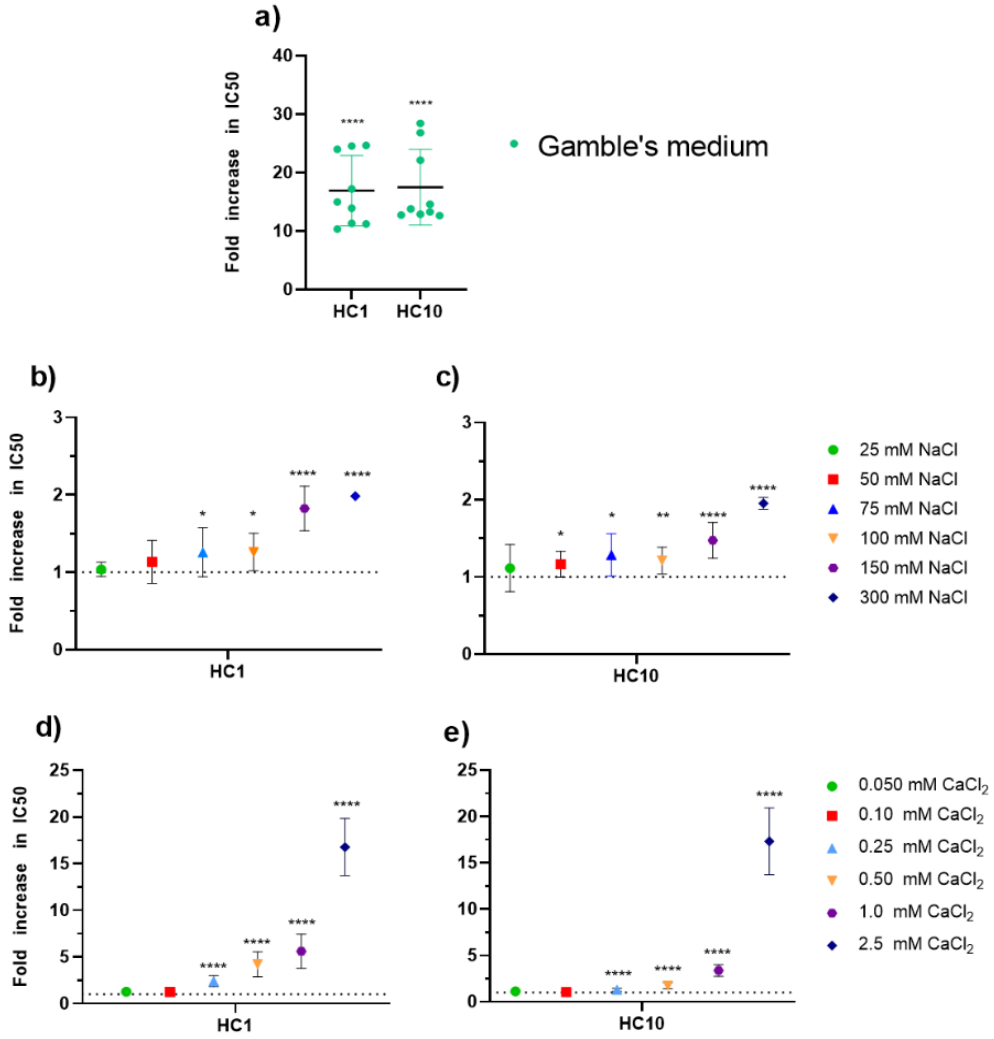


Figure V.1: Salt sensitivity of HC1 and HC10. **a**) The activity of HC1 and HC10 against *P. aeruginosa* PAO1 strongly decreases in simulated lung salt conditions (Gamble's medium) **b,c**) The anti-pseudomonal activity of HC1 and HC10 slightly decreases in the presence of high (≥ 75 mM) concentrations of NaCl **d,e**) The addition of $CaCl_2$ has a clear, negative impact on the antimicrobial activity of HC1 and HC10. At physiological concentrations (2.5 mM), the activity strongly decreased. Data are presented as the mean ± the standard deviation. Data were analysed using a one sample t-test to test for significant difference from 1. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, and **** $p \leq 0.0001$. All experiments were carried out in biological triplicate.

At 100 mM NaCl, the concentration of salt present in lung fluid, the activity only minorly decreased compared to the activity in non-supplemented medium, with an 1.3-fold increase in IC_{50} for HC1, and 1.2-fold increase for HC10 [343]. In 150 mM NaCl, approximately the concentration found in human blood, the IC_{50} increased by a factor of 1.8 for HC1, and 1.5 for HC10 [351]. The effect of $CaCl_2$ on the AMP antimicrobial activity was more outspoken than that of NaCl. For both peptides, a significant decrease in activity was found from 0.25 mM $CaCl_2$ onwards (**Figure V.1d, V.1e**). At 2.5 mM $CaCl_2$, the antimicrobial activity strongly decreased, with an average 17-fold increase in IC_{50} for both HC1 and HC10. As this is also the concentration found in human lung fluid, the presence of divalent salts presumably causes the significant decrease in activity in lung conditions [343].

V.3.3 LPS neutralization by HC1 and HC10

The chromogenic LAL assay was used to study the ability of HC1 and HC10 to neutralize free-circulating, standard *E. coli* endotoxin. The assay makes use of an extract of blood cells from the Horseshoe Crab, which contains a pro-enzyme that is activated in the presence of endotoxins, or LPS [352, 353]. After a catalytic cascade of reactions, the coloured product *p*-nitroaniline is released, which can be quantified spectrophotometrically. HC1 was able to neutralize the added endotoxin in a concentration-dependent manner. At concentrations as low as 4 µM, 89 % LPS neutralization was observed (**Figure V.2**). LPS neutralization for HC10, however, could not accurately be calculated and is therefore not included in the graph below. Upon addition of HC10, a high background signal was obtained, even in endotoxin-free peptide controls, indicating that HC10 autonomously induces the LAL pro-enzyme.

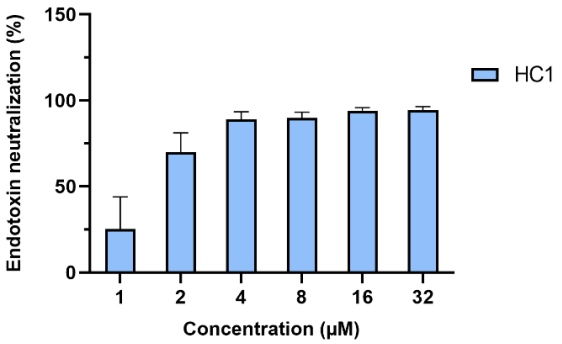


Figure V.2: **LPS-neutralization by HC1, as determined by a chromogenic LAL assay.** HC1 shows a concentration-dependent neutralization of standard *E. coli* endotoxin, with high neutralization from 4 µM onwards. Data for HC10 are not included in the graph, as this peptide intrinsically induced the LAL enzyme cascade, leading to high background signals. The experiment was carried out in biological triplicate. Data are represented as the mean + standard deviation of all technical and biological replicates.

V.3.4 Binding of HC1 and HC10 to lipid A and effect of $CaCl_2$.

Lipid A is the pyrogenic part of LPS, responsible for triggering the innate immune response via Toll-Like Receptor 4 [354]. Structurally, it is the most conserved part of LPS, consisting of a hydrophilic amino disaccharide backbone, and a hydrophobic domain of fatty acids tails [354, 355]. To study the affinity of the AMPs to the Lipid A toxic center of LPS, a fluorescent displacement assay using BC was carried out [356]. BC, cadaverine linked to the fluorescent dye Bodipy TR, will bind to lipid A via electrostatic interactions, which decreases its fluorescent signal. When BC is displaced from its interaction with lipid A due to competition with other LPS-binding compounds, dequenching of its fluorescence will occur [355, 356]. Both AMPs show a concentration-dependent increase in fluorescence, compared to the non-peptide treated control (**Figure V.3a, V.3b**). Displacement of BC by the AMPs occurs fast, and plateaus after 20 min of treatment.

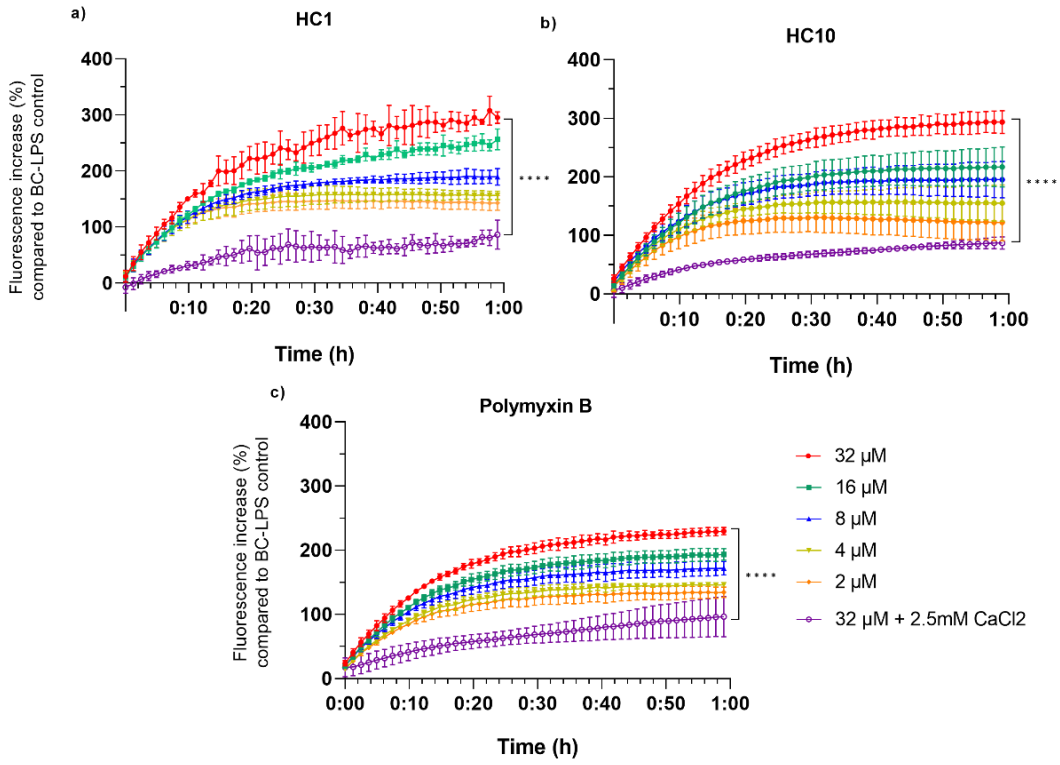


Figure V.3: **Bodipy-TR Cadaverine displacement assay**. **a,b)** HC1 and HC10 are able to displace BC in a concentration-dependent manner, signified by the increase in fluorescence over time. As a control, samples with BC and LPS (100 ng/mL) without peptide treatment were included. At 32 µM of AMP, there is close to a 300 % increase in fluorescence compared to the control. **c)** BC displacement assay for the peptide antibiotic reference polymyxin B. The LPS-binding of polymyxin B follows a similar concentration-dependent trend over time, but for 16 and 32 µM the polymyxin-induced LPS-binding is lower than for HC1 and HC10. Fluorescence was read during a one hour cycle. Data are represented as the mean ± standard deviation. Datasets at the last timepoint were compared with a one-way ANOVA test, **** $p \leq 0.0001$. The assay was carried out in biological triplicate.

At MIC concentrations (2 µM), there is already a high amount of AMP-LPS binding, signified by the over 100 % increase in fluorescence. At 32 µM, there is a 295 % increase in fluorescence for HC1, and a 294 % increase for HC10. For polymyxin B (**Figure V.3c**) the LPS-binding trend is similar, however, the increase in fluorescence at 32 µM only reaches approximately 220 %, significantly lower ($p \leq 0.5$) than for HC1 and HC10. To test the effect of divalent cations on the lipid A binding, the assay was repeated in 2.5 mM

$CaCl_2$ supplemented buffer. In the presence of divalent salts, the LPS binding is noticeably lower for HC1, HC10, and polymyxin B, as the fluorescence increased only marginally at high concentrations of 32 µM.

V.3.5 Inhibition of LPS-induced macrophage activation

To determine whether the LPS-AMP binding also decreases macrophage activation, a Griess reaction was performed. When macrophages are activated upon contact with LPS, they release the unstable nitric oxide, which is quickly converted into the more stable nitrite [357]. Hence, nitrite, which can be detected by the chromogenic Griess reaction, is a useful parameter for macrophage activation. HC1 and HC10 showed a concentration-dependent effect on the nitrite release by LPS stimulated murine macrophages (**Figure V.4**). At lower concentrations, the inhibition of nitrite production was more variable than at higher concentrations (**Figure V.4**). However, at 32 µM, both peptides consistently achieved close to 100 % inhibition of nitrite formation. To ensure that any observed effects were not due to decreased cell viability, the effect of the AMPs, either in monotreatment or in co-treatment with LPS, and LPS (100 ng/mL), on RAW264.7 cell viability was checked with a resazurin assay. No significant change in viability was observed (**Figure SV.1**).

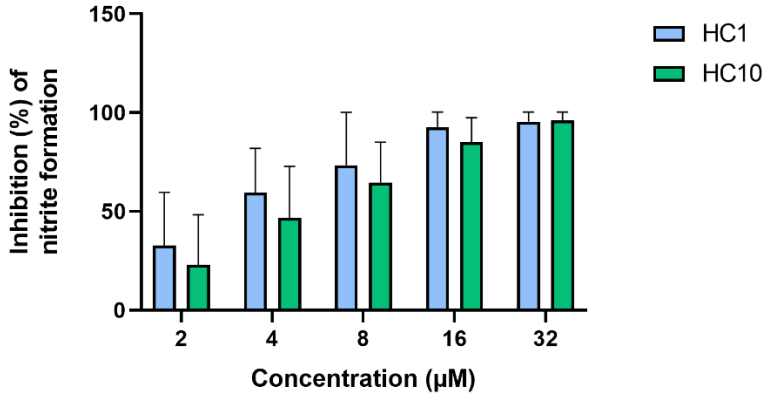


Figure V.4: Effect of HC1 and HC10 on nitrite production by LPS stimulated RAW264.7 macrophages as determined by a Griess reaction. HC1 and HC10 have a concentration-dependent effect on the macrophage activation after LPS addition, presumably through their LPS-binding ability. Both peptides achieve high, near 100 % inhibition of nitrite formation at concentrations of 32 µM. As a positive control, LPS stimulated macrophages without peptides were used. The experiment was carried out in biological triplicate. Data are represented as the mean + standard deviation.

V.3.6 Inhibition of pro-inflammatory cytokine release

The effect of the AMPs on the release of the pro-inflammatory cytokines TNFα and IL-6 by murine macrophages was investigated using both ELISA and qPCR (**Figure V.5**). Upon co-treatment with LPS, both HC1 and HC10 downregulate the expression of TNFα and IL-6 in a concentration-dependent manner, while, at near-MIC concentrations (2-4 µM), the effect on the cytokine release was not statistically significant. The decrease in pro-inflammatory cytokine production was consistently high and significant at 32 and 16 µM. qPCR experiments for IL-1β and IL-12β showed comparable results (**Figure SV.2**). In addition, the ELISA experiment showed that at 32 µM, HC1 also moderately induces the expression of TNFα in absence of LPS stimulation, indicating that at high concentrations HC1 has intrinsic immunomodulating effects, unrelated to its LPS-binding activity (**Figure V.5a**). The same effect was not seen for HC10, or for IL-6 expression (**Figure V.5c**). Noticeably, this TNFα induction by HC1 was not observed in the qPCR experiments

(**Figure V.5b, V.5d**). As the ELISA measured cytokine levels after 24 h, while the qPCR experiments analysed the expression of cytokine mRNA after 4 h of treatment, the time difference in exposure to the peptides could explain this effect.

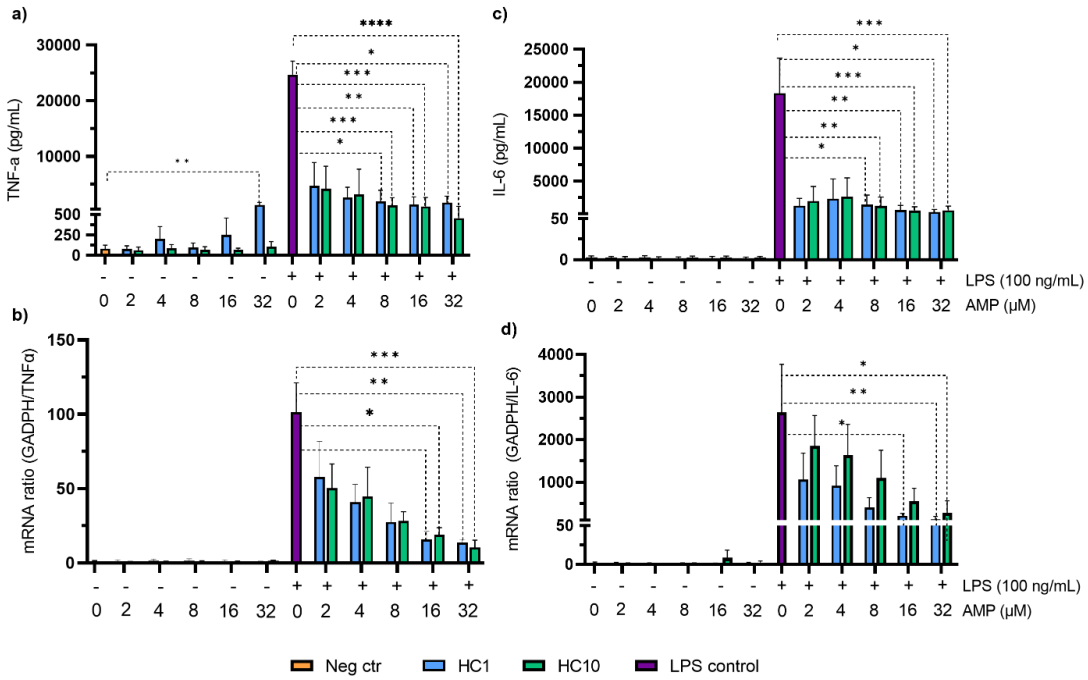


Figure V.5: Effect of HC1 and HC10 on the release of the pro-inflammatory cytokines TNFα and IL-6. a,b) The release of TNFα by RAW264.7 macrophages after peptide monotreatment or LPS-AMP was investigated using ELISA (24 treatment) and qPCR (4 hour treatment). **c,d**) The release of IL-6 by RAW264.7 macrophages after peptide monotreatment or LPS-AMP was investigated using ELISA (24 treatment) and qPCR (4 hour treatment). LPS treated cells (100 ng/mL) were included as positive controls. Data are represented as the mean + standard deviation, and statistically analysed using a Kruskal-Wallis test. * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, *** = $p \leq 0.0001$. All experiments were carried out in biological triplicate.

Important to note, however, is that only one reference gene (*GAPDH*) was used for qPCR analyses. Guidelines on the performance of and reporting on qPCR experiments (MIQE) advise the use of minimum two reference genes for more reliable data normalization in case of interference of the experimental conditions with the expression of the housekeeping genes [358]. Nevertheless, earlier AMP research has regularly used *GAPDH*

as singular reference gene, and statistical analysis of the Ct scores (2^{-ct}) of the housekeeping gene showed no significant difference ($p > 0.05$) between any of the AMP or LPS treated groups and the negative cell control (One-Way ANOVA, Figure SV.3) [359-362]. This tentatively indicates absence of any effect of LPS or AMP treatment on GAPDH expression in the murine macrophage cell line, although the addition of a second housekeeping gene (e.g. *β-actin*) should be considered for future qPCR experiments.

V. 4 Discussion

V.4.1 AMPs as anti-pseudomonal therapeutics

As an opportunistic pathogen that has the ability to cause invasive, difficult-to-treat infections, *P. aeruginosa* poses a significant health threat to immunocompromised individuals [332, 363]. Due to growing resistance rates, the need for new, functional antibacterials is high [255]. Most *P. aeruginosa* infections are characterized by a strong inflammatory response, which not only leads to acute tissue damage, but is also related to poor disease outcome and the bacterium's ability to cause chronic, persistent infections [364, 365]. It has been opted that therapeutics with immunomodulating effects could positively influence the course of a *P. aeruginosa* infection [364]. Due to their wide range of bioactivities, AMPs have gathered substantial interest as potential next-generation antimicrobials [69, 366]. Since many AMPs can target both the bacterial colonization and inflammation processes during infection, these new peptide therapeutics seem promising to effectively combat Gram-negative bacteria. [69, 366]. Despite a surge in AMP research and development, infections with Gram-negative pathogens such as *P. aeruginosa* remain largely untargeted by AMPs in clinical trials [115, 272, 367].

V.4.2 LPS binding is involved in HC1 and HC10 activity

Two assays were used to detect LPS-AMP binding in our study: a LAL assay and a BC displacement assay. The LAL assay has been commonly used to study endotoxin-neutralization of AMPs [368-370]. However, due to high background signals caused by

HC10, presumably through intrinsic activation of the LAL proenzyme, the LAL assay gave unreliable results for HC10. We used an additional BC displacement assay to confirm the presence of AMP-LPS interactions, a technique that is used less frequently, yet offers a valid alternative for the LAL assay when studying LPS-AMP binding [355]. The BC displacement assay confirmed the binding of HC1 and HC10 to lipid A, the toxic centre of LPS. Moreover, despite a lower antimicrobial activity against *P. aeruginosa* compared to polymyxin B, the binding of *P. aeruginosa* LPS was higher at 32 µM for HC1 and HC10.

The importance of this LPS-AMP binding for the AMP activity was further supported by the results of our screening of the two black soldier fly AMPs, HC1 and HC10, against a panel of different *P. aeruginosa* isolates. The susceptibility of *P. aeruginosa* RP73, a chronic cystic fibrosis isolate, to the peptides was noticeably lower [333]. *P. aeruginosa* RP73 contains low-inflammatory lipo-oligosaccharide molecules that possess under-acylated lipid A structures. In contrast to some other cystic fibrosis isolates, RP73 does not contain LPS mutations that typically confer increased resistance to AMPs, such as the addition of aminoarabinose to the free phosphate groups of the glucosamine backbone [266, 371]. However, under-acylation of LPS molecules has in the past also been linked to increased AMP and polymyxin resistance in various Gram-negative bacteria [372-375]. For *P. aeruginosa* specifically, strains with penta-acylated LPS structures are less susceptible to polymyxin B due to decreased hydrophobic interactions [375]. Our research suggests that the antibacterial activity of HC1 and HC10 cecropins is also influenced by the acylation pattern of lipid A, although other mutations in *P. aeruginosa* RP73 can play a role in its decreased AMP susceptibility. Nevertheless, as *P. aeruginosa* isolates often acquire LPS mutations during chronic infections, screening of AMPs against different clinical isolates ranging from various stages of infection, should not be overlooked during pre-clinical *in vitro* examination of anti-pseudomonal peptides.

V.4.3 LPS binding decreases inflammatory responses

To verify whether LPS-binding by AMP also had effect on the inflammatory response caused by LPS, a series of experiments were performed. The release of various pro-

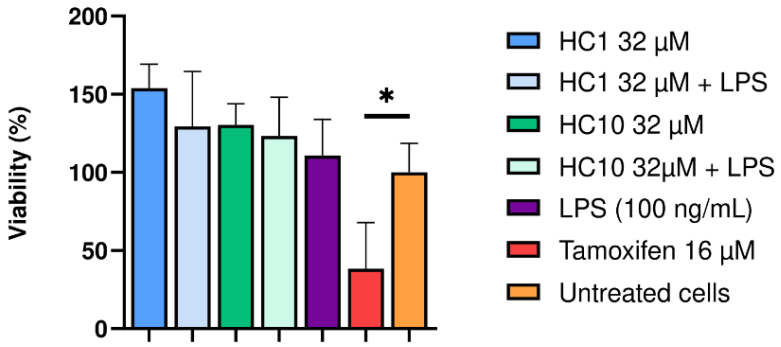
inflammatory mediators, including nitric oxide, IL-6, and TNFα, by murine macrophages after AMP and LPS co-treatment was investigated. Both HC1 and HC10 reduced the inflammatory effects caused by LPS, indicating that the AMP-LPS complex is less prone to triggering the immune cascade. Similar anti-inflammatory effects through LPS-binding have been described for other cecropin AMPs as well, including Papiliocin and SibaCec [376, 377]. The underlying mechanism is thought to involve a change in the aggregate structure of free LPS by AMP interaction. LPS-binding AMPs are either able to break up the LPS micelles in solution, creating smaller, unorganized particles, or increase LPS aggregation, forming large multilamellar structures. In both cases, LPS becomes less available for LPS binding protein, which in turns leads to a decrease in CD14 binding and decreased activation of immune cells [338, 378]. Interestingly, HC1 also moderately increased TNFα levels after 24 h, while IL-6 levels remained unaffected. A similar pro-inflammatory response has also been reported for the human AMP LL-37 [379].

V.4.4 Salt sensitivity decreases LPS binding and challenges AMP development

One of the challenges in the clinical development of AMPs, is their frequent loss of activity in physiological salt conditions [380]. A broadly accepted explanation of AMP's salt sensitivity is the decrease of electrostatic interactions with the anionic phosphate groups of the bacterial membranes, due to the occupation of the AMP binding sites by positive salt ions [380-382]. Others, however, have suggested that salt stability of alpha-helical peptides is dependent on their conformational rigidity; high concentrations of salt can interfere with the stability and/or formation of the alpha helices [383]. Here, salt sensitivity for both HC1 and HC10 was also noted. Although the AMPs were only minorly affected by high NaCl concentrations, the presence of divalent cations such as Ca^{2+} strongly decreased their antimicrobial activity. The BC assay confirmed that in physiological concentrations of $CaCl_2$, the LPS-AMP binding was highly impacted. This suggests that LPS serves as an important anchor for the AMPs upon contact with the microbial membrane and that the LPS-AMP binding is involved in the cecropin's antimicrobial activity mechanism [382]. To overcome this salt sensitivity, many structural

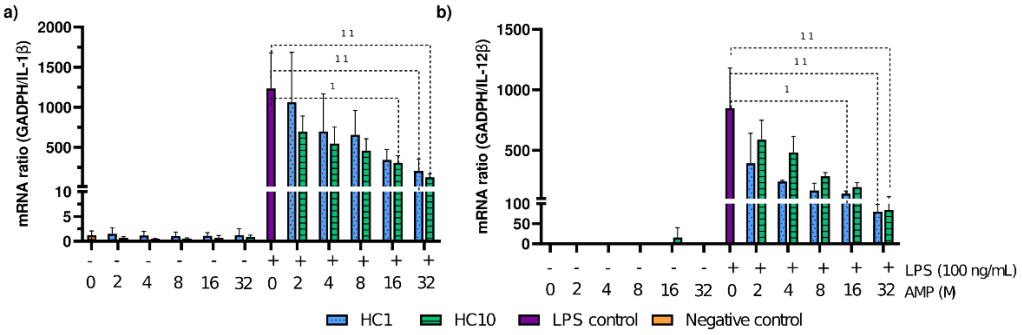
modifications have been proposed. Amino acids such as histidine and tryptophan can be replaced by bulkier residues such as β-naphtylalanine [384, 385]. This can lead to a deeper insertion of the AMP into the microbial membrane, protecting it partially from cation competition [384]. Introducing lipophilic tags, including cholesterol or vitamin E, at the C-terminus or N-terminus of the peptide has also been proven successful in past research [334]. Apart from structural modification, designing innovative formulations for AMP delivery can also increase salt resistance. Nanomedicine-based formulations such as liposome encapsulation and polymer-peptide conjugation are possible strategies to protect AMPs from salt interactions before they reach their target location [386, 387]. For polymyxin antibiotics, these formulations have been proposed for inhalation therapy, shielding the peptide from the challenging lung environment [387]. For the HC1 and HC10 this could also be a valid strategy to work towards usable antibiotics against *P. aeruginosa* lung infections. Overall, despite promising properties such as strong antimicrobial activity and endotoxin-neutralizing properties, it is clear that both HC1 and HC10 will need optimization before any further routes to clinical applications can be considered.

Figure SV.1 Effect of HC1 and HC10 on RAW264.7 cell viability



SV.1 The effect of HC1, HC10 and/or LPS on the RAW264.7 viability. The highest dose of HC1 and HC10 (32 µM) used in experiments was added to RAW264.7 macrophages, with or without the addition of LPS (100 ng/mL) to study the effect on cell viability after 48h of incubation. Data are represented as the mean + SD of the cell viability compared to a control of untreated cells. Tamoxifen was included as a positive control. Data were analysed using a one-way ANOVA test.

Figure SV.2 Effect of HC1 and HC10 on IL-1β and IL-12β production in LPS stimulated RAW264.7 cells



SV.2 The effect of HC1 and HC10 on pro-inflammatory cytokine production in LPS-stimulated RAW264.7 cells. a) The release of IL-1β by RAW264.7 macrophages after peptide monotreatment or LPS-AMP co-treatment for 24 h was investigated using qPCR b) The release of IL-12β by RAW264.7 macrophages after peptide monotreatment or LPS-AMP co-treatment for 24 h was investigated using qPCR. LPS treated cells (100 ng/mL) were included as positive controls. Data are represented as the mean + SD, and statistically analysed using a Kruskal-Wallis test. * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, *** = $p \leq 0.0001$. All experiments were carried out thrice.

Figure SV.3 Cycle threshold values (ct) of the housekeeping gene GAPDH for the different experimental conditions used in the qPCR set-up. One-Way ANOVA analysis indicated no significant difference ($p > 0.05$) between any of the tested groups. 2^{-ct} values are plotted. HKG = housekeeping gene

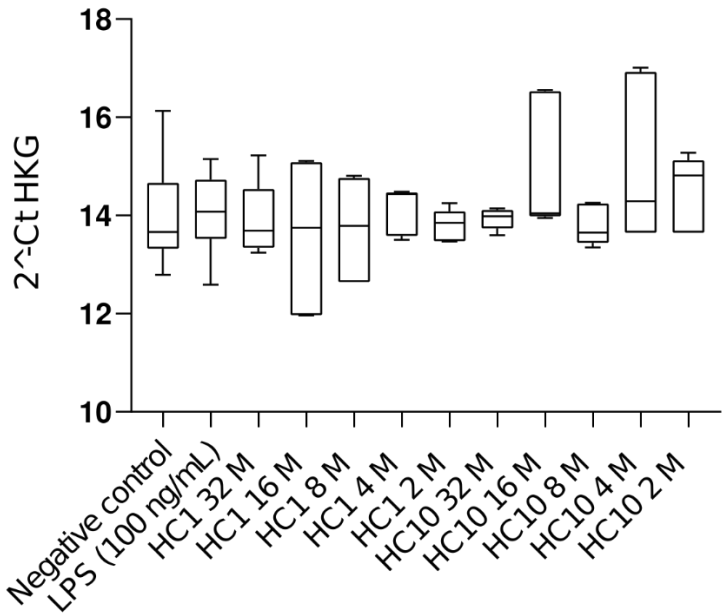


Table SV.1 Composition of Gamble's medium

Composition	Gamble's solution (mM)	Gamble's solution (g/L)
Magnesium chloride	1	0.095
Sodium chloride	105	6.019
Potassium chloride	4	0.298
Di-sodium hydrogen phosphate	0.88	0.126
Sodium sulfate	0.44	0.063
Calcium chloride di-hydrate	2.5	0.368
Sodium acetate	7.14	0.574
Sodium bicarbonate	31	2.604
Sodium citrate di-hydrate	0.38	0.097

CHAPTER VI

In vivo evaluation of HC10

Van Moll, L., Geerts N., De Smet, J., De Vooght, L., Van Der Borght, M., Cos, P., *In vivo* evaluation of the anti-pseudomonal and LPS-neutralizing activity of the black soldier fly cecropin HC10. Unpublished.

VI.1 Introduction

From all possible sites, the lungs are most frequently affected in case of a *P. aeruginosa* infection [388, 389]. *P. aeruginosa* pneumonia is the most common among all Gram-negative nosocomial lung infections, and its prevalence is especially high in intensive care hospital settings [388, 389]. The mortality associated with *P. aeruginosa* nosocomial lung infections is also higher than almost any other bacterial pneumonia, mostly due to its propensity to cause disease in immunocompromised hosts [389]. Community-acquired *P. aeruginosa* lung infections are much less common and are mostly seen in elderly patients with comorbidities such as chronic obstructive pulmonary disease [389]. *P. aeruginosa* pneumonia can manifest itself as a rapidly lethal infection in those with an impaired immune system, or can persist for decades long in a biofilm-like state, for example in the lungs of patients with CF [389]. In addition, *P. aeruginosa* can spread to the blood, leading to bacteraemia and potentially, multi-organ failure [390].

Despite the promising, high *in vitro* activity of many AMPs against *P. aeruginosa*, only a select few peptides are being tested in clinical trials specifically targeted against Gram-negative systemic or lung infections [380, 391]. As mentioned before (chapter II), the *in vitro* antimicrobial activity of AMPs is often poorly correlated with their *in vivo* activity in mammalian infection models [392]. This difficult translation of promising *in vitro* results to *in vivo* settings presents a significant bottleneck in preclinical AMP research and development. AMP development is often halted after poor clinical performance or toxicity, or the AMP use is re-targeted towards different, mostly local applications, which are less susceptible to the interactions present in the more complex matrices of the blood or the lungs [393]. Nevertheless, early animal testing of AMPs remains a necessary step to gauge the actual *in vivo* potential of these peptides to determine the next course of action for AMP development. Many different *in vivo* models have been applied in preclinical AMP testing, ranging from simple invertebrate models to more complex, mammalian animal models such as *P. aeruginosa* lung infection models.

In recent years, various AMPs have been investigated in murine lung infection models of *P. aeruginosa* with the end goal of developing new AMP inhalation therapies [394]. In early research, the peptides are usually delivered in simple liquid form, by intratracheal or intranasal instillation of the mice [394]. Sometimes, however, AMPs are delivered to the lungs in more elaborate formulations, such as lipid or solid nanoparticles, to improve the stability and residence time of the AMP in the lungs [394-396]. Peptides are usually structurally altered before *in vivo* administration, however, sometimes the native peptide is tested without any prior modifications, such as the human LL-37 cathelicidin [394, 397]. AMPs are mostly tested in acute models of *P. aeruginosa* lung infection, and activity is generally evaluated within a 24-h timespan [284, 398-402]. Presumably, as chronic models are more difficult to establish due to the high virulence of *P. aeruginosa* and often require the incorporation of the bacteria into alginate or agar beads, acute models are preferred in early animal research studies [403]. Although mice are highly useful to model complex diseases such as bacterial pneumonia, care must be taken when interpreting results. Relevant mouse-human differences exist within the lung, including anatomical, cellular and immunological differences [404].

Invertebrate models can be included in AMP research to bridge the gap between *in vitro* experiments and the use of mouse models. The larvae of the greater wax moth, *G. mellonella*, are commonly used in early drug research to test both antimicrobial activity or toxicity of novel compounds [405, 406]. This invertebrate model offers various advantages over the more complex mammalian animal models. The larvae are inexpensive and easy to maintain, less susceptible to legal or ethical concerns, and applicable for a wide range of bacterial infection models [406]. The activity of several AMPs, including LL-37, has already been investigated in *P. aeruginosa* infection models of *G. mellonella*, all showing increased larval survival after AMP treatment [407-409].

In this chapter, the *in vivo* activity of one selected cecropin (HC10) is investigated. Firstly, the inter-batch differences in antimicrobial activity of HC1 and HC10 are discussed, illustrating the selection of HC10 for following experiments. Then, the activity of HC10 in

three different *in vivo* models is analysed, being (**i**) a *P. aeruginosa* infection model of *G. mellonella*, (**ii**) a mouse model of an acute *P. aeruginosa* lung infection, and (**iii**) in an LPS-induced mouse model of lung inflammation.

VI.2 Materials and methods

VI.2.1 Antimicrobial screening of different AMP batches

During the course of the *in vitro* experiments, five different batches were ordered at two different producers: Proteogenix (France) and Genscript (The Netherlands). Antimicrobial activity of each batch against *P. aeruginosa* PAO1 (ATCC 15692) was investigated upon arrival. Stock solutions and dilutions of the peptides were made as mentioned in III.2.1. The antimicrobial screening experiment was carried out as described in III.2.3. For batch 1 and 2, the AMPs were added *in duplo* for each replicate, for batch 3-5, the peptides were tested in technical triplicate.

VI.2.2 *Galleria mellonella* infection model

Last-instar larvae of the greater wax moth (*G. mellonella*) were bought from a local vendor (Anaconda Reptiles, Kontich) and stored in containers containing wood chips at room temperature until further use. Larvae showing no signs of melanisation and weighing between 200 and 250 mg were selected for the experiments and randomly allocated into groups of ten larvae. *P. aeruginosa* PAO1 was cultured in MHB to early logarithmic phase (OD_{600} between 0.1 and 0.7), centrifuged (3000 rpm, 10 min) and washed with PBS. The bacterial culture was diluted in PBS to obtain an inoculum of approximately 10^3 CFU/mL. Next, the larvae were infected with 10 µL of *Pseudomonas* suspension in the left hind proleg using a 20 µL Hamilton Syringe as described earlier by Cools et al. (2019), which corresponds to the minimal lethal dose of 10 CFU/larva [410]. Thirty min after infection, the larvae were treated with 10 µg HC10/larva in the opposite hind proleg. Non-infected control groups injected with PBS, non-infected controls injected with HC10, and *P. aeruginosa* infected controls were included, as well as unmanipulated larvae. All larvae were incubated at 37°C in the dark, and every 24 h,

survival was evaluated. Larvae were considered deceased when (i) no movement occurred after external stimuli, or (ii) dark pigmentation due to melanisation was present. The experiment was performed in biological duplicate, and data were pooled to obtain sample sizes of 20 larvae.

VI.2.3 Mice

Wild-type female C75BL/6J mice were purchased from Janvier Labs (France) at six weeks old and allowed to acclimatize to the laboratory environment at least one week before experiments. The mice were housed under standard pathogen-free conditions in individually ventilated cages with HEPA filters with a 12 h light/dark cycle, at a temperature of 20-25°C and an average humidity of 50-60%. Food and water were provided *ad libidum*, and paper shreds and cardboard houses were present as cage enrichment and shelter. During the experiments, the welfare of the mice was observed and graded at least daily, using a functional battery observation (FOB) scoring system (SVI.1). All mice were weighted at the start and end of each experiment.

VI.2.4 Acute toxicity pilot mice study

To determine the highest tolerated dose of HC10 to use in further experiments, a pilot toxicity study was carried out. Mice were randomly allocated into four groups of three mice each and were intranasally treated with either 15 µg HC10/mouse, 50 µg/mouse, 150 µg/mouse, or PBS as the control vehicle. For the intranasal treatment, the mice were lightly anesthetized using isoflurane (5%, Alivira) and held in a supine position. Fifty µL of HC10 solution was gently pipetted onto both nostrils of the mice, and the animals were held for one additional minute afterwards to allow complete inhalation of the peptide solution. After the treatment, all mice were followed up at 2-h intervals for 8 h and scored using the FOB system. The next day, 24 h after treatment, the mice were humanely killed by an intraperitoneal pentobarbital injection with a 28G needle (150 mg/kg, Kela). A FlexiVent™ system (SCIREQ) was used to study the lung function of the mice, and afterwards, the lungs were collected during an autopsy for further qPCR analysis.

VI.2.5 *P. aeruginosa* murine lung infection model

To study the antibacterial activity of HC10 against *P. aeruginosa*, a murine lung infection model was used. A stationary phase culture of *P. aeruginosa* PAO1 (ATCC 15692) was diluted 1:5 into fresh MHB medium and grown at 37°C with shaking at 200 rpm (New Brunswick Innova 4300) for 2 additional h to reach logarithmic growth phase. Next, the culture was centrifuged at 3000 g for 10 min and the bacterial pellet was resuspended in sterile PBS to obtain a culture with a concentration of approximately 6 x 10^7 CFU/mL. Mice were sedated by light isoflurane anaesthesia (5%, Alivira) and 50 µL of bacterial inoculum was instilled intratracheally. During the procedure, the mice were put on an intubating platform with an elastic band around their incisors. Then, the tongue of the mice was held with tweezers while the inoculum was gently pipetted into the back of the mice's throat. Two hours after bacterial infection, six mice were treated intranasally (see VI.2.2) with 50 µg HC10 per mouse. The other six mice (infection control) received 50 µL of PBS intranasally. Afterwards, the mice were followed-up at regular intervals and 4 h post-treatment, the mice were sacrificed by an intraperitoneal pentobarbital injection (200 mg/kg, Kela), as the maximum threshold FOB score for a humane endpoint had been reached. The mice were autopsied and the lung, liver, and spleen were collected for further analysis of bacterial burden and cytokine measurement.

VI.2.6 LPS-induced acute lung injury mouse model

The *in vivo* LPS-neutralizing activity of HC10 was studied using an LPS-induced murine model of acute lung injury. LPS from *P. aeruginosa* (Sigma-Aldrich) dissolved in PBS was instilled intratracheally as described above at a concentration of 20 µg/mouse. Next, six mice received an intranasal treatment with 50 µg HC10 immediately after LPS administration, while the infection control group (n = 6) received PBS. A control group of 3 non-infected mice was included as well. Afterwards, the mice were put back in their cages and a FOB analysis was carried out at 2-h intervals for 8 h long. 24-h post LPS administration, the mice were humanely killed using an intraperitoneal pentobarbital injection (200 mg/kg, Kela). A FlexiVent™ system (SCIREQ) was used to study the lung

function of the mice, and afterwards, the lungs were collected during an autopsy for further qPCR and oedema analysis (see below).

VI.2.7 FlexiVent analysis

Selected mice were subjected to lung capacity measurements using FlexiVent™ FX equipment (SCIREQ, Canada), a system that can measure the respiratory mechanics of the airway tract and tissue (**Figure VI.1a**) [411]. After applying a volume-driven perturbation to the subject's airway, parameters such as pressure, volume and flow are measured by the equipment and analysed by the software [411]. This perturbation can be a single frequency oscillation waveform matching the breathing rate and tidal volume of a normal mouse (='snapshot'), or a combination of perturbations at varying frequencies ('prime perturbation') [412]. For the respiratory measurements, mice were euthanized with pentobarbital, intubated with a 19G blunt tip canula and connected to the FlexiVent™ ventilator (**Figure VI.1b**). Increasing concentrations of the bronchoprovocative agent methacholine (0 mg/mL – 50 mg/mL, Sigma) were delivered to the mice's lungs via nebulization, and various lung function parameters were characterized, including **i**) Newtonian airway resistance of the conducting airways (R_N), **ii**) tissue damping (G) which reflects the energy lost in the airway tissue due to friction, **iii**) elastance (E_{rs}), an index for airway stiffness, and **iv**) the total airway resistance (R_{rs}), which includes resistance from the conducting and peripheral tract, lung tissue and chest walls [412, 413].

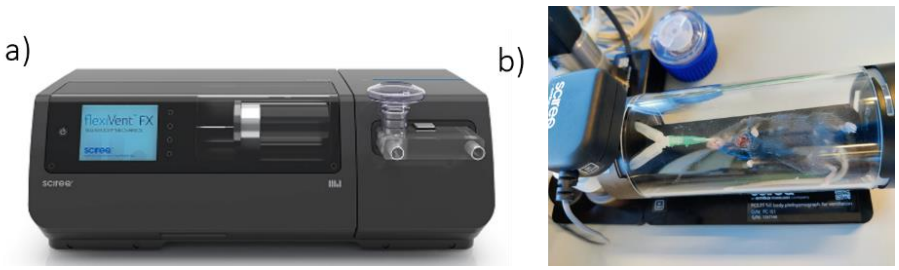


Figure VI.1 FlexiVent™ system. a) The FlexiVent™ equipment used for lung capacity measurements. **b**) The intubation platform where subject mice are connected to the system.

VI.2.8 RNA extraction and cytokine expression analysis by qPCR

To study the effect of HC10 on cytokine expression in the lungs, a qPCR analysis was conducted. Briefly, during the autopsy the post-caval lobe and the inferior lobe of the right lung were collected and stored in RNAlater® (Sigma) at -80°C. Before RNA extraction, the RNAlater® was removed and the lungs were homogenized in 1 mL of TRIzol® reagent using a Tissueruptor (Qiagen). The RNA was extracted following the standard manufacturer's protocol, and RNA concentration was determined using a Nanodrop™ 2000 system (Thermo Scientific). Afterwards, the RNA samples were treated with ezDNAse (Invitrogen) and cDNA was constructed using the Superscript IV reverse transcriptase (Invitrogen). The SensiFAST SYBR® No-ROX One-Step kit (Bioline) was used to amplify the TNFα and IL-6 genes, using mouse-specific primers (see V.2.10). *GAPDH* was used as the house-keeping gene control. The Lightcycler 480 II system and corresponding software (Roche Diagnostics) were used to perform the qPCR experiments, and settings were identical to those described earlier (V.2.9). Relative gene expression, normalized to *GAPDH*, was calculated using the Pfaffle method [344].

VI.2.9 Determination of bacterial burden

For the *P. aeruginosa* murine infection model, the bacterial burden was investigated 4 h post HC10 treatment. In short, the left lung, the left liver lobe, and the spleen were collected during the autopsy. Organs were mixed in 1 mL of PBS using a Tissueruptor (Qiagen), and serially diluted in sterile plastic tubes. Selected dilutions were plated on TSA agar plates. Plates were incubated overnight at 37°C and a standard plate count was performed to determine the bacterial burden.

VI.2.10 Determination of lung oedema

For the LPS inoculation model, the upper right lobe of the lungs was collected during the autopsy and weighed. Next, the lung lobes were places in Eppendorf tubes and heated at 60 °C for 72h. The weight after drying was compared to the starting organ weight to determine the relative water weight, indicative for oedema.

VI.2.11 Data analysis

All data were analysed and visualized using GraphPad Prism 9. For the *G. mellonella* experiments, Kaplan-Meier curves were constructed and analysed using log rank tests. For the mice experiments, the comparison of the bacterial burdens in the lung was done using a student's t-test. FlexiVent data from the LPS inoculation experiment were analysed using a one-way ANOVA test (normally distributed data with equal variances), while qPCR data were analysed using a Welch's ANOVA test due to unequal variances between the data sets.

VI.2.12 Ethics statement

All *in vivo* mice experiments were carried out in accordance with the guidelines of the University of Antwerp and the European Directive for Laboratory Animal care (Directive 2010/63/EU). The Animal Ethics Committee of the University of Antwerp reviewed and approved all mice experiments prior to the start of the study (file number: 2022-66).

VI.3 Results

VI.3.1 Inter-batch differences in AMP antimicrobial activity

During the course of the *in vitro* experiments, various batches of peptide were ordered at two different producers. While batch 1 and 2 showed comparable activity values (MIC values of both peptides in the low micromolar range), batch 3-4 of the HC1 peptide performed remarkably worse against *P. aeruginosa* with a sharp increase in MIC value (16-32 µM) (**Figure VI.2**). Notably, batch 5 that was ordered simultaneously with batch 4, showed results comparable to the earlier batches. The divergent activity of HC1 for batch 3 and 4 were communicated to Proteogenix, but no conclusive answer to the decrease in antimicrobial activity was found. As the inter-batch difference in activity was less outspoken for HC10, this peptide was chosen for the following *in vivo* experiments.

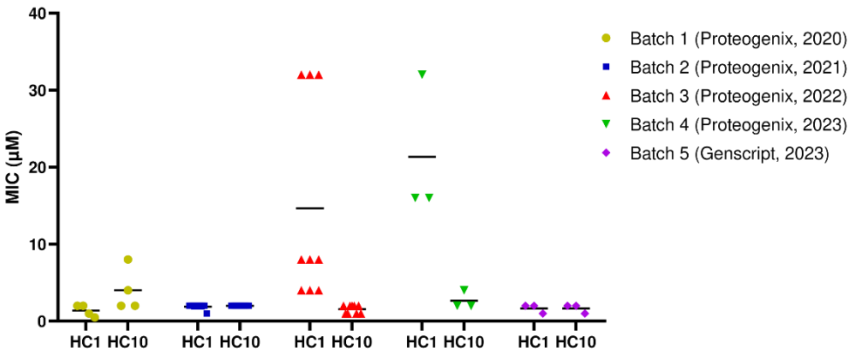


Figure VI.2 Anti-pseudomonal activity of five different batches of HC1 and HC10. After each arrival of a batch of new raw peptide material, the activity against *P. aeruginosa* was investigated. Batch 3 and 4 (Proteogenix) both showed a decrease in antimicrobial activity for the HC1 peptide, while the activity of HC10 remained unaffected. All other batches had comparable, consistent minimum inhibitory values.

VI.3.2 *Galleria mellonella* infection model

The anti-pseudomonal activity of HC10 was investigated in a *G. mellonella* model by monitoring larval survival over a 48-h timespan post bacterial infection. The AMP treated group had significantly improved survival with 80% of larvae being alive after 48 h, compared to only 20% of larvae in the infected control group (Figure VI.3a, VI.3b). The peptide control group (non-infected, HC10 treated) did not show any mortality or signs of melanisation during the experiment, indicating absence of acute larval toxicity.

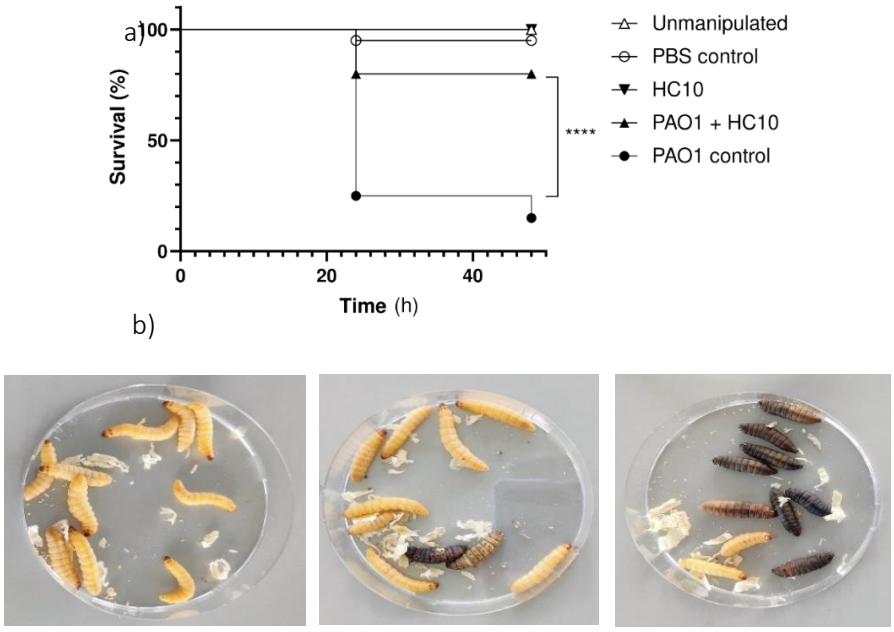


Figure VI.3 Activity of HC10 in a *P. aeruginosa* infection model of *Galleria mellonella*. a) Larvae were (**i**) infected with *P. aeruginosa* PAO1 at a dose of 10 CFU/ larva, (**ii**) infected with *P. aeruginosa* and treated with 10 µg HC10/larva, or (**iii**) only treated with HC10. A PBS control and a control of unmanipulated larvae were also included. The HC10 treatment significantly improved larval survival after bacterial infection. Data represent the survival (%) of two pooled groups of larvae (n = 20) and were analysed by a Mantel-Cox log ranking test. **** = $p \leq 0.0001$ **b**) *G. mellonella* larvae 24 h after infection (repeat 1): Non-infected, HC10 treated larvae (left), *P. aeruginosa* infected and HC10 treated larvae (middle), and *P. aeruginosa* infected control (right).

VI.3.3 Acute toxicity pilot study in a mouse model

Three different doses of HC10 were administered intranasally to investigate any intrinsic, unwanted toxic side effects of the AMP within a 24-h timespan. Group 1 received only PBS as control vehicle, group 2 received 15 µg HC10/mouse, group 3 received 50 µg AMP/mouse, and group 4 received the highest dose of 150 µg HC10/mouse. The dose of 15 µg/mouse did not cause any signs of illness or distress in any of the three mice per group (FOB score = 0 throughout the experiment). One mouse of group 2 (50 µg/mouse), however, showed mild to medium signs of discomfort, including increased breathing rate, with a maximum FOB score of four after 2 h. One mouse of group 4 (150 µg/mouse), developed serious symptoms of illness and distress, showing reduced breathing, a hunchback position, and overall poor condition and apathy, with a score of eight after 2 h (**Figure VI.4a**). This reduced wellbeing is also in line with the reduced consumption of water 24 h post-treatment in group 4. Presumably, the sick mouse drank less water, leading to a lower total water consumption (**Figure VI.4b**). Both mice with symptoms were visually recovered after 24 h. However, FlexiVent™ analyses indicated a long-lasting impact on all tested lung functions for one of the mice of the highest dose group. This mice showed a clear increase in lung elastance and tissue damping (**Figure VI.4c, 4d**) as well as total and conducting airway resistance (data not shown). Lung elastance (inversely related to compliance) is a measure of the stiffness of the total airway tract, and is measured using a snapshot (i.e. single frequency oscillation wave) protocol. Tissue damping, however, refers to the energy dissipated in the alveoli as measured with a prime perturbation protocol [412].

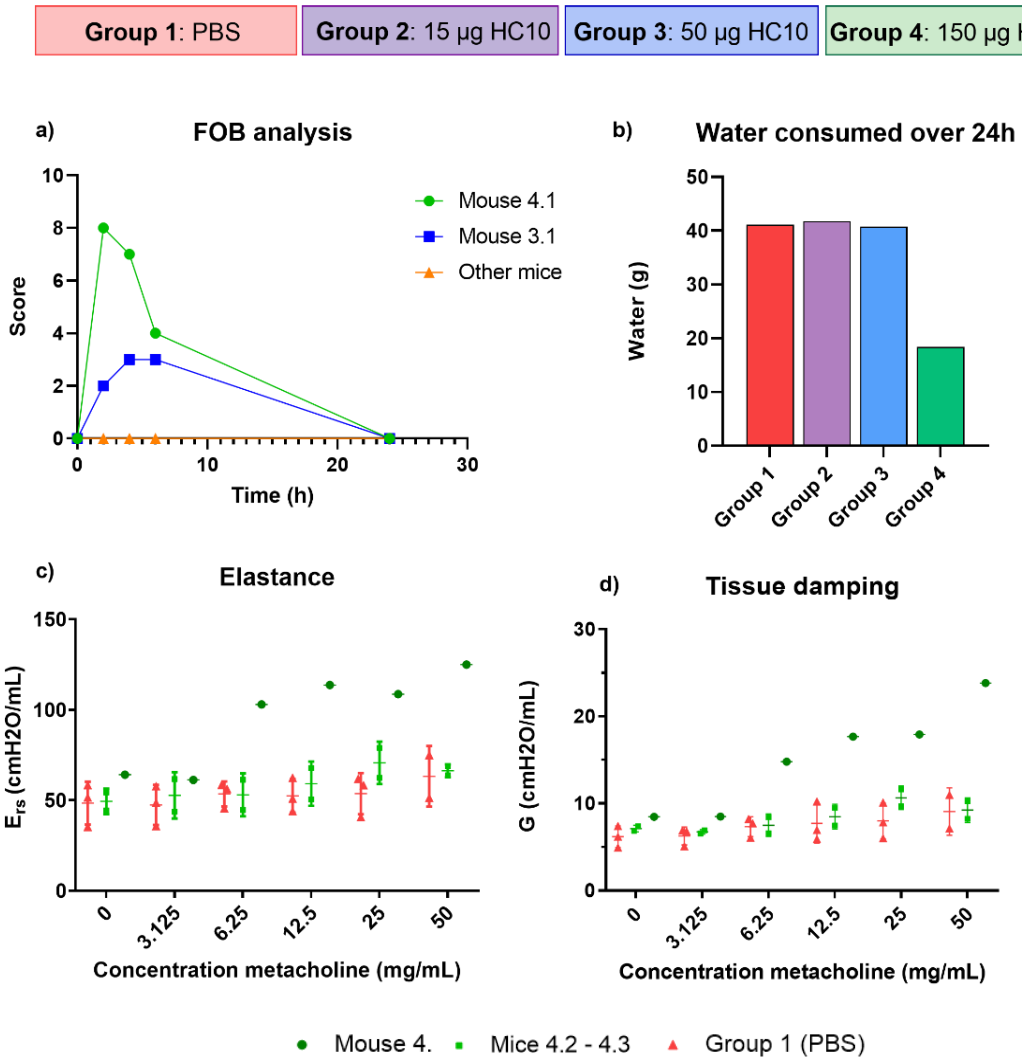


Figure VI.4 Acute toxicity pilot study. a) Evolution of the wellbeing of the treated and control mice 24 h after HC10 treatment, graded via a functional observational battery (FOB) scoring system. Group 4 received the highest dose of 150 µg HC10 /mouse, while group 3 received the middle dose of 50 µg/animal. One mouse from group 4 (mouse 4.1) developed serious symptoms of illness and stress, while another mouse from group 3 (3.1) developed mild symptoms of distress. **b**) Total water consumption (g) during the 24 h following HC10 treatment. **c**) Maximal respiratory elastance of the control group (group 1) and the mice treated with the highest HC10 dose (group 4). One mouse (4.1) showed increased tissue elastance compared to the other mice of group 4. **d)** Maximal tissue damping of the control mice (group 1) and the mice treated with the highest HC10 dose (group 4). One mouse of group 4 (4.1) showed increased tissue damping. All data are indicative and were not analysed statistically due to small sample groups.

QPCR analysis of TNFα and IL-6 expression in the lungs of group 4 did not show a marked increase compared to the PBS control group, suggesting that the effect on the lung capacity was not due to an inflammatory response caused by HC10. Mice and organ weight were also not affected in the HC10 treated groups (data not shown). Based on these observations, the middle dose of 50 µg/mouse HC10 was chosen for further *in vivo* experiments.

VI.3.4 Activity of HC10 in a *P. aeruginosa* lung infection model

Anti-pseudomonal activity of HC10 was investigated in a murine model of an acute bacterial lung infection. Two h after intratracheal infection with *P. aeruginosa* PAO1, the mice were treated intranasally with HC10 (50 µg/mouse), and 4 h after treatment, the effect of HC10 was investigated. The bacterial count in the left lung of the mice (n = 6) was significantly reduced by HC10 treatment by 0.80 ± 0.11 $\log_{10}$ ($p < 0.01$) compared to the infection control group (n = 6) (**Figure VI.5a**). The high bacterial load recovered in the left lung after 6 h of infection (± 5 x 10^7 CFU/g), and the dissemination to the liver (± 5 x 10^4 CFU/g), and spleen (± 2 x 10^4 CFU/g) illustrates the acute and severe character of the *P. aeruginosa* lung infection. The HC10 treatment did not have a significant effect on the bacterial burden in the liver and spleen (**Figure VI.5a**).

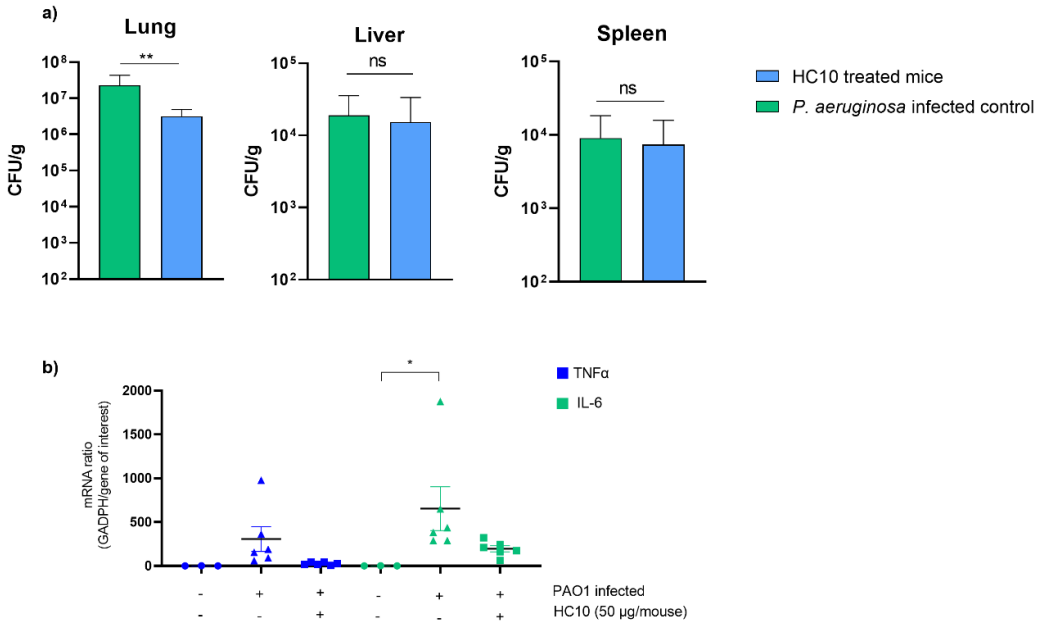


Figure VI.5 Antimicrobial activity of HC10 in an acute mouse model of a *P. aeruginosa* lung infection. a) The bacterial burden in the left lung, the left liver lobe and the spleen was investigated 4 h after HC10 treatment (6 h post-infection). HC10 reduced the bacterial count in the lung but did not prevent dissemination to the liver and spleen. Data represent the mean + standard deviation from six mice in each group and were analysed using a student's t-test. **b**) HC10 lowered the TNFα and IL-6 production in the lungs during bacterial infection, although the effect was not statistically significant. Data represent the individual data points, as well as the mean ± standard deviation from six mice in both the positive control and HC10 treated group, and 3 mice in the negative control group. Data were analysed using a Welch's ANOVA * = $p < 0.05$, ** =$p < 0.01$, ns = not significant

Next to the bacterial burden, the effect of HC10 on two pro-inflammatory cytokines was investigated. Although AMP treatment seemed to have a reducing effect on IL-6 and TNFα expression in the lungs, the effect was not statistically significant (**Figure VI.5b**). Notably, the infected control mice showed high variation in cytokine production. Organ weight and mice weight were also not affected significantly (data not shown). FlexiVent analysis was not carried out for this experiment.

VI.3.5 Activity of HC10 in an LPS-induced lung inflammation model

To verify whether the LPS-neutralizing activity, and hence, the anti-inflammatory effect, of HC10 is also present *in vivo,* a murine model of LPS-induced inflammation was used. Twenty-four hours after LPS administration, the effect of HC10 was investigated using FlexiVent analysis, oedema measurements, and qPCR. Mice that were administered 20 µg of LPS had a significant impairment of all tested lung parameters except the resistance of the conducting airways (Figure VI.6a-6d), and an upregulation of both IL-6 and TNFα compared to the PBS control mice (Figure VI.6e), indicating a successful LPS-induced lung injury. The HC10 treatment, however, did not significantly improve any of the lung functions, which all remained on the same level as the positive LPS control (Figure VI.6a-6d).

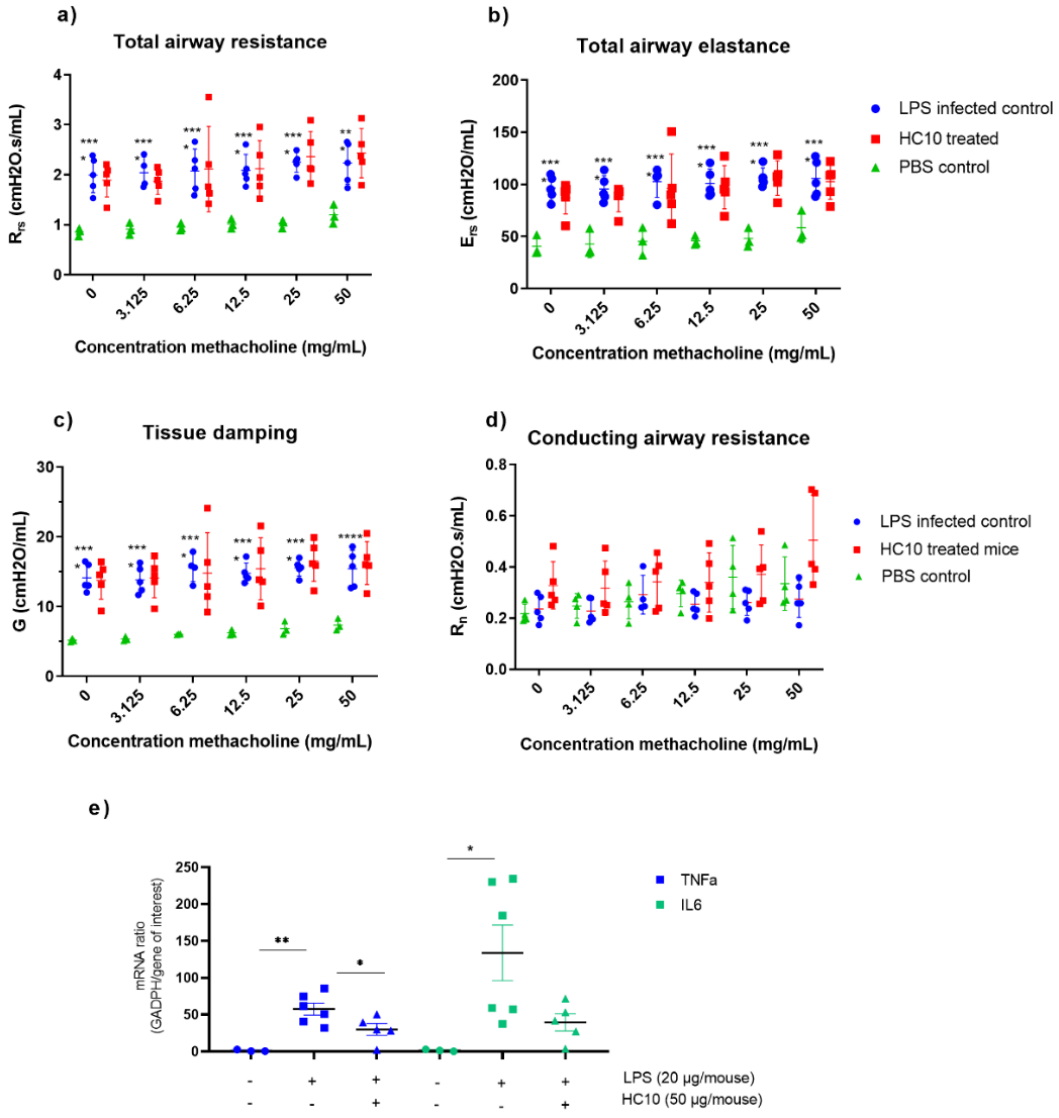


Figure VI.6 Activity of HC10 in a murine LPS-induced lung inflammation model. The effect of HC10 on the lung capacity and TNFα and IL-6 production in lung tissue 24 h after LPS administration. **a-d**) HC10 did not significantly improve any of the tested lung functions, including the total airway resistance and elastance, the tissue damping and resistance of the conducting airway. **e**) HC10 lowered the TNFα production in the lungs, but the effect was not significant for IL-6. Data represent the individual data points, as well as the mean ± standard deviation from six mice in the LPS control and HC10 treated group, and 3 mice in the negative control group. Data were analysed using a one-way ANOVA (FlexiVent data) or Welch's ANOVA (qPCR). * = $p < 0.05$, ** =$p < 0.01$, *** = $p < 0.001$, and **** = $p < 0.0001$.

HC10 did, however, have significant reducing effect on the TNFα levels in the lung tissue. A similar decreasing trend was seen for IL-6, but here the effect was not statistically significant. The effect on lung oedema, indicative for the presence of lung inflammation, was investigated by comparing the water weight of the upper right lobe between the HC10 treated and non-treated mice. However, no significant decrease of oedema was noted (data not shown). Overall, HC10 did not have major anti-inflammatory effects at a dose of 50 µg peptide/mouse after LPS administration, with only a minor decrease of TNFα levels but no improvement of any other tested lung parameters.

VI.4 Discussion

VI.4.1 Choice of peptide and dose for *in vivo* experiments

In this chapter, the *in vivo* anti-pseudomonal and LPS-neutralizing activity of HC10 was investigated. The HC10 peptide was chosen over HC1, as the latest batch of HC1 peptide at that time (batch 3, Proteogenix) had a significantly lower activity against *P. aeruginosa*, while this inter-batch difference in antibacterial activity was not seen for HC10. Noticeably, the next batch ordered at Proteogenix (batch 4) showed the same decrease in activity for HC1, while a batch ordered simultaneously at a different producer (Genscript), had activity values for both AMPs comparable to those of the earlier batches (1-2). These inter-batch differences can significantly complicate the research on AMPs. Often, increased self-aggregation of a peptide can explain a lower batch activity. AMP aggregation can be influenced by the presence of both peptide and non-peptide impurities, such as the TFA used for resin cleavage [414]. Moreover, process parameters such as temperature, pH, agitation, pressure and the lyophilization process can influence peptide aggregation [414]. However, sample purity was comparable for all peptide lots (>90%), and no changes in the synthesis protocol were reported. In addition, this change in activity was not observed for HC10. The reason behind the inter-batch differences in activity for HC1 remain unresolved. For the peptide dosage, the middle dose of 50 µg/mouse was chosen. Only moderate signs of distress were noted in one mouse after AMP treatment at this dose. Adverse effects were mostly breathing-related (e.g. difficulty

breathing after intranasal instillation), which was confirmed by subsequent FlexiVent analysis for the highest dose group (150 µg/mouse). As AMPs are amphiphilic compounds, they have a high surface tension activity with a tendency to foam in solution. Hence, the consistency of the highly concentrated AMP solutions might be responsible for the noted adverse effects, due to interference with the normal breathing mechanics.

VI.4.2 Activity of HC10 in *Galleria mellonella* model

In a first step of the *in vivo* experiments, the activity of HC10 was evaluated in a *G. mellonella* infection model. Larvae treated with HC10 had a clear, increased survival compared to the *P. aeruginosa* infection control. The increased survival of the HC10 treatment group indicates that the AMP successfully lowered or eradicated the initial bacterial inoculum and retained activity in the hemolymph environment of the insect. In addition, treatment with HC10 alone did not decrease larval survival, signifying absence of acute toxicity in the larval model. Regardless of a positive experimental outcome, results should be carefully interpreted; the administered AMP dose was relatively high (10 µg/larva or 50 µg/g bodyweight), whereas the bacterial inoculum was low (10 CFU/larva), increasing HC10's chances at successful antimicrobial activity. However, compared to data published by Dean et al. (2011) who performed similar wax moth infection experiments with comparable set-up parameters (AMP and bacterial dose), HC10 performs better than the LL-37 peptide [408]. In this study, the AMP treatment only led to a survival level of 30% (D-LL-37), or 60% (LL-37) after 48 h, compared to a survival level of 85% for HC10. Survival rates for the infection control were similar. Toxicity and antimicrobial susceptibility noted in the wax moth model have been correlated for other drugs to those seen in mammals such as rodents and humans [406]. Nevertheless, the translational value of the model is limited, and larvae lack complexity necessary to study specific infectious conditions, such as bacterial pneumonia. Hence, the *G. mellonella* model is mainly used as an additional step in the transition from *in vitro* to *in vivo* assays, rather than a substitution of rodent models [406].

VI.4.3 Anti-pseudomonal activity of HC10 in a pneumonia mouse model

In a murine model of *P. aeruginosa* pneumonia, HC10 caused an average of 0.80 ± 0.11 log_{10} reduction (= 84 %) of bacteria in the lungs after a 4-h treatment window at a dose of 50 µg/mouse. Bacterial dissemination to the liver and spleen still took place and mice showed clear signs of septic shock. This indicates that HC10 mostly acts locally after intranasal treatment, and that at a high bacterial inoculum (3 x 10^6 CFU/mouse) HC10 is not able to reverse the progressive, acute infection caused by *P. aeruginosa*. Longer lasting infection models could be of use to study the antimicrobial kinetics of HC10 over time, as the severity of the *Pseudomonas* infection did not allow continuation of the experiment past the 4 h treatment window. However, short timeframe studies are still highly relevant, especially for AMPs that are prone to proteolytic degradation and have a low residency time in the lungs. The antimicrobial activity of LL-37, for example, was reported to be lower after a 24-h timepoint than after 4 h of treatment [399]. In contrast, the proteolytic-stable Esc(1-21)—1c peptide was able to kill off more bacteria in a 24-h timespan due to its higher enzymatic stability (Table VI.1) [399]. Nevertheless, despite the earlier reported notable decrease in activity in lung simulated conditions *in vitro*, HC10 still maintains significant antibacterial activity *in vivo*. Equating the antibacterial activity of HC10 to other *in vivo* tested AMPs is challenging, as differences in set-up of the mouse model (e.g. mouse strain, AMP dose, application route and treatment duration), complicate an accurate one by one comparison. For reference, the performance of other α-helical AMPs after a single dose in murine models of *P. aeruginosa* pneumonia and relevant parameters are listed below in Table VI.1.

Table VI.1 Anti-pseudomonal activity of various α-helical peptides tested in murine models of *P. aeruginosa* lung infections. The log_{10} reduction in bacterial count in lung tissue is given as a parameter for antimicrobial efficiency. Relevant experimental parameters are included in the table for comparison. * = estimated, calculations based on graphical information.

AMP	Log reduction (CFU) in lungs	Treatment time (h)	Application route	Dose (µg/mouse)	Strain	Reference
HC10	0.80	4	Intranasal	50	PAO1	This study
Esc(1–21)	0.81*	4	Intratracheal	2	PAO1	[399]
Esc(1–21)-1c	0.81*	4	Intratracheal	2	PAO1	[399]
	2	24				
LL-37	0.57*	4	Intratracheal	2	PAO1	[399]
	0.60*	6		1		[415]
	0.30*	24		2		[399]
	0.40*	24		1		[415]
D-BMAP18	No activity	24	Intratracheal	10-40	RP73	[398]
WLBU2	0.90	6	Intratracheal	1	PAO1	[415]
WLBU2	1.43*	24	Intratracheal	1	PAO1	[415]
	0.92*	24		1		[416]
D8	1.05*	24	Intratracheal	1	PAO1	[416]

Compared to other studies, we were the only ones to opt for an intranasal AMP treatment as opposed to intratracheal administration. Intranasal treatment was chosen for its less invasive character. However, with intranasal drug delivery, not all of the administered peptide will reach the targeted region of the lower respiratory tract and lungs [417]. Reports on lung penetration after intranasal delivery range from 48% to 19% [417, 418]. Moreover, intranasal delivery can lead to more variable lung deposition and increased uptake via the stomach [417, 418]. Hence, it can be expected that only a

fraction of the 50 µg HC10 administered to the mice reached the lower regions of the lungs, making accurate comparison with other studies difficult.

VI.4.4 *In vivo* anti-inflammatory activity of HC10

The effect of HC10 on the production of inflammatory cytokines TNFα and IL-6 in the left lung was investigated in both the *P. aeruginosa* and LPS murine models. In both models, HC10 showed a reducing trend on TNFα and IL-6 production. In the *P. aeruginosa* pneumonia model, inflammation was strongly present, as shown by the upregulation of TNFα and IL-6. Mice also showed clear outward signs of inflammation (e.g. piloerection, shivering, and lethargy) [419]. HC10 was able to mitigate the inflammation effect to some extent (although not significantly), but it was not able to completely reverse the disease progression at the tested dose. Noted anti-inflammatory effects of HC10 can be caused by either the antibacterial killing or the additional LPS-neutralization. Next, the effect of HC10 was tested in an LPS-induced model of acute respiratory stress. Inflammation induced in this model was lower than that seen in the bacterial infection model. HC10 was administered immediately after LPS to mimic the conditions tested earlier *in vitro*. TNFα and IL-6 production was lowered, although the effect was not significant for IL-6. Nevertheless, the cecropin AMP seems to retain at least part of its LPS-neutralizing activity *in vivo*. Discrepancies between the *in vitro* and *in vivo* effect can be explained by either i) the lower lung penetration after intranasal treatment, ii) the AMP-salt interactions in the physiological conditions of the lungs, iii) biological degradation of HC10 in the lungs, and iv) dose differences in HC10 and LPS between *in vitro* and *in vivo* studies. *In vivo* anti-inflammatory activity has been noted for other α-helical AMPs as well (including LL-37), but activity is mostly tested in systemic, LPS-induced models of sepsis [420, 421].

VI.4.5 Concluding remarks

The activity of the cecropin peptide HC10 was investigated in (i) a *P. aeruginosa* infection model of *G. mellonella*, (ii) a mouse model of an acute *P. aeruginosa* lung infection, and (iii) in an LPS-induced mouse model of lung inflammation. HC10 retained antibacterial killing activity against *P. aeruginosa in vivo.* A mild decreasing trend on the pro-inflammatory cytokine production of TNFα and IL-6 was noted in the mice models as well, although the overall effect was less outspoken then earlier measured *in vitro*. Nevertheless, HC10 retains some of its promising activity against *P. aeruginosa in vivo*, highlighting its potential as a starting point for further anti-pseudomonal drug research and development.

Table SVI.1 Functional observational battery sheet

I. Pick up cage, don't touch animals

- 1) Body position:
  0: (S): Sitting or normally standing (walking, exploring)
  0: (R, rearing): Standing on hind limbs
  1: (hunchback): Back is rounded, even when walking
  2: (apathic): sitting or lying (not asleep) but without interest in surroundings
  3: (lying): Lying on side
  4: (flattened): Animal is spread out with abdomen pressed to floor
  5: (catalepsy): Animal is in a cataleptic-like state, must maintain an unnatural
  posture
- 2) Respiration:
  0: Normal
  1: Tachypnea or bradypnea
  2: Weak breathing (breathing very little)
- 3) Fur condition
  0: Normal
  1: Slightly raised/rough hair (localized)
  2: Overall bad fur-condition
- 4) Overall condition:
  0: normal appearance
  1: poor appearance
  2: very poor appearance
- 5) Occurrence of stereotype behaviour or abnormal behaviour? (no=0, yes=1)
  Stereotype: turning around, squeaking, shaking head, and other repetitive behaviour.
  Abnormal: e.g., squirming, running backwards, labored movement, disregarding stereotypical activity.

II. Touch/Pick up animal

- 6) Palpebral closure:
  0: Normal (eyes are open)
  1: Slightly sagging/half shut (eyelids slightly lowered)
  2: Shut (eyelids are closed)

Table SVI.1 Functional observational battery sheet

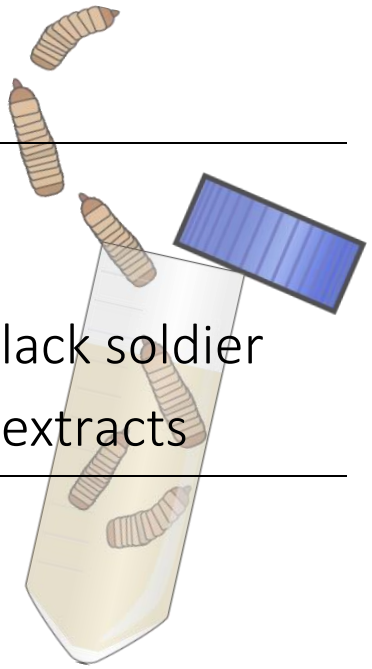
Involuntary movement/nerve symptoms:
0: None/normal
1: Head tremor (when picked up)
2. Tilted head or circular pacing
3. Head tremor (spontaneous) or extended contraction of limbs (rigid)
4. Generalized tremors or sudden jumping (all limbs off the floor)
5. Seizures/convulsions

- 8) Handling/state of activity:
  0: Normal resistance (none-slight)
  1: Overly excited/aroused
  2: Apathic, can be picked up easily without resistance or attempts to escape

Humane endpoints are applied when mice score >9 on the FOB analysis, or when an acute weight loss of >20% occurs.

CHAPTER VII

Antimicrobial activity of the black soldier fly's larval gut symbionts and extracts



Parts of this chapter are adapted from:

(**i**) Gorrens, E., Van Looveren, N., Van Moll, L., Vandeweyer, D., Lachi, D., De Smet, J., Van Campenhout, L., *Staphylococcus aureus* in Substrates for Black Soldier Fly Larvae (*Hermetia illucens*) and Its Dynamics during Rearing. Microbiology Spectrum, 2021. 9(3): p. e02183-21.

(**ii**) Gorrens, E., Van Moll, L., Frooninckx, L., De Smet, J., Van Campenhout, L., Isolation and Identification of Dominant Bacteria From Black Soldier Fly Larvae (*Hermetia illucens*) Envisaging Practical Applications. Frontiers in Microbiology, 2021. 12.

VII.1 Introduction

The BSF transcriptome has proven to be a valuable source of new, active AMPs. Apart from transcriptome analysis, other techniques have been successfully applied to exploit the BSF for the discovery and characterization of antimicrobials. Insect extracts can be useful to discover not only active peptides, but also non-gene encoded compounds of interest such as lipids and chitin [422]. Lauric acid and monolaurin, for example, have been identified in BSFL extracts and have marked, broad-spectrum microbicidal activity [422-424]. Chitin is a structural component of insects' exoskeleton and its deacylated form chitosan also has broad spectrum antibacterial activity [425, 426]. Extracts can be prepared from fully body insects and larvae or, when isolating AMPs, from the hemolymph. As the production of AMPs can be upregulated by microbial contact, immunization of the larvae before extraction (e.g. by inoculating their feed or by direct injection with pathogens) can increase the peptide content in the extracts significantly [422]. Extracts can be of interest for the isolation, purification and characterization of the active compounds, or they can be used for downstream applications as a whole. Insect extracts are often investigated as potential feed and/or food additives for their antimicrobial, and thus preservative, functions [427, 428]. Interestingly, recent notions of the antimicrobial activity of BSF frass have appeared [429]. Frass is the left-over residue after insect rearing, including insect excrements, feed substrate, and insect parts [429].

Apart from the insect and their frass, the microbes living in symbiotic relationships with the BSF can be studied for their antimicrobial properties as well. As detailed in chapter II, many bacterial and fungal families are known producers of antimicrobial compounds. Soil *Streptomyces* bacteria, for example, have delivered many antibiotics used in clinical practice today. [430] Currently, however, focus is shifting to less mined microbial niches such as insect symbionts [3]. Microorganisms living in or on insects can be involved in their defence against foreign invaders during all stages of life, from pupal development to adult insect [3]. Only one study on the antimicrobial activity of BSF symbionts was

published prior to the work in this chapter. Here, Correa et al (2019). identified anti-staphylococcal activity for the fungal BSF symbiont *Chrysosporium multifidum* and isolated the related compounds, being α-pyrone derivatives and a diketopiperazine [431]. The microbiome of the BSF has been the focus of countless studies, and core gut genera have been described, with *Enterococcus*, *Morganella*, and *Providencia* being the most prevalent [432]. A screening of a large BSF symbiont collection, however, had not yet been reported before the start of this work. Since then, however, more studies reporting on the antimicrobial activity of a large collection of BSFL symbionts have surfaced, including one by Tegtmeier et al. (2021) [433].

In this chapter, the antimicrobial activity of a previously established collection of highly abundant gut symbionts of the BSF is tested. In addition, BSF larval extracts are probed for their antimicrobial activity. A first set of extracts is produced in-house by subsequent chemical extraction, while another set of extracts has been acquired via an industrial collaborator.

VII.2 Materials and methods

VII.2.1 Black soldier fly rearing

The BSFL used for most of the experiments in this chapter were obtained from the on-campus laboratory scale rearing facility (Sustainable Biomass and Chemistry, Thomas More University of Applied Sciences, Geel). Details of the rearing cycle have been described previously by Gorrens et al. (2021) [434]. Briefly, at day 0, 1 g of BSF eggs were added to 200 g of nursing substrate, consisting of chicken feed (AVEVE, Leuven) and tap water in a 1:1 ratio (w/v). Unhatched eggs were taken out of the rearing trays 4 days later for maximal larval age uniformity. Then, larvae were transferred to a new rearing box with 600 g nursing substrate [chicken feed and water in a 60:40 ratio (w/v)]. After 8 days, the instar larvae were divided over three separate smaller trays with 100 g of fresh substrate (either chicken starter feed or a fiber-rich substrate), to a larval density of 3.3 larvae/cm² (± 500 larvae/box). From here on, 100 g substrate was administered to the

larvae every 2 days until day 16. Larval samples (30 g) were taken at day 8 and day 16 (Figure VII.1). All rearing trays were covered with a mesh lid to allow air flow. Larvae were reared in a climate chamber (Pharma 600, Weiss Technik) at a temperature of 27 °C and 60% relative humidity [434].

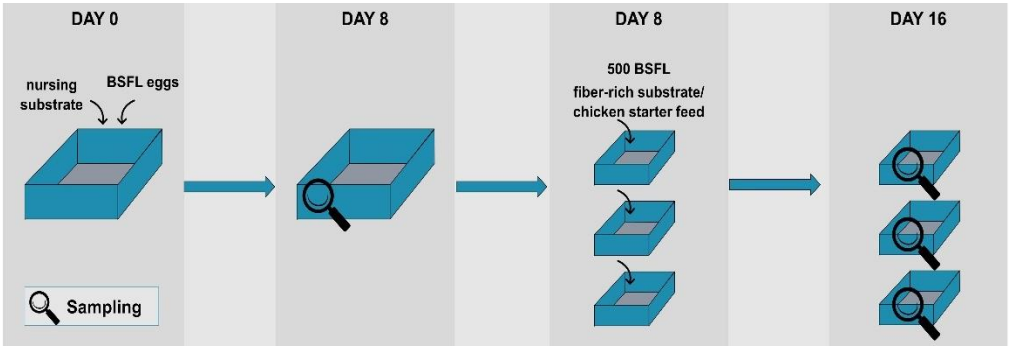


Figure VII.1 Rearing and sampling strategy of the black soldier fly larvae, Gorrens et al. (2021) [434]. Larvae were sampled either on day 8 and 16 (for the collection of gut symbionts) or only on day 16 (for the production of larval extracts) of the rearing cycle.

VII.2.2 Isolation of abundant gut microorganism from the black soldier fly[1]

Isolation of highly abundant gut symbionts of the BSF has been detailed earlier in Gorrens et al. (2021) [434]. Samples of 30 g of larvae were taken either at day 8 or at day 16 of the rearing cycle. Next, larvae were surface-disinfected using 70% ethanol as described by Wynants et al. (2018) [435]. The BSFL were then aseptically homogenized in PBS with a home type mixer (Bosch CNHR 25). Serial dilutions (1/10) from all larval samples were prepared and plated on Plate Count Agar (PCA, Biokar Diagnostics). Only dilutions ranging from 10^{-5} until 10^{-9} were used, as the goal was to focus on the abundant (and thus dominant) microbes present in the BSF gut. Agar plates were incubated at 30 °C up to 3 days. Five to ten morphologically different colonies were selected with sterile inoculation loops and streaked onto new agar plates. These plates were then incubated at 30 °C for 24–48 h. Next, one individual colony was picked from each plate and inoculated in Luria Bertani (LB) broth (10.0 g/L peptone (Biokar Diagnostics), 5.0 g/L yeast extract (VWR),

[1] Work mentioned in VII.2.2 was performed by a fellow ENTOBIOTA researcher (Ellen Gorrens)

5.0 g/L NaCl), and grown overnight at 30 °C shaking (Orbital mini shaker, VWR) at 150 rpm. Glycerol stocks were prepared and stored at -80 °C.

VII.2.3 Antimicrobial activity screening of black soldier fly gut symbionts

To determine the antimicrobial activity of the BSF gut symbionts, isolates obtained in VII.2.2 were screened against a broad panel of microorganisms, including: *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis, BCCM LMG 18735)*, P. aeruginosa* (PAO1, ATCC 15692), *S. aureus* (BCCM LMG 8064)*, B. cereus* (BCCM LMG 6923) *and C. albicans* (ATCC MYA-5876). Prior to the activity screen, the bacteria were grown overnight in LB broth, shaking at 150 rpm at 37 °C (Orbital mini shaker, VWR)). The next day, turbidity of the cultures was adjusted to a McFarland unit of 4 (± 10^9 CFU/mL). Antimicrobial activity was determined by a deferred inhibition assay in the version of Lee et al. (2008) with a few modifications [436, 437]. Briefly, isolates were spotted on PCA using sterile toothpicks at a ratio of four isolates per plate. The agar plates were incubated 2-3 days at 30 °C to allow the isolate to grow. After incubation, the bacteria were added to the plates using a soft agar overlay technique: soft LB agar 0.75% (w/V) was inoculated with the desired indicator strain at 5% (V/V) and added homogenously on top of the isolate spots. After solidification, the agar plates were incubated for another 24 h at 37 °C. Afterwards, zones of inhibition were observed and scored based on both their diameter and clarity. To determine the diameter of the zone, the diameter of the isolate spot was subtracted from the total diameter of the inhibition zone. The clarity was qualitatively assessed and defined as either high ('+++'), indicating absence of growth, medium ('++'), indicating presence of some colonies throughout the zone, and low ('+') indicating diffuse growth or the presence of many colonies throughout the zone. Each isolate was tested in biological duplicate.

VII.2.4 Identification of black soldier fly gut symbionts[2]

To determine the identity of the unknown BSF gut isolates, random amplification of

[2] Work of VII.2.4 was mostly performed by fellow ENTOBIOTA researchers (Ellen Gorrens and Jeroen De Smet)

polymorphic DNA (RAPD) was performed on the whole strain collection. Briefly, RAPD was performed on bacteria suspended in milli-Q water using the universal primer GTG5. PCR was carried out using DreamTaq polymerase (5 U/µl, Life Technologies), 10 nM deoxynucleotide triphosphates (Thermo Fisher Scientific), and DreamTaq Green buffer (Thermo Fisher Scientific). PCR settings included 10 cycles of denaturation (45 s, 95 °C), annealing (45 s, 36 °C), and extension (2 min, 75 °C), followed by 25 additional cycles with an annealing temperature of 50 °C. Post-cycle elongation was performed for 5 min at 72 °C. PCR products were visualized on a 1.5% agarose gel with SYBR Safe DNA gel staining (Thermo Fisher Scientific). Band fragment heights were identified using GelAnalyzer. Then, Matrix Assisted Laser Desorption Ionization Time-Of-Flight (MALDI-TOF) mass spectrometry was performed on isolates with different RAPD banding patterns for identification at Lavetan (Geel). *E. coli* and *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) were included as bacterial controls. The obtained spectra were compared against a spectral database (MBT Compass Library, Bruker). The spectral compatibility was expressed as a log-score between 0.00 and 3.00. A log-score of minimum 2.00 is needed for accurate identification at species level [438]. Isolates with a log-score below 2.00 were identified via amplification and sequencing of the 16S ribosomal RNA gene (bacterial isolates) or the fungal internal transcribed spacer region (fungal isolates) at Eurofins Genomics. The obtained sequence data were aligned by BioEdit software and a BLAST search against type materials in GenBank was performed.

VII.2.5 Preparation of black soldier fly larval extracts

Whole body extracts were prepared from 30 g fifth instar larvae (sampled at day 16 or rearing cycle), following methods adapted from Choi et al. (2012) and Park et al. (2014) [161, 439]. Larvae were disinfected and homogenized as described earlier (VII.2.2) in a cooled mixture of methanol/water/acetic acid (90/9/1 (v/v/v)) supplemented with 1 mM EDTA. The larval paste was homogenized in a stomacher bag for one minute and transferred to falcon tubes. The mixture was left to stand for 24 h at 4 °C to allow the extraction to take place. Next, the falcon tubes were centrifuged at 1600 g for 20 min

(Multifuge 3SR, Marshall Scientific) and the extract was collected after filtration in round bottom flasks. The larval mass left behind was subsequently extracted with ethyl acetate and dichloromethane. Next, the supernatant of the extract was evaporated using a Rotavapor (Buchi) at 40°C for 20 min. The remaining residue was stored at 4°C and later tested for antimicrobial activity (VII.2.6).

VII.2.6 Antimicrobial screening of black soldier fly larval extracts

BSFL extracts were tested for antimicrobial activity using an agar spot assay. The panel of tested microorganisms consisted of: *S.* Typhimurium (BCCM LMG 18732), *P. aeruginosa* (PAO1, ATCC 15692), *S. aureus* (BCCM LMG 8064), *E. coli* (BCCM LMG 2092), and *C. albicans* (ATCC MYA-5876). Bacteria were grown and diluted as described earlier and added to the plates using a soft agar overlay technique (see VII.2.3). Twenty µL of extracts were spotted on the PCA plates, and 4 technical replicates per extract were included per plate. Kanamycin (100 µg/mL) was included as a positive control. The solvent (methanol, ethyl acetate, or dichloromethane) was included as a vehicle control. Bacteria were grown and diluted as described earlier and added to the plates using a soft agar overlay technique (see VII.2.3). Plates were incubated overnight at 37 °C. Following incubation, the presence of any inhibition zones was noted visually. Experiments were carried out in biological duplicate.

VII.2.7 Antimicrobial screening of black soldier fly industrial stick water

In collaboration with an international project partner, the antimicrobial activity of industrially obtained BSFL water extracts was tested. Briefly, the company supplied (i) extracts in concentrated liquid form ('stick-water', 20% dry matter) and (ii) powder extracts obtained through spray drying (90% dry matter) the liquid extract. Details of the extraction protocol were not disclosed. Before antimicrobial screening, the bacterial burden of both extracts was first tested by a standard protocol. The dried extract was dissolved in sterile demineralized water at 40 mg/mL, and both extracts were plated on TSA. As bacterial contamination was detected, both extracts were filtered using 0.22 µm cellulose filters (Merck). To test antimicrobial activity of the extracts, the antimicrobial

screening assay detailed in III.2.3 was used. The microbe test panel consisted of *S. aureus* (ATCC 6538), *B. cereus* (ATCC 14579), *E. coli* (ATCC 8739), *P. aeruginosa* (ATCC 9027), and *C. albicans* (B59630). As references, doxycycline (Sigma-Aldrich; *S. aureus*, *E. coli*, *P. aeruginosa*, *B. cereus*), and flucytosine (Sigma-Aldrich; *C. albicans*) were used. Three technical replicates were included per assay, and two to three independent biological tests were performed. One batch for each extract was tested.

VII.3 Results

VII.3.1 Antimicrobial activity of black soldier fly gut symbionts

Prior to microbial identification, the antimicrobial activity of the culture collection of abundant BSFL gut microorganisms was tested qualitatively. In total 178 isolates were obtained (172 bacterial isolates, and 6 fungal isolates). From these isolates, the majority showed at least some indication of antibacterial activity (Table VII.1). Noticeable, activity against *C. albicans* was less present. Activity was graded based on (i) the clarity of the inhibition zone, and (ii) size of the inhibition zone. Antimicrobial activity, however, was highly variable; isolates later identified as the same species sometimes showed different results in the antimicrobial screening. Moreover, the inhibition zones were usually small and not completely clear (indicating only partial growth inhibition).

Table VII.1: Presence or absence of antimicrobial activity for the black soldier fly gut isolates

178 isolates	Activity?	
	Yes	No
S. aureus	104	74
B. cereus	91	87
S. Enteritidis	100	78
P. aeruginosa	111	67
C. albicans	42	136

Only eight isolates showed interesting, consistent antimicrobial activity, being: six fungal isolates (all later identified as *Trichosporon asahii* strains), and two bacterial isolates, both *Chryseobacterium arthrosphaerae*. The *Trichosporon* species showed strong activity

against both *S. aureus* and *C. albicans*, with remarkably large inhibitions zones (diameter 25 mm) of high clarity. *C. arthrosphaerae* had pronounced anti-pseudomonal activity, with a small (4 mm) yet clear inhibition zone (Figure VII.2).

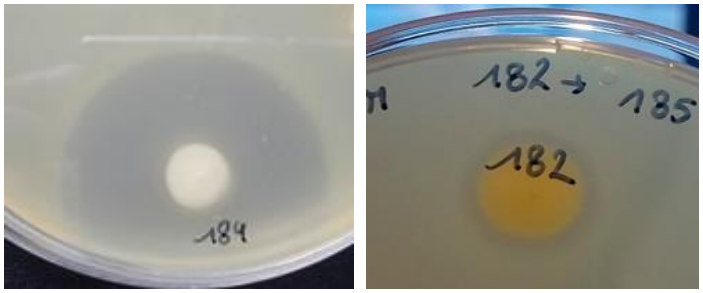


Figure VII.2 Antimicrobial screening of black soldier fly gut symbionts. Left: activity of *Trichosporon asahii* against *S. aureus*. The *Trichosporon* isolate showed a large and clear zone of inhibition. Right: *Chryseobacterium arthrosphaerae* showed a small, yet clear zone of inhibition against *P. aeruginosa*.

VII.3.2 Antimicrobial activity of black soldier fly extracts

Antimicrobial activity of full body BSF larval extracts was tested against a panel of Gram-positive and Gram-negative bacteria. Samples were prepared via sequential solvent-extraction using acidified methanol, ethyl acetate and dichloromethane, to collect compounds of varying polarity. Although the activity analysis was mostly quantitatively (i.e. absence or presence of activity), two parameters were included to grade the activity, being clarity and size of the inhibition zone (Table VII.2, Figure VII.3). The methanol extracts had strong activity against *S. aureus*, low activity against *C. albicans*, and no activity against any of the Gram-negative strains. The ethyl acetate extract had broad antibacterial activity, although activity was more variable among the different replicates. The dichloromethane extract had no clear antimicrobial activity.

Table VII.2 Antimicrobial activity of full body black soldier fly larval extracts. Activity of extracts was tested against a broad panel of microbes. Extracts were made in three different solvents (methanol = MeOH, ethyl acetate = EtAc, and dichloromethane = DCM). Activity was scored on both the clarity of the inhibition zone (+ indicating low clarity with growing colonies throughout the inhibition zone, ++ indicating medium clarity, and +++ indicating a completely clear zone with absence of microbial growth) and diameter of the inhibition zone. The inhibition zone size is given as the average ± standard deviation of all biological and technical replicates.

Indicator strain	MeOH		EtAc		DCM	
	Clarity	Size (mm)	Clarity	Size (mm)	Clarity	Size (mm)
P. aeruginosa	-	-	++	9 ± 3	-	-
E. coli	-	-	+	6 ± 2	-	-
S. Typhimurium	-	-	+	3 ± 3	-	-
S. aureus	+++	14 ± 1	++	12 ± 2	-	-
C. albicans	+	10 ± 1	-	-	-	-

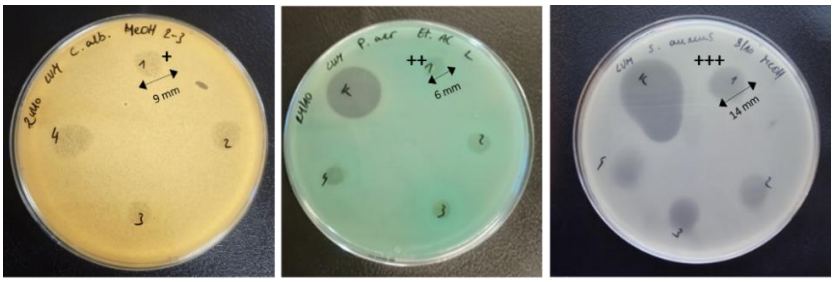


Figure VII.3 Visual representation of antimicrobial activity of larval extracts. Left: activity of methanolic extracts against *C. albicans*, with a low clarity (+). Middle: activity of ethyl acetate extracts against *P. aeruginosa* with medium clarity (++). Right: activity of methanolic extracts against *S. aureus* with high clarity (+++).

VII.3.3 Antimicrobial activity of industrial larval extracts

In a collaboration with an industrial partner, the antimicrobial activity of BSF larval extracts obtained on a large, industrial scale, was investigated. The extraction was obtained with water as a vehicle, and was delivered both in liquid and dried form. For the concentrated liquid extract, signs of activity against *S. aureus* were noted (Table VII.3),

with an indicative IC_{50} value of 38.40 ± 2.07 mg/mL. Due to divergent results in the third independent screening, an exact IC_{50} value could not be calculated. No activity was noted for the dried extract at the highest tested dose of 20 mg/mL, at which point the water solubility limit had been reached (data not shown).

Table VII.3 Antimicrobial activity of industrially obtained black soldier fly larval liquid extracts. Some degree of anti-staphylococcal activity was found, but activity against any of the other strains was absent.

	Activity (IC50[a]) of liquid extract (mg/mL)				
	S. aureus	*B. cereus*	*E. coli*	*P. aeruginosa*	*C. albicans*
Repeat 1	37.62	> 60	> 60	> 60	> 60
	39.39	> 60	> 60	> 60	> 60
	36.67	> 60	> 60	> 60	> 60
Repeat 2	36.05	> 60	> 60	> 60	> 60
	36.94	> 60	> 60	> 60	> 60
	36.78	> 60	> 60	> 60	> 60
Repeat 3	>60	ND[b]	ND	ND	ND
	42.36	ND	ND	ND	ND
	>60	ND	ND	ND	ND
Average ± SD	38.40 ± 2.07[c]	> 60	> 60	> 60	> 60

[a]: IC_{50} value = concentration leading to 50% growth inhibition of the tested organism, [b]: ND = not done

[c:] This is an approximation of the average IC50 value against *S. aureus*. Two technical repeats (> 60 mg/mL) of the third screening were left out to be able to calculate an IC50 value.

VII.4 Discussion

VII.4.1 Black soldier fly symbionts to the rescue?

Insects have proven to be a valuable source of a structurally diverse repertoire of antimicrobial agents. Not only the insects themselves, but also their symbiotic microorganisms have been gathering interest as producers of bioactive compounds [3]. A large collection of highly abundant gut bacteria of the BSF were screened for their antimicrobial activity using an agar overlay assay. The results showed two noteworthy trends. (i) The majority of the isolates presented some degree of antibacterial activity,

although inhibition zones were usually small and only partially clear. Microorganisms are known to possess several defence strategies against other, competing microbes. This can include the production of antimicrobial secondary metabolites, but zones of inhibition can also be caused by nutrient competition or toxic waste build-up [440-442]. (ii) High variation was seen in antimicrobial activity in between biological repeats for the same species. Inconsistent results can be explained by the nature of the agar overlay assay, as it is prone to experimental variation caused by, for example, poor drying of the isolate spot, deviations in concentration of the bacterial inoculum or non-homogenous application of the competitor strain [442]. Alternative assays often require the prior extraction of the microbial symbionts, being more time consuming and less accessible than the agar overlay screening [443].

Two symbiont species did show consistent zones of inhibition with high clarity. *C. arthrosphaerae* is a Gram-negative bacterium producing the yellow pigment flexirubin [444, 445]. Some proof of antimicrobial activity of flexirubin against *E. coli* and *B. cereus* has been reported. However, the underlying mechanism is as of yet not confirmed, and anti-pseudomonal activity has not been described either [446]. Thus, it remains unclear which compounds contribute to the clear halo noted in the antimicrobial activity assay. Similarly, little is known about the production of antibacterial metabolites by *T. asahii*. Recently, Ider et al. (2020) isolated a new AMP, called oranicin P16, from *T asahii*. Activity against the Gram-positive bacterium *Kocuria rhizophila* was reported [447]. Interestingly, the authors also tested culture supernatant of *T. asahii* against *S. aureus* and *C. albicans*, but found no activity here [447]. The significant activity against important human pathogens *S. aureus* and *C. albicans* seen in our experimental set-up, however, warrants the further study of the *Trichosporon* antimicrobial effects.

VII.4.2 Full body extracts of black soldier fly larvae

When focussing on the active compounds produced by the larvae themselves, two main approaches are usually considered. Via transcriptome analysis, gene-encoded antimicrobial compounds can be found [130, 164, 448]. This technique was explored in

the previous chapters and led to the characterization of the AMP library. Secondly, compounds of interest can be found through characterization and purification of insect extracts. In this chapter, focus was laid on the antimicrobial activity of whole-body extracts of non-immunized larvae. Acidified methanol was used as the main extraction solvent, as it has proven to be useful to extract small, polar compounds such as peptides from larval extracts [422, 439]. Notable activity against *S. aureus* was seen for the methanolic extracts. Similar findings have been previously reported by Choi et al. (2014). In their study, activity of methanolic extracts against *S. aureus* was attributed to the presence of hexanedioc acid. However, activity in our experiment could be caused by a multitude of other low-molecular weight compounds as well, and purification of the extracts was not performed [439]. Ethyl acetate and dichloromethane were mostly used for defatting purposes [422]. Interestingly, the ethyl acetate fraction showed broad spectrum antibacterial activity, a result that is in line with previous findings of Park et al. (2014) [439]. Ethyl acetate is a solvent with medium polarity, able to extract a wide spectrum of compounds [449]. No activity was seen for the dichloromethane extract, indicating that either (**i**) active lipids had been extracted by the ethyl acetate solvent or (**ii**) no active lipids were extracted in the chosen experimental set-up.

Another set of antimicrobial screening experiments was performed for industrially obtained BSFL extracts at the request of one of the project partners. This company is mostly focussed on exploiting BSFL as high-quality, sustainable feed for agriculture. The focal point of their research is the aquaculture of salmonids and shrimps. Salmonid and shrimp cultures are often impacted by the presence of fish pathogens (e.g. *Aeromonas* or *Vibrio* species) [450]. Hence, BSFL extracts with antimicrobial activity could be of interest as feed additives. Unfortunately, only mild anti-staphylococcal activity was found for the liquid extract, whereas salmonid and shrimp pathogens are mostly Gram-negative [450]. Interestingly, the dried powder derived from the liquid extract had no antimicrobial activity at the highest tested dose. This could indicate that the drying, atomization process negatively impacted the antimicrobial activity of the unidentified

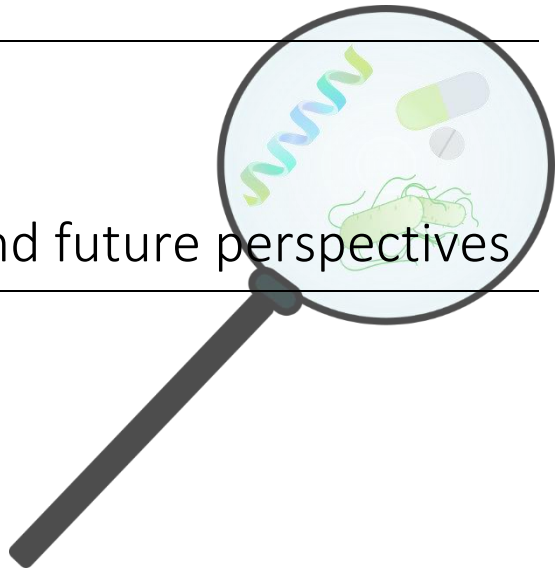
compound(s). Testing additional batches of extracts could prove useful to consolidate the antimicrobial activity findings.

VII.4.3 Conclusive remarks

In this chapter, antimicrobial activity of a collection of BSFL symbionts was investigated. The high antimicrobial activity against *S. aureus* and *C. albicans* of the fungal symbiont *T. asahii* was especially interesting. However, identification and/or purification of any related antibacterial compounds has not yet been successfully performed. In addition, two sets of BSFL extracts were screened for their antimicrobial activity. While our in-house methanolic and ethyl acetate extracts showed antibacterial activity, the industrial "stick water" extract did only exhibit very weak anti-staphylococcal activity, and the drying process of the extract likely negated the antibacterial activity.

CHAPTER VIII

General discussion and future perspectives



VIII.1 General discussion

Given the alarming rise of AMR, the research and development of new, innovative antimicrobial compounds remains pressing. Any new antibiotic should ideally possess some of the following characteristics: low propensity for resistance development, synergy with traditional antibiotics, have a new mode of action and/or belong to a new structural class, good pharmacokinetic and pharmacodynamic properties, and lack of toxicity [451]. Nevertheless, most recently approved antibiotics are derivatives or combinations of agents already in use and lack true innovation. Now, researchers are turning to underexplored (natural) sources in hopes to find the key to the AMR crisis. Research on insect-derived antimicrobials, for example, has seen a steadfast increase since the 90's (Figure VIII.1).

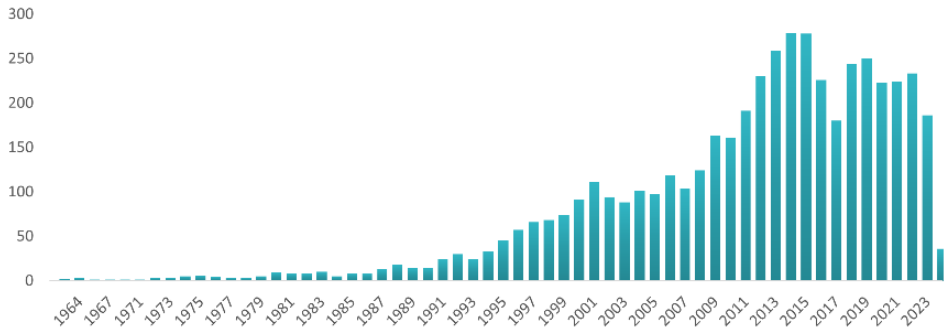


Figure VIII.1 Research articles indexed in PubMed containing the MeSH terms 'insect(s)' and 'antimicrobial'. With a total of 5.745 results, research on insect-derived antimicrobials has been significantly increasing. Although the search terms present a generalization, they give an indication of a changing research field.

In this thesis, the BSF has been the focal point of the research for new antimicrobials. As voracious and polyphagous feeders, the BSFL are able to efficiently decompose biowaste [452]. They also preferably lay eggs near a rotting food source [453]. Hence, the BSF is type example of an insect that comes into contact with a wide variety of microorganisms. Living in a close relation with microbes, the BSF has a remarkable infection resilience [166]. This has fuelled the hypothesis among researchers that the BSF could be an exceptional producer of antimicrobial compounds, including AMPs [130]. This thesis has

formulated two main research objectives that investigate this hypothesis more in detail (chapter I). First of all, the goal was to perform an **antimicrobial evaluation and biological characterization of BSF AMPs.** Secondly, a smaller research objective was created, being **the exploration of the antimicrobial potential of the BSF larval gut symbionts and larval extracts.**

VIII.1.1 Black soldier fly antimicrobial peptide library

In 2018, Vogel et al. described the AMP repertoire of the BSF for the first time using transcriptome analysis [130]. This research elucidated that the BSF has the second largest AMP library ever found in insects, and the largest of any dipteran species [130, 454]. The library consists of 66 peptide sequences in total, being 13 lysozyme transcripts and 53 AMP sequences [130]. Later research by Moretta et al. (2020) largely confirmed the size and content of this library by similar transcriptome experiments [164]. In a collaboration with the University of Giessen and the Fraunhofer Institute (Germany), a large part of the BSF AMP library was evaluated for its antimicrobial activity using a high-throughput screening platform (chapter III). Aside from the activity of some defensin AMPs against the Gram-positive *S. aureus* bacterium, the majority of activity was situated in the Gram-negative antibacterial spectrum. Near all tested cecropins (12 out of 13) showed potent activity against *E. coli* and *P. aeruginosa* in the low micromolar range. The remarkable activity against Gram-negative bacteria of the BSF cecropin family was confirmed in recent research by Peng et al. (2023). Interestingly, however, these authors describe a cecropin repertoire that is even more expansive than described in our research; they found 33 putative cecropin sequences through BSF genome analysis, and confirmed the transcription of 30 of these sequences [454]. Apart from their outspoken Gram-negative activity spectrum, absence of mammalian cell toxicity at antimicrobial concentrations was also reported. Noteworthy is also the absence of antimicrobial activity for a large part of the AMP library. As these peptides are still actively transcribed by BSFL, it is not likely that they result from functionally inactive pseudogenes [133]. Rather, they might have antimicrobial activity against microbes not included in the test panel, require

protein modifications not included in the chemical solid-phase synthesis protocol, or fulfil other immune or non-immune related functions [133, 136]. In addition, the chosen experimental set-up (including DMSO solubilization in the antimicrobial screening) might have influenced the peptides' natural folding state, and hence, their antimicrobial activity.

VIII.1.2 Selection of HC1 and HC10

To continue the biological characterization experiments, two peptides were selected from the cecropin family. As all cecropins had near-identical performance in the activity and toxicity tests, selection was made based on structural divergence of the peptides. HC1, a 44 AA long AMP, has a primary sequence much in line with the majority of other cecropins, ending in a -PQ motif. HC10, however, is 47 AA long and ends on a -QQRHA motif, unique within BSF cecropins. Interestingly, Peng et al. (2023) found that while all cecropins genes are situated on the BSF chromosome 5, HC10 is located on chromosome 3 [454]. Further experiments found absence of hemolysis for HC1 and HC10 at antimicrobial concentrations, the most common form of cell toxicity caused by AMPs [106]. Structural characterization has been performed mostly *in silico*. Prediction models with the highest confidence score forecasted a double alpha-helical structure for HC1 and HC10. This structure, with an N-terminal amphipathic helix and C-terminal hydrophobic helix, separated by a short hinge region, is characteristic for cecropins [455, 456]. Explorative CD experiments confirmed the double alpha helical-character of HC1, whereas HC10 took on a random coil formation. In cecropins, a confirmational change is often induced by the proximity of biological material such as the bacterial membrane. However, addition of LPS seemed to have no immediate effect on the HC10 structure in the preliminary CD experiments. The exact condition-dependency of the cecropin's secondary structure, including the effect of salt on the AMPs confirmation, remains to be elucidated. Next, membrane permeabilization experiments were performed. Results confirmed that HC1 and HC10 are able to target and permeabilize both outer and inner bacterial membranes. Bactericidal concentrations of AMPs were correlated to

concentrations where a high degree of membrane damage occurred. Thus, membrane permeabilization is likely an import part of the AMPs' mechanism of action, although additional (intracellular) mechanisms of action cannot be ruled out.

VIII.1.3 Anti-pseudomonal activity of HC1 and HC10

For the remainder of the *in vitro* and *in vivo* characterization experiments (Chapter IV - VI), *P. aeruginosa* was chosen as the pathogen of interest. As an opportunistic pathogen, *P. aeruginosa* is mostly a risk to immunocompromised and hospitalized people. Infections usually target the lungs, but other types of infections (e.g. skin, blood or urinary tract infections) are also seen. This bacterium is notoriously difficult to combat in the clinical practice despite available treatment regimens. *P. aeruginosa* possesses a remarkable intrinsic resistance to antibiotics and a large set of virulence factors [252]. In addition, AMR is rising significantly. Currently, the peptide antibiotic colistin is used as a last-resort treatment of *P. aeruginosa*, often in combination with other drugs [267]. However, colistin is known to come along with several toxic side effects including nephrotoxicity [457]. Anti-pseudomonal peptides without this toxicity could gain a prominent place in the treatment and/or prevention of *P. aeruginosa*. Apart from HC1 and HC10, AMPs with anti-pseudomonal activity have been described in the past and were recently summarised by Chen et al. (2023) [273]. Although most of these peptides work at least partially through membrane-targeting mechanisms, others can counteract *P. aeruginosa* virulence. For example, the human LL-37 reduces quorum sensing, whereas the bacteria-derived acidocin reduces release of pyoverdine, pyocyanin, elastase and *P. aeruginosa* proteases [458]. Anti-biofilm activity is also an important asset of new anti-pseudomonal therapies. Biofilm production greatly contributes to *P. aeruginosa's* disease burden, and many conventional antibiotics perform poorly when added to bacterial biofilms [459]. HC1 and HC10 did not manage to significantly eradicate *P. aeruginosa* biofilms, although they managed to prevent biofilm formation in a concentration-dependent manner (chapter IV). The lack of biofilm eradication is likely due to matrix interference, such as electrostatic interactions with alginate. Not all cationic, helical AMPs are prone to these

interactions to the same extent. The mouse-derived cathelicidin peptide (CRAMP), for example, is able to completely eradicate a *P. aeruginosa* biofilm at 20 µM [460]. Both HC1 and HC10, however, do retain notable activity against *P. aeruginosa* persisters, which play an important role in the antibiotic tolerance of biofilms (chapter IV) [459]. At bactericidal concentrations (≥ 8 µM) both HC1 and HC10 caused at least a 2 log reduction when added to the persistent bacteria that had been previously treated with a high dose of ciprofloxacin.

Apart from biofilm killing, other AMP aspects could be useful when treating *P. aeruginosa* infections. In chapter IV, synergy of HC1 and HC10 with conventional antibiotics and resistance induction were additionally studied. In contrast to the general understanding of membrane permeabilizing AMPs, no clear synergistic action with traditional antibiotic classes was found for HC1 and HC10 (FIC > 0.5). In a recent overview by Wesseling et al. (2022), the synergy of polymyxins and AMPs with antibiotics against Gram-negative bacteria was highlighted [461]. Various AMPs have FIC values well below 0.5, the general accepted cut-off value for synergy [461]. Here, the membrane perturbing mechanisms of the cationic peptides is voiced as the underlying cause of synergy. These widespread proclamations of AMP synergy seem to be in contrast not only with our findings, but those of He et al. (2015) as well [282]. In general, there is a need for more AMP specific, optimized synergy assays as well as uniform guidelines on FIC interpretation and reporting when using the checkerboard assay. Repetition of synergy experiments by independent laboratories (i.e. ring-trials) could be helpful to decipher the true degree of synergy of AMPs. Next to synergy, resistance induction was investigated. Here, it was confirmed that HC1 and HC10 indeed do not give rise to spontaneous resistance induction in *P. aeruginosa* after 20 days of consecutive exposure. Although these results are indeed promising, this does not mean that AMPs are completely exempt from any form of resistance occurring over time. It has, for example, been shown that by structural modification of LPS, Gram-negative bacteria can become less susceptible to cationic peptides [321].

VIII.1.4 Neutralization of bacterial endotoxins

LPS is an important structural component of the outer layer of Gram-negative bacteria, while also functioning as a virulence factor. LPS can trigger the immune system and set an inflammatory cascade into motion. As many AMPs target Gram-negative bacteria via binding to LPS, these interactions can have a positive effect on the infection outcome by mediating the LPS-related inflammation. Hence, the specific LPS-binding of HC1 and HC10 and effects on pro-inflammatory parameters was investigated in chapter V. A concentration-dependent LPS binding of both AMPs has been found, and this LPS interaction also leads to a lower *in vitro* macrophage activation. These characteristics could be valuable when treating LPS-driven inflammatory conditions such as sepsis [462]. A general LPS interaction model has been proposed by Chu et al. (2020) for cecropins with a helix-hinge-helix structure [463]. Here, LPS contact is first established via electrostatic interactions between the N-terminal amphipathic helix. Then, the C-terminal hydrophobic helix will insert itself within the lipid A part of LPS through hydrophobic interactions [463]. The AMP-LPS binding, however, can be a double-edged sword. For some AMPs, LPS binding induces extreme peptide aggregation on the OM, leading to poor membrane traversing and inactivation of the AMP [464]. Next to LPS-neutralization, AMPs can also exhibit other immunomodulating properties, such as immune cell recruitment, stimulation of chemokine production and upregulation of chemokine ligands, or mast cell degranulation [336, 465]. These effects, however, have so far not yet been studied for HC1 and HC10.

In chapter V, the effect of mono- and divalent salts on AMP activity was additionally investigated. The presence of physiological concentrations of divalent cations was found to interfere significantly with the cecropins' antibacterial effect. As LPS-binding also decreased in physiological salt concentrations, divalent ions such as Mg^{+2} and Ca^{+2} likely interfere with the electrostatic attraction forces between the AMPs and the outer bacterial leaflet [463]. Decrease of antimicrobial activity in salt environments is a known limitation for many natural AMPs. Different techniques have been applied successfully to

increase AMPs' salt stability, while maintaining or even enhancing the antibacterial activity. Increasing the lipophilicity of the peptide termini, for example by β-naphthylalanine end tagging, can increase salt sensitivity [463].

VIII. 1.5 Confirmation of activity in *in vivo* models

Activity of HC1 and HC10 was investigated in three different animal models (chapter VI). As both cecropins performed fairly similar in prior experiments, only one AMP (HC10) was chosen for *in vivo* evaluation to spare the number of animals needed. First, a *G. mellonella* model was used to probe for HC10's activity and toxicity. *G. mellonella* is an invertebrate animal model that, despite its translational limitations, can offer a valuable bridge between *in vitro* experiments and mice infection models [406]. After positive evaluation in this model, three different mice experiments were set-up. Before infection experiments, acute toxicity of HC10 was tested at three different doses during a 24 h timespan. Doses were chosen to cover a broad concentration range. For the highest dose (150 µg /mouse) and middle dose (50 µg /mouse) some side effects were noted, especially laboured respiration. It was hypothesized that this was a mechanical toxic effect, related to the structural nature of the AMP and its quality to foam in solution. For the following experiment, the medium HC10 dose was chosen. A *P. aeruginosa* lung infection model was used to test the antibacterial activity, as pneumonia is the most common manifestation of *P. aeruginosa* [388]. The main goal of this experiment was to investigate whether bacterial killing still occurred in a physiological environment, and if HC10 could protect the mice from a rapidly progressing *P. aeruginosa* infection. Significant antibacterial activity was found for HC10. However, HC10 was not able to reverse the bacterial spread to the liver and spleen, and the infection could not be completely reversed by HC10 in the tested conditions. LPS-neutralization and related anti-inflammatory effects were evaluated in a mice model of acute LPS-induced lung injury. Here, mild trends of inflammation reduction were noted. However, due to high experimental variation in the LPS control group, the net effect of HC10 on cytokine reduction was not clear. It must be noted that only the cytokines TNFα and IL-6 were

tracked as parameters for inflammation, where other inflammation markers could have been useful to get a broader understanding of the AMP's effect. Overall, HC10 did retain parts of its activity in *in vivo* conditions at the tested dose, and the experiments mostly confirmed HC10's potential as a starting point for further anti-pseudomonal research and antibiotic development. After a broad *in vitro* and *in vivo* biological characterization and evaluation of BSF AMPs, chapter VI concluded the first research objective (Figure VIII.2).

VIII.1.6 Looking beyond antimicrobial peptides

In the last research chapter (chapter VII), a second objective was investigated. The previous research has been focussed on the investigation of BSF AMPs found through transcriptome analysis. Although this is a highly useful and extensive approach that enables the identification of a full peptide library, other techniques have been applied to identify AMPs from insects as well. One main road for AMP discovery is the use of insect-derived extracts. Hemolymph extracts of BSFL have led to the discovery of several AMPs (e.g. DLP4, CLP1) years before the first BSF transcriptome study [167, 171]. Insect extracts can also be of interest for the isolation of other compounds with antimicrobial activity, including lipids and chitin. In our research, antimicrobial activity against *S. aureus* was seen for methanolic extracts of full body larvae, whereas the ethyl acetate extracts showed broad spectrum antibacterial activity. Isolation of related compounds was not performed, as the research of library of AMP material was prioritized. Next to insect extracts, antimicrobial screening of the insect symbionts is a possible route to discover new compounds of interest from insects. Symbionts can play an important role in the defence system of an insect. Fungus-growing ants, part of the Hymenoptera order, have mostly been the topic of interest in insect symbiont research [3]. Cyphomycin, isolated from *Streptomyces* symbionts of *Cyphomyrmex* ants, is one of the most well studied and promising compounds from insect microbes to date [3, 188]. For *H. illucens*, not much research on the antimicrobial activity of its symbionts was performed prior to this study. In chapter VII, the antimicrobial screening of dominant gut microorganisms of the BSF was described. The most promising activity was seen for the fungal *T. asahii* symbionts,

which displayed strong activity against *S. aureus* as well as moderate activity against *C. albicans*. To date, identification and purification of active compounds remains to be performed. Chapter VII concluded the second research objective (**Figure VIII.2**).

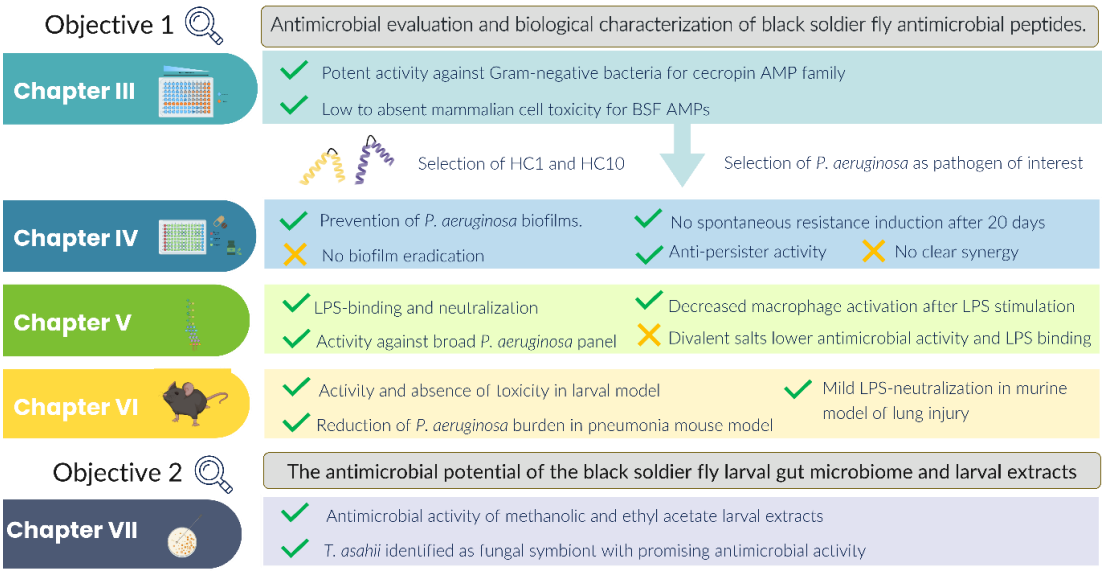


Figure VIII.2 Graphical summary of main findings of this research study and its related objectives.

VIII.2 Strengths and limitations of this study

This research has successfully explored the BSF as a source of antimicrobial compounds, focussing hereby on both the insect itself and on its microbial symbionts. For the first time, a large BSF AMP collection was evaluated for its antimicrobial activity and toxicity. Earlier studies on BSF AMPs have mostly concentrated on the exploitation of these peptides for non-medical related applications. Our study, however, has investigated the BSF AMPs through the lens of antibiotic drug development. Two promising AMPs have been characterized extensively and this research has exposed some of their advantages as well as shortcomings, opening roads for future research. We have added to the growing collection of anti-pseudomonal peptides, and substantiated the claim that AMPs indeed hold promise as future therapeutics. Nevertheless, this research comes along with several limitations. Priority was given to a broad, *in vitro* and *in vivo* evaluation to gauge the scope of the AMPs' activity. However, this also means that a lot of the

peptides' characteristics were not studied in detail. For example, while we have unveiled the membrane permeabilizing effects of HC1 and HC10, the exact nature of their membrane targeting mechanisms is not known, and other modes of action have not been investigated. In addition, many biological functions of HC1 and HC10 have not been researched, including immunomodulating properties beyond LPS binding, antiviral activity, effect of the AMPs on *Pseudomonas* cell adhesion and invasion, effect on *Pseudomonas* virulence factors other than LPS,… As the focus has been put on the antibacterial activity characterization, questions surrounding enzymatic stability and (lung) residency time of the cecropins remain mostly unaddressed. Moreover, the possible allergenicity of the AMP candidates remains unexplored to date. Although some AMPs have progressed down the clinical pipeline in their natural form (e.g. LL-37), most AMPs require some degree of structural modification to optimize their drugability. Lastly, to narrow the scope of the research, lung infections caused by *P. aeruginosa* were chosen as the topic of interest. However, the AMPs have shown a broader Gram-negative activity spectrum. The antimicrobial potential of HC1 and HC10 could be exploited towards the treatment (or prevention) of other bacteria and/or infections as well.

VIII.3 Future direction of this research

Research on the BSF antimicrobials has not yet been finished and will be continued after the end of this thesis. Here, some short-term and long-term perspectives are discussed. These include both planned research objectives, as well as hypothetical perspectives.

VIII.3.1 Short-term research perspectives

In a first step of the follow-up research, some previously performed experiments could be repeated or broadened. For example, CD analysis could be repeated with or without the addition of a higher LPS dose, inactivated bacteria, or physiological concentration of (divalent) salt to gain a better understanding of the AMPs' confirmation in a biological matrix. Next, biofilm experiments could be repeated in combination with chelating agents such as EDTA, to study whether this combination can increase biofilm eradication

by the cecropins [466]. The immunomodulating properties of HC1 and HC10 could be explored further by studying the AMPs' effect on a broader panel of inflammatory mediators, including cytokines, chemokines, and complement proteins in cell culture models [467]. With the eye on the use of BSF cecropins as therapeutics for *P. aeruginosa* pneumonia, several new research questions could be addressed as well. These include:

- What is the residency time of HC1 and HC10 in human lung fluid? How fast are the peptides broken down by lung proteases?
- Do the peptides retain antimicrobial activity in sputum conditions?
- What is the effect of HC1 and HC10 on the adhesion of *P. aeruginosa* to lung epithelial cells?
- Are the peptides able to target and kill intracellular *P. aeruginosa* niches?
- Do the peptides elicit allergenic responses?

Firstly, not much is known about the biostability of the cecropins in relevant lung matrices. Although the *in vivo* experiments showed significant antimicrobial activity in murine lungs, it has not been determined how much of the AMP was left after the 4 h treatment window. *In vitro* experiments could help gain insight in the lung stability of the peptides. For example, by incubating HC1 and HC10 with bronchoalveolar fluid, the stability of the peptides over time could be tracked using SDS-PAGE or chromatography and spectrometry techniques. Antimicrobial activity could additionally be studied in more elaborate lung matrices such as sputum, which contains a wide variety of biomolecules (DNA, lipids, mucins, polysaccharides,…) [468]. For now, only the effect of physiological salts has been studied. Next, the effect of the AMPs on specific bacterial-cell interactions could be studied in cellular infection models. In addition, it is not yet known whether HC1 and HC10 are able to target *Pseudomonas* intracellularly. *P. aeruginosa* is able to form intracellular niches of bacteria that evade the immune system and persist during antibiotic treatment [469]. Eradicating these populations of bacteria could be an additional asset when treating *P. aeruginosa*. Lastly, the allergenicity of HC1

and HC10 remains to be explored. *In silico* analysis (e.g. using the Structural Database of Allergenic Proteins or the Epitope Prediction and Analysis Tools) can give a first indication of the AMPs' immunogenicity. Follow-up *in vitro* assays such as lymphocyte or basophil activation tests can be used for result validation [470].

Another short-term research objective is the identification and purification of the compound(s) responsible for the *T. asahii* activity. Two main roads of action can be considered. Extractions can be prepared of either *Trichosporon* culture supernatant or of the agar zone where activity against the bacterial species was noted. Then, after antimicrobial activity of the extracts has been confirmed, the extracts can be fractionated and analysed with chromatography techniques [3]. Mass spectrometry can be used to identify the compound structure. Alternatively, active metabolites of *T. asahii* can be identified via transcriptome analysis.

VIII.3.2 To modify or not to modify?

After finalising the AMP characterisation experiments, ideally (i) one peptide candidate should be chosen for further development, and (ii) a decision should be made on how to move forward with the AMP research. As HC1 and HC10 have mostly performed similarly activity-wise, the most logical choice of AMP is HC10, as this peptide has already been tested *in vivo*. Commonly, AMPs are structurally modified before going over to the preclinical development phase. Indeed, most peptide-based antimicrobials that are investigated in clinical trials today are derivatives of natural AMPs [107, 380, 471].

VIII.3.2.1 Rational peptide optimization

Currently, the biggest shortcoming of the BSF AMPs is their salt sensitivity. While relevant activity of HC10 has still been found *in vivo*, the dose to obtain antimicrobial killing and LPS-neutralization is presumably much lower when salt stability is increased. By lowering the AMP dose, any unwanted side effects *in vivo* can also be decreased. Moreover, biofilm eradication can likely be enhanced by decreasing ionic interactions. Structural modifications can also be applied to increase the AMPs' biological residency time. To

improve HC10's performance, a rational drug design approach can be taken [472]. Strategies to increase salt resistance have been briefly discussed in chapter V. Various other routes of structural modification can be considered as well. Briefly, structural changes that are regularly applied include (**i**) AMP truncation, (**ii**) modifying the peptide backbone by incorporating D-AA or unnatural AA, (**iii**) end-tagging the AMP terminals, (**iv**) cyclization, or (**v**) pegylation of AMPs [473, 474]. Shortening of the AMP sequence can also decrease the synthesis cost, an additional advantage of AMP modification [475]. Rational, structural optimization of AMPs, however, can be challenging as it requires a fundamental understanding of the peptide's structure-activity relationship. In addition, different desired activity outcomes (e.g. activity, toxicity, stability,…) can call for conflicting target modifications [59]. Newly designed peptides need to be synthesized and tested experimentally, which can be time-consuming and costly.

VIII.3.2.2 Using machine learning in peptide design

Apart from the rational design of cecropin mimetics by introducing targeted modifications, machine learning in combination with predictive models can be used. Large amounts of AMP derivatives can be generated computationally while forecasting their activity *in silico* in a high-throughput fashion. The characterized cecropin AMPs can serve as templates for the construction of BSF AMP-derivative libraries. For instance, Yoshida et al. (2018) developed a template-based evolutionary algorithm that generates new AMPs with increased fitness through successive rounds of introducing mutations and deletions in a starting sequence [476, 477]. Another option, for example, is the creation of a library of truncated cecropin sequences through online available tools (e.g. CAMPR3 database) [478]. These newly created peptides can then be evaluated through available predictive pipelines (e.g. AMPlify) [479]. *In silico* prediction models can function as a time-efficient alternative to the laborious wet lab screening experiments of AMP libraries. However, as the accuracy of the predictive algorithms is highly dependent on the quality of the input data, *in vitro* validation of the top hits will remain indispensable. As opposed to template-based design, *de novo* design of AMPs is gaining popularity as well. Here,

generative models that have been familiarized with the natural protein landscape through deep learning can create a diverse set of entirely new AMPs [480].The earlier mentioned AMP WLBU2 (Chapter VI) that is currently under clinical investigation as anti-biofilm therapy for *P. aeruginosa*, has been engineered *de novo* [481].

VIII.3.2.3 Formulating antimicrobial peptides

An alternative (or complementary) approach to structural modification is the use of innovative formulation technologies. Drug delivery systems can sequester AMPs, shielding them from interactions and increasing their tissue distribution, hereby increasing their therapeutic potential [107, 473, 482]. To this end, nanocarriers such as lipid, metal, polysaccharide and polymeric nanoparticles are being investigated [482-484]. Mostly, AMPs are encapsulated by the drug carriers, but surface attachment of the peptide to the delivery system is being investigated as well. Next, AMPs can be formulated into microgels through co-assembly with polymers. By encapsulating the peptides in a (hydro)gel matrix, they are protected from undesired interactions and controlled drug release can be obtained [482, 483]. These formulations are mostly investigated as either wound dressings or antimicrobial coatings. Lastly, covalent conjugation with other molecules (e.g. biomacromolecules such as polysaccharides or polymer-AMP conjugates) is also a possible formulation strategy to increase activity or stability, decrease toxicity, or to obtain targeted delivery (e.g. AMP-antibody conjugation).

VIII.3.3 Long-term research perspectives

Ultimately, one main goal of the continuation of this research is to harness the antibiotic potential of the BSF AMPs to create added value for patients in the clinic. This thesis has specifically zoomed in on the activity of HC1 and HC10 against *P. aeruginosa* pneumonia. The antimicrobial effects of the cecropins could be of use in both the **treatment**, as well as the **prevention** of *P. aeruginosa* lung infections. When treating lung infections, the AMPs could be administered systemically (e.g. through intravenous injections and/or infusions) or locally via inhalation therapy. As AMPs are known to have poor systemic bioavailability, direct administration to the lungs could be the main plan of approach.

Recent advances in the development of AMPs for inhalation therapies have been summarized by Wang et al. (2022) [394]. Peptides for inhalation therapy are mostly formulated in liquid solutions and administered via nebulization, as opposed to dry powder inhalation medications [394]. Aerosolization, however, puts the peptide antimicrobials under various forms of stress, including mechanical shear stress [394]. Overall, the development of AMPs for inhalation is complex, and it requires specific formulation expertise. Previously mentioned nanoparticle carriage systems are also being investigated for inhalation AMP formulations [485]. Currently, colistin is available as inhalation therapy for chronic *P. aeruginosa* infection for CF patients. For AMPs, the inhalation administration route is not yet commonly applied, and formulation research is in its early stages. Nevertheless, some successes have been reported. For example, the synthetic AMP SET-M33 has been formulated in a dextran nanoparticle system for pulmonary administration in early preclinical research [396]. Murepavadin, an anti-pseudomonal peptidomimetic, has shown promise in recent clinical trials as aerolization therapy, where earlier clinical test of systemic murepavadin administration were cut short due to renal toxicity [486].

Next to treatment, AMPs could also play a key role in the prevention of pneumonia. *P. aeruginosa* is the main causative agent of VAP and linked to a high mortality rate [256, 487]. AMP coatings of endotracheal tubes are increasingly investigated as innovative, preventive measures against VAP [487]. Coatings can be formed by immobilizing the peptides covalently to the tube surface, either by direct interaction (e.g. via binding domains within the peptide itself) or by using a linker compound (e.g. polydopamine layer) [487]. Alternatively, AMPs can be loaded into a polymeric matrix coating the tubes, such as hydrogels, collagen, or polymeric-lipid encapsulation layers [487, 488]. The former technique is aimed at obtaining direct killing upon microbial contact, while the latter also allows for a gradual release of the AMP into the surrounding tissue [482]. Development of a BSF cecropin coating for endotracheal tubes is a current, ongoing project that has been based on the results obtained in this thesis.

Although not currently on the agenda, the BSF AMPs could also be of value in the treatment other *P. aeruginosa*-related diseases. Wound infections, for example, are often caused by *P. aeruginosa* colonization [489]. As AMPs often exhibit wound healing qualities next to antibacterial activity, they are increasingly investigated as treatments for skin infections (e.g. in hydrogel formulations) [490]. Next, the cecropin AMPs could also be of interest in combatting other (Gram-negative) pathogens. Similarly, the use of HC1 and HC10 in other prevention strategies for infectious diseases could be explored. Preventive medicine is important to lower the disease burden and antibiotic usage. Apart from endotracheal tube coatings, peptides could be of use for a wide variety of biomedical devices, including catheters, implants, sutures,… [488]. Moreover, AMPs can be used as additives in contact lens fluid, used in antibacterial wound dressings, and mouth washes to prevent caries [491-493]. It is clear that the promising activity of the BSF cecropins cannot only be exploited for the treatment of *P. aeruginosa* lung infections, but that they can play a key role in other applications as well.

VIII.4 Final conclusion

This body of work has explored the BSF as a source of novel antimicrobial compounds. Two main findings of this thesis are the **i**) potent, strong activity against selected Gram-negative pathogens of BSF cecropins, and **ii**) the high anti-staphylococcal activity of the *T. asahii* symbiont strain. These findings are interesting on two fronts: firstly, they provide a valid foundation for the future research and development of antimicrobial drugs, and secondly, they contribute to our fundamental understanding of the antimicrobial defence systems in the BSF. With the broad antimicrobial screening of the BSF peptide library, this thesis has added to the large collection of AMPs with known antibacterial activity. The biological activity profile of two selected cecropins, HC1 and HC10, has been characterized in-depth. The characteristics of the cecropins are largely in line with those described for other cationic, α-helical peptides in literature. These include LPS neutralization, membrane permeabilization, fast bactericidal activity, and divalent salt sensitivity. Other characteristics, such as biofilm eradication and synergistic action, were not found for HC1 and HC10. As these peptides showed promising activity against *P. aeruginosa* (with HC10 having confirmed *in vivo* activity), they can serve as interesting starting points for future antipseudomonal therapeutics. However, some key points of the cecropins, such as their enzymatic stability and biological residency time, remain unaddressed to date. Future research will focus on the development of antibacterial cecropin coatings on endotracheal tubes for the prevention of VAP. Moreover, the compounds responsible for the *T. asahii* activity remain to be elucidated.

Summary

With its ever growing incidence and related mortality, antimicrobial resistance is estimated to be one of the most urgent health issues in the upcoming decades. Many infectious diseases are becoming partially or completely unresponsive to treatment with the antibiotics available in the clinical practice. Hence, developing new antimicrobial compounds that are able to eradicate these drug-resistant pathogens is of utmost importance. Nature has long proven to be a valuable source of structurally diverse antimicrobials. One of the natural niches that has recently been receiving growing attention for the discovery of new antibiotics, are insects. Insects have a well-developed defence system, which can be exploited in the search for new active compounds. Of interest are both the compounds produced by the insect itself, as the compounds produced by the microbial symbionts of the insect. In this thesis, the black soldier fly has been the focal point of research on new antimicrobials.

The black soldier fly (*Hermetia Illucens*, BSF) is a widespread fly known for its exceptional capacity of decomposing organic waste. It is a type example of an insect that lives in close relation with a broad scala of microorganisms. The BSF has the second largest repertoire of antimicrobial peptides (AMPs) ever recorded in insects. AMPs are evolutionary conserved host defence peptides that play a key role in the immune system of all living organisms. Before the start of this research, the activity of its AMP library had not been characterized in detail (objective 1). In addition, the antimicrobial activity of the BSF symbionts had not yet been extensively investigated (objective 2).

In chapter III, the broad antimicrobial screening of the BSF peptide library against a panel of human pathogens is described. Here, notable activity against Gram-negative pathogens *E. coli* and *P. aeruginosa* was found for the family of α-helical peptides (cecropins). In addition, low cell toxicity against human fibroblasts was noted. Two peptides (HC1 and HC10) were chosen for further characterization experiments. These cecropin peptides harbour a helix-hinge-helix structure, have low hemolytic activity, and

membrane permeabilizing effects. In the chapter IV, activity of HC1 and HC10 against *P. aeruginosa* was investigated more in detail. As an opportunistic pathogen with high acquired and intrinsic antibiotic resistance, pseudomonal infections can be a true menace for immunocompromised and hospitalised patients. The anti-biofilm activity of HC1 and HC10 was tested. Both cecropins are able to prevent biofilm formation *in vitro*, whereas biofilm eradication was not accomplished. Activity against antibiotic persister cells of *P. aeruginosa* was also noted at bactericidal concentrations of AMPs. Next, chapter IV also describes synergy experiments, where the combination of the AMPs with conventional antibiotics was tested. However, no significant synergy has been found for HC1 and HC10. Lastly, by performing serial passage mutagenesis experiments, it was shown that *P. aeruginosa* does not spontaneously acquire resistance to HC1 and HC10 after 20 days of exposure to low AMP concentrations. In chapter V, the binding of the AMPs to lipopolysaccharide (LPS), an important toxin and virulence factor of *P. aeruginosa*, is described. Binding to LPS is presumably a key step in the AMP's mechanism of action. In addition, by binding LPS, the inflammatory activation of macrophage cells *in vitro* was lowered, as noted by a decreased release of nitrite oxide and cytokines TNFα and IL-6. The AMPs also showed antimicrobial activity against a broad panel of P. aeruginosa (environmental and clinical) isolates. However, chapter V also discusses an important shortcoming of HC1 and HC10. The antimicrobial activity is significantly lowered in the presence of physiological concentrations of divalent cations (Mg^{+2} and Ca^{+2}). These salts also interfere with the AMP-LPS binding. In chapter VI, the *in vivo* activity of HC10 was investigated in both a larval *Galleria mellonella* model, as in murine models. Absence of toxicity was noted for HC10 in the larval model, whereas dose-dependent laboured breathing was detected upon intranasal treatment of the mice. In the *P. aeruginosa* mouse model, significant antibacterial activity was noted in the lungs, although the systemic disease progression could not be reversed. Effects of the LPS binding on cytokine production was additionally investigated. However, anti-inflammatory effects (as measured by TNFα and IL-6 concentrations) was less clear; only mild (mostly non-significant) reduction was noted. Overall, the antibacterial activity and toxicity character

of selected BSF cecropins has been thoroughly investigated in this thesis. Notable strengths as well as some AMP limitations have been found, and HC1 and HC10 show promise as candidates for further anti-pseudomonal drug research and development.

Lastly, an additional objective was investigated in chapter VII. During research of a fellow ENTOBIOTA PhD candidate, a collection of dominant gut microorganisms was established. All isolates were subjected to a broad antimicrobial screening. Although mild antimicrobial activity was found widespread throughout the collection, high, consistent activity was mostly seen for *T. asahii* species. This fungus showed strong activity against *S. aureus* and moderate activity against *C. albicans*. Compounds responsible for the noted activity remain to be identified in follow-up research.

Samenvatting

Door de groeiende incidentie en mortaliteit van antimicrobiële resistentie, wordt geschat dat dit één van de meest urgente gezondheidsproblemen van de komende decennia wordt. Veel infectieziekten reageren gedeeltelijk of helemaal niet meer op behandeling met de antibiotica die in de klinische praktijk beschikbaar zijn. Daarom is het van het grootste belang om nieuwe antimicrobiële middelen te ontwikkelen die in staat zijn om deze resistente kiemen te bestrijden. De natuur heeft al lang bewezen een waardevolle bron te zijn van structureel diverse antimicrobiële stoffen. Een van de natuurlijke niches die de laatste tijd steeds meer aandacht krijgt voor de ontdekking van nieuwe antibiotica, zijn insecten. Insecten hebben een goed ontwikkeld afweersysteem dat kan worden benut bij de zoektocht naar nieuwe actieve verbindingen. Van belang zijn zowel de verbindingen die door het insect zelf worden geproduceerd, als de verbindingen die door de microbiële symbionten van het insect worden geproduceerd. In dit proefschrift stond de zwarte soldaatvlieg centraal in het onderzoek naar nieuwe antimicrobiële stoffen.

De zwarte soldaatvlieg (*Hermetia Illucens*, ZSV) is een wijdverspreide vlieg die bekend staat om zijn uitzonderlijke vermogen om organisch afval af te breken. Het is een typisch voorbeeld van een insect dat in nauwe relatie leeft met een breed scala aan micro-organismen. De ZSV heeft het op één na grootste repertoire antimicrobiële peptiden (AMP) dat ooit bij insecten is gevonden. AMP zijn evolutionair geconserveerde verdedigingspeptiden die een sleutelrol spelen in het immuunsysteem van alle levende organismen. Voor de start van dit onderzoek was de activiteit van de peptidenbibliotheek nog niet in detail gekarakteriseerd (onderzoeksobjectief 1). Daarnaast was er nog weinig geweten over de antimicrobiële activiteit van de symbionten van de ZSV (onderzoeksobjectief 2).

In hoofdstuk III wordt de brede antimicrobiële screening van de ZSV peptidebibliotheek tegen een panel van menselijke pathogenen beschreven. Hier werd opmerkelijke activiteit tegen Gram-negatieve ziekteverwekkers *E. coli* en *P. aeruginosa* gevonden voor

de familie van α-helicale peptiden (cecropines). Daarnaast werd een lage celtoxiciteit tegen menselijke fibroblasten vastgesteld. Twee peptiden (HC1 en HC10) werden gekozen voor verdere karakteriseringsexperimenten. Deze cecropinepeptiden hebben een helix-scharnier-helixstructuur, een lage hemolytische activiteit en membraanpermeabiliserende effecten. In hoofdstuk IV werd de activiteit van HC1 en HC10 tegen *P. aeruginosa* meer in detail onderzocht. Als opportunistische ziekteverwekker met een hoge verworven en intrinsieke antibioticaresistentie kunnen pseudomonale infecties een bedreiging vormen voor immuungecompromitteerde en gehospitaliseerde patiënten. De anti-biofilmactiviteit van HC1 en HC10 werd getest. Beide cecropines zijn in staat om biofilmvorming *in vitro* te voorkomen, terwijl uitroeiing van een bestaande biofilm niet werd vastgesteld. Activiteit tegen antibioticapersistente cellen van *P. aeruginosa* werd wel waargenomen bij bactericide AMP concentraties. Vervolgens worden in hoofdstuk IV ook synergie-experimenten beschreven, waarbij de combinatie van de AMP met conventionele antibiotica werd getest. Er werd echter geen significante synergie gevonden voor HC1 en HC10. Tot slot werd door het uitvoeren van seriële passage mutagenese experimenten aangetoond dat *P. aeruginosa* niet spontaan resistentie tegen HC1 en HC10 verwerft na 20 dagen blootstelling aan lage AMP concentraties. In hoofdstuk V wordt de binding van HC1 en HC10 aan lipopolysaccharide (LPS), een belangrijk toxine en virulentiefactor van *P. aeruginosa*, beschreven. Binding aan LPS is vermoedelijk een belangrijke stap in het werkingsmechanisme van de AMP. Bovendien werd door binding aan LPS de activatie van macrofaagcellen *in vitro* verlaagd, zoals bleek uit een verminderde afgifte van nitrietoxide en cytokines TNFα en IL-6. De AMP vertoonden ook antimicrobiële activiteit tegen een breed panel van *P. aeruginosa* (omgevings- en klinische) isolaten. In hoofdstuk V wordt echter ook een belangrijke tekortkoming van HC1 en HC10 besproken. De antimicrobiële activiteit wordt aanzienlijk verminderd in aanwezigheid van fysiologische concentraties divalente kationen (Mg^{+2} en Ca^{+2}). Deze zouten interfereren ook met de binding van AMP-LPS. In hoofdstuk VI werd de *in vivo* activiteit van HC10 onderzocht in zowel een larvaal *Galleria mellonella* model als in muismodellen. Er werd geen toxiciteit vastgesteld voor HC10 in het larvale model,

terwijl dosisafhankelijke ademhalingsmoeilijkheden werden waargenomen bij intranasale behandeling van de muizen. In het *P. aeruginosa* muismodel werd significante antibacteriële activiteit in de longen waargenomen, hoewel de systemische ziekteprogressie niet omgekeerd kon worden. Daarnaast werd het effect van de LPS-binding op de cytokineproductie onderzocht. De ontstekingsremmende effecten (zoals gemeten door TNFα en IL-6 concentraties) waren echter minder duidelijk; er werd slechts een milde (meestal niet-significante) reductie waargenomen. In het algemeen zijn de antibacteriële activiteit en toxiciteit van geselecteerde BSF-cecropines in dit proefschrift grondig onderzocht. Er zijn zowel opmerkelijke sterke punten als enkele AMP-beperkingen gevonden en HC1 en HC10 zijn veelbelovend als kandidaten voor verder onderzoek en ontwikkeling op het gebied van antipseudomonale geneesmiddelen.

Tot slot werd in hoofdstuk VII een aanvullende doelstelling onderzocht. Tijdens onderzoek van een mede-doctoraatsonderzoeker van ENTOBIOTA werd een verzameling dominante darm micro-organismen aangelegd. Alle isolaten werden onderworpen aan een brede antimicrobiële screening. Hoewel milde antimicrobiële activiteit wijdverspreid in de collectie werd aangetroffen, werd een hoge, consistente activiteit vooral gezien bij *T. asahii* soorten. Deze schimmel vertoonde een sterke activiteit tegen *S. aureus* en een matige activiteit tegen *C. albicans*. De verbindingen die verantwoordelijk zijn voor de opgemerkte activiteit moeten nog worden geïdentificeerd in vervolgonderzoek.

Curriculum vitae

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2017 - 2019 Master in pharmaceutical sciences: drug development
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LIST OF SCIENTIFIC PUBLICATIONS

a) International, peer-reviewed

2024 Mahieu, L.*, **Van Moll, L.***, De Vooght, L., Delputte, P., Cos, P. (2024). *In vitro* modelling of bacterial pneumonia: a comparative analysis of widely applied complex cell culture models. FEMS Microbiology Reviews, 2024. 48(2). https://doi.org/10.1093/femsre/fuae007

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2023 **Van Moll, L.**, Wouters, M., De Smet, J., De Vooght, L., Delputte, P., Van Der Borght, M., Cos, P. (2023). In-depth biological characterization of two black soldier fly anti-Pseudomonas peptides reveals LPS-binding and immunomodulating effects. mSphere, 8(5), e00454-00423. https://doi.org/doi:10.1128/msphere.00454-23

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a) National, not peer-reviewed

2023 Van Moll, L. (2023) De zwarte soldatenvlieg: ten strijde tegen antibioticaresistentie. Eos wetenschap online.

CONTRIBUTIONS TO SCIENTIFIC MEETINGS AND CONFERENCES

a) Poster presentations

2023 Van Moll, L., De Vooght, L., De Smet, J., Van Der Borght, M., & Cos, P. (2023). The black soldier fly as a source of anti-Pseudomonal peptides [conference poster]. FEMS, Hamburg, Germany.

Wouters, M., Van Moll, L., De Smet, J., Van Der Borght, M., De Vooght, L., & Cos, P. (2023). The black soldier fly as a source of anti-Pseudomonal peptides [conference poster]. Gordon research conference, Lucca, Italy.

2021 De Smet, J., Gorrens, E., Van Moll, L., Cos, P., & Van Campenhout, L. (2020). The potential of dominant micro-organisms isolated from the larvae of *H. illucens* for microbiological control [conference poster]. Insects to Feed the World Virtual Conference.

b) Oral presentations

2022 Van Moll, L., De Smet, J., Paas, A., Dorothee, T., Vilcinskas, A., Cos, P., & Van Campenhout, L. (2022). Insects in the fight against antibiotic resistance: *in vitro* characterization of back soldier fly antimicrobial peptides [conference lecture]. Insecta, Giessen, Germany.

Van Moll, L., Van Moll, L., De Smet, J., Paas, A., Dorothee, T., Vilcinskas, A., Cos, P., & Van Campenhout, L. (2022). Insects in the fight against antibiotic resistance: black soldier fly antimicrobial peptides. Departemental research day, University of Antwerp, Wilrijk, Belgium.

2021 Vandeweyer, D., Lachi, D., Van Moll, L., Gorrens, E., De Smet, J., & Van Campenhout, L. (2021). Horizontal transfer of food pathogens

ADDITIONAL COURSES AND WORKSHOPS

2019 Intermediate excel, Bit by Bit, University of Antwerp

2020 Laboratory animal sciences, FELASA C, University of Antwerp

2023 Linux introduction, CalcUA, University of Antwerp

GUIDANCE OF GRADUATE STUDENTS

2022 *In vitro* characterization of two black soldier fly antimicrobial peptides. Ewa Janicki, Master of Drug Development Pharmaceutical Sciences), University of Antwerp, Wilrijk

2023 A first sight into the mode of action of the antimicrobial peptides HC1 & HC10, Marziha Rahman, Master of Drug Development (Pharmaceutical Sciences), University of Antwerp, Wilrijk

“ἔοικα γοῦν τούτου γε σμικρῷ τινι αὐτῷ τούτῳ σοφώτερος εἶναι, ὅτι ἃ μὴ οἶδα οὐδὲ οἴομαι εἰδέναι.”

- Plato, Apologia 21d

“I seem, then, in just this little thing to be wiser than this man at any rate, that what I do not know I do not think I know either.”

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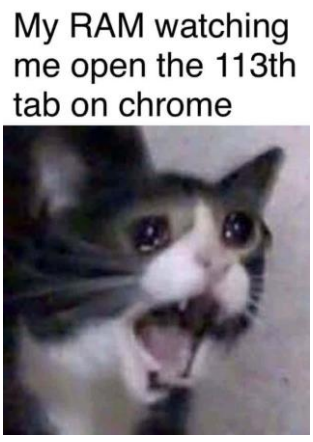
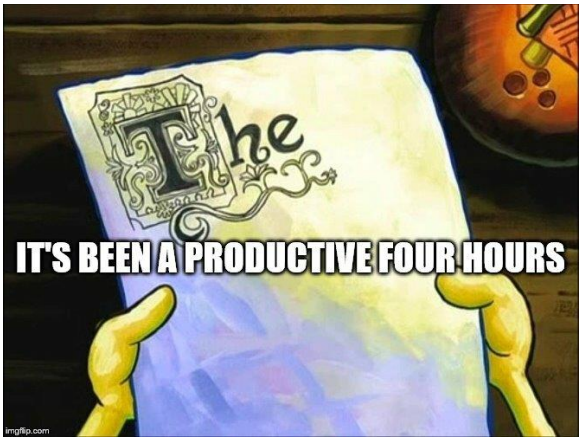
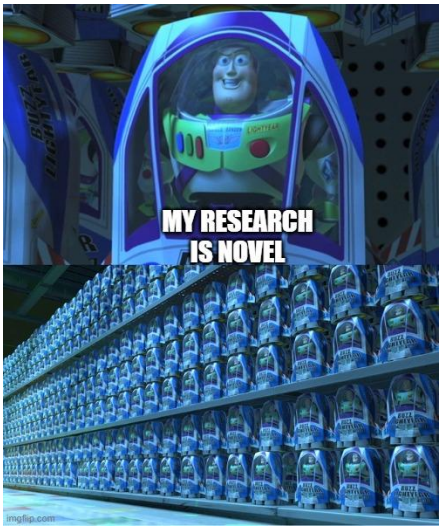
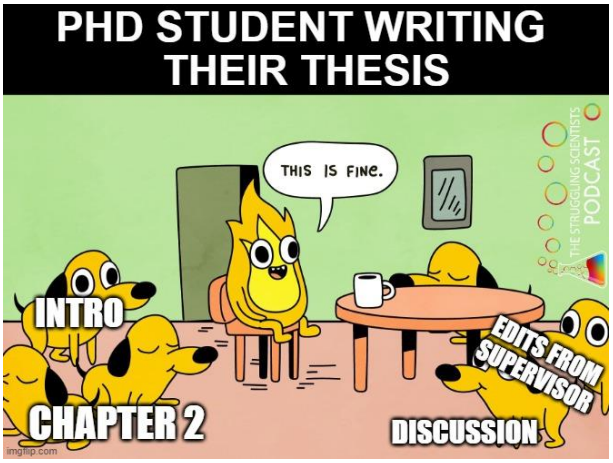
EPILOGUE

A brief overview of the writing process: a story of mixed emotions









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