Antimicrobial peptide resistance: infection dynamics, cross-sensitivity to insect immune effectors, and evolution of pharmacodynamics

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إهـداء

إلى والدى رحمه الله

اللهم اجعل ثواب هذا العمل فى ميزان حسناته وضاعف له قدر ما تشاء

إلى أمى الحبيبة

أدام الله أنفاسك العطرة

وما كفانا دعواتك أبداً

إلى خالى الحبيب وأبى الروحى

إلى سندى أخى الغالى

إلى رفيق الدرب وحبيب الروح زوجى

إلى أبنائى الأعزاء جويدان وإلياس

بارك الله فيكما

إلى عائلتى وأصدقائى وأحبائى فى الله

إلى أساتذتى

(Dedication in Arabic language)

Contents

1. Summary

Host defence and parasite pathogenicity interactions determine infection outcomes. Antimicrobial peptides (AMPs) are conserved components of immune defences of multicellular organisms. In this thesis, the fitness costs of the evolution of resistance toward the host's own AMPs were investigated.

In chapter 1, the experiment focused on the *in vivo* fitness cost of evolving AMP resistance. The mealworm beetle, *Tenebrio molitor,* was used as a host. The opportunistic pathogen *Staphylococcus aureus* selected against one or two of the beetle abundant AMPs was injected into *T. molitor*. The AMP-resistant parasite persistence and virulence were investigated. The infection dynamics of an array of AMPs-selected bacteria versus sensitive unselected control strains were monitored. Our laboratory, previously, showed that the AMP-selected strains harboured a mutation in either *nsa* or *pmt* operons. To measure the genetic costs of evolving resistance to these immunological stressors, the bacterial loads, were reanalyzed based on the resistance driving mutation. The induced host mortality rate inferred the virulence of the AMP-resistant pathogen. The findings in **chapter 1** showed that, unexpectedly, a pathogen resistant to one or two of the host's own AMPs neither had increased bacterial load nor affected the host's survival rate. Costs of resisting AMP were presumably high, suggesting that intrinsic costs might constrain natural AMP resistance. As AMP-resistant pathogen showed limited infection success, this might explain the hampered AMP resistance evolution observed in environmental bacteria.

Despite being resistant to one or two of the host's own AMPs, *S. aureus* did not perform well in the beetle. The thesis investigated reasons that might cause such unexpected limited pathogenicity. The study, herein, hypothesized that an AMP-resistant pathogen might be more susceptible to eradication by host's other immune effectors. However, in **chapter 1**, all AMP-resistant pathogens were shown to be phagocytosis resistance except those harbouring mutation in both *nsa* and *rpo* operons.

In **chapter 2,** RNA interference-based knockdown study was performed to investigate the collateral sensitivity of AMP-resistant pathogen towards the host's other immune effectors. The results highlighted that AMP-selected *S. aureus* strains are cross-sensitive towards phenoloxidase dependent immune responses, except the *nsa-rpo* mutants, which were, uniquely, resistant to phenoloxidase activity. Furthermore, collateral sensitivity to other AMPs of the *in vivo* defence cocktail was investigated. Strains harbouring mutations in *pmt* or both *nsa* and *rpo* operons showed cross-sensitivity to one or more component of the *in vivo* AMP defence cocktail.

In conclusion, resistance against AMPs and phagocytosis neither influenced the host's morbidity nor mortality because it is balanced by collateral sensitivity to other immune effectors. Despite being essential for infection success in vertebrates, AMP resistance does not provide a survival advantage to *S. aureus* in a host environment that is dominated by AMPs because of collateral sensitivity to other immune effectors.

It was interesting to investigate whether bacterial evolution of AMP resistance triggers fitness-adaptive modifications to their growth parameters and pharmacodynamic parameters *in vitro*. In **chapter 1**, the defensin- (tenecin 1) selected *S. aureus* showed an extended lag phase compared to the sensitive unselected control. However, the maximum velocity of bacterial multiplication was not affected by AMP resistance evolution.

In **chapter 3**, a four-parameter pharmacodynamic model was used to analyze time-kill curves of AMP-resistant *S. aureus* strains. The comparative analysis of pharmacodynamic curves of pexiganan, melittin and tenecin-resistant *S. aureus* demonstrated that the Hill coefficient, kappa, describing the steepness of the pharmacodynamic curve evolves. Kappa was usually ignored or set to a constant value in most pharmacodynamic models. Such a finding is crucial, as it was shown, previously, that the kappa relates to the probability of resistance evolution against AMPs. Medical practitioners routinely perform pharmacodynamic analysis for various drugs. However, the results in **chapter 3** clarified that pathogens showed different pharmacodynamics evolution patterns, which should be considered, for example, when optimizing the choice of a new drug candidate in cycling therapy. Moreover, this finding could be extended to all kind of host-pathogen relationship. Pharmacodynamic parameters evolution should not be over-simplified to minimum inhibition concentration (MIC) when studying co-evolution to host's AMP defence cocktail or other antimicrobials; this might enrich our perception of drug resistance evolutionary dynamics.

Collectively, the thesis revealed that AMP resistance displayed, mostly, high fitness costs both *in vivo* and *in vitro*. This impaired pathogen fitness plays a decisive role in supporting AMPs as therapeutics. Some AMP-resistant mutants showed increased infection success than others. Therefore, for any AMPs-based therapy, a survey of fitness costs of possible evolving resistant pathogen mutants should be evaluated. Weighing the pros and cons of AMPs as therapeutics and avoiding unintentional evolution of felicitous AMP-resistant superbugs are tremendously important.

Zusammenfassung

Wechselwirkungen zwischen der Abwehrreaktion des Wirtes und Pathogenität des Parasiten determinieren den Ausgang einer Infektion. Antimikrobielle Peptide (AMPs) spielen bei der Immunabwehr von mehrzelligen Organismen eine essenzielle Rolle. In der vorliegenden Arbeit wurden die Fitnesskosten der Resistenzevolution gegenüber Wirt AMPs untersucht.

Kapitel 1 fokussiert auf die In-vivo-Fitnesskosten für die Evolution von AMP-Resistenzen. Als Wirt wurde der Mehlwurmkäfer *Tenebrio molitor* verwendet. Der opportunistische Erreger *Staphylococcus aureus* wurde in *T. molitor* injiziert und selektierte gegen ein oder zwei der im Käfer vorhandenen AMPs. Untersucht wurden die Beständigkeit und Virulenz des AMP-resistenten Parasiten. Ich habe die Infektionsdynamik eines Array-AMP-ausgewählten Bakteriums gegenüber empfindlichen nicht selektierten Kontrollstämmen überwacht. Unser Labor hat zuvor gezeigt, dass die AMP-ausgewählten Stämme eine Mutation in *nsa*- oder *pmt*-Operons aufwiesen. Um die genetischen Kosten für die Entwicklung einer Resistenz gegen diese immunologischen Stressoren zu messen, wurde die Bakterienladung basierend auf der Mutation, die die Resistenz antreibt, erneut analysiert. Wir haben die Virulenz des AMP-resistenten Pathogens als induzierte Wirtssterblichkeitsrate abgeleitet. Die Ergebnisse in Kapitel 1 zeigten, dass ein Krankheitserreger, der gegen ein oder zwei AMPs des Wirts resistent war, unerwartet weder die Bakterienladung erhöht noch die Überlebensrate des Wirts beeinflusst hatte. Die Kosten für die Resistenz gegen AMPs waren vermutlich hoch, was darauf hindeutet, dass die Eigenkosten die natürliche AMP-Resistenz einschränken könnten. Da AMP-resistente Pathogene einen begrenzten Infektionserfolg zeigten, könnte dies die erschwerte Entwicklung der AMP-Resistenz erklären, die bei Umweltbakterien beobachtet wurde.

Obwohl *S. aureus* gegen ein oder zwei AMPs des Wirts resistent war, zeigte es im Käfer keine außerordentliche Pathogenität. Wir untersuchten Gründe, die eine solch unerwartet begrenzte Pathogenität verursachen könnten. In der vorliegenden Studie wurde die Hypothese aufgestellt, dass ein AMP-resistenter Erreger anfälliger für die Ausrottung durch die anderen Immuneffektoren des Wirts ist. In Kapitel 1 wurde jedoch gezeigt, dass alle AMP-resistenten Pathogene eine Phagozytose-Resistenz aufweisen, mit Ausnahme derjenigen, die sowohl in nsa- als auch in rpo-Operons Mutationen aufweisen.

In Kapitel 2 wurde eine auf RNA-Beeinträchtigung basierende Knockdown-Studie durchgeführt, um die Kollateralempfindlichkeit von AMP-resistenten Pathogenen gegenüber den anderen

Immuneffektoren des Wirts zu untersuchen. Die Ergebnisse zeigten, dass ein AMP-selektierter *S. aureus* Stamm gegenüber Phenoloxidase-abhängigen Immunantworten kreuzempfindlich ist, mit Ausnahme der *nsa-rpo* Mutanten, die eindeutig gegen Phenoloxidase aktivität resistent waren. Darüber hinaus wurde die Kollateralempfindlichkeit gegenüber anderen AMPs des In-vivo-Abwehrcocktails untersucht. Stämme, die Mutationen in *pmt*- oder sowohl *nsa*- als auch *rpo*-Operons enthielten, zeigten eine Kreuzempfindlichkeit gegenüber einer oder mehreren Komponenten des *in vivo*-AMP-Abwehrcocktails.

Zusammenfassend lässt sich sagen, dass die Resistenz gegen AMPs und Phagozytose weder die Morbidität noch die Mortalität des Wirts beeinflussen, da sie durch die Kollateralempfindlichkeit gegenüber anderen Immuneffektoren ausgeglichen wird. Obwohl die AMP-Resistenz für den Infektionserfolg bei Wirbeltieren wesentlich ist, bietet sie *S. aureus* in einer Wirtsumgebung, die aufgrund der Kollateralempfindlichkeit gegenüber anderen Immuneffektoren von AMPs dominiert wird, keinen Überlebensvorteil.

Es war interessant, ob die AMP-Resistenz die Bakterienwachstumsparameter und die pharmakodynamischen Parameter beeinflussen könnte. In Kapitel 1 konnte ich zeigen, dass das von *S. aureus* ausgewählte Defensin (tenecin 1) eine verlängerte Verzögerungsphase im Vergleich zur empfindlichen nicht ausgewählten Kontrolle hervorruft. Die maximale Geschwindigkeit der Bakterienvermehrung wurde jedoch durch die Entwicklung der AMP-Resistenz nicht beeinflusst.

In Kapitel 3 wurde ein pharmakodynamisches Vier-Parameter-Modell verwendet, um Time-Kill-Kurven von AMP-resistenten *S. aureus*-Stämmen zu analysieren. Die vergleichende Analyse der pharmakodynamischen Kurven von pexiganan-, melittin- und tenecin-resistenten *S. aureus* zeigte, dass sich der Hill-Koeffizient Kappa, der die Steilheit der pharmakodynamischen Kurve beschreibt, evolviert. Kappa wird in den meisten pharmakodynamischen Modellen ignoriert oder auf einen konstanten Wert gesetzt. Ein solches Ergebnis ist entscheidend, wie zuvor gezeigt wurde, weil sich das Kappa auf die Wahrscheinlichkeit der Resistenzentwicklung gegen AMPs bezieht.

Ärzte führen routinemäßig pharmakodynamische Analysen für verschiedene Medikamente durch. Die Ergebnisse aus Kapitel 3 verdeutlichten jedoch, dass Krankheitserreger unterschiedliche pharmakodynamische Evolutionsmuster aufweisen, die beispielsweise bei der Optimierung der Auswahl eines neuen Medikamentenkandidaten in der periodischen Therapie berücksichtigt werden sollten. Darüber hinaus könnte dieser Befund auf alle Arten von Wirt-Pathogen-Beziehungen ausgedehnt werden. Die Entwicklung der pharmakodynamischen Parameter sollte für MIC nicht zu

stark vereinfacht werden, wenn die Koevolution zum AMP-Abwehrcocktail des Wirts oder anderen antimikrobiellen Mitteln untersucht wird. Dies könnte unsere Wahrnehmung der evolutionären Dynamik der Arzneimittelresistenz bereichern.

Insgesamt ergab die Arbeit, dass die AMP-Resistenz sowohl *in vivo* als auch *in vitro* meist hohe Fitnesskosten aufweist. Diese beeinträchtigte Fitness von AMP-Pathogenen spielt eine entscheidende Rolle bei der Unterstützung von AMPs als Therapeutika. Einige AMP-resistente Mutanten zeigten einen höheren Infektionserfolg als andere. Daher sollte für jede AMP-basierte Therapie eine Untersuchung der Fitnesskosten möglicher sich entwickelnder resistenter Pathogenmutanten ausgewertet werden. Es ist enorm wichtig, die Vor- und Nachteile von AMPs als Therapeutika abzuwägen und die unbeabsichtigte Entwicklung von AMP-resistenten Superbugs zu vermeiden.

2. General Introduction

2.1. Antibiotic resistance impedes medical treatment

The beneficial effect of antibiotics, since Alexander Fleming's discovery of penicillin (Fleming, 1941) in controlling infections related morbidity and mortality is undeniable (Andersson & Hughes, 2010). The astounding golden era did not persist for half a century. Unfortunately, the number of pathogenic bacterial species resistant to antibiotics is continuously growing. Beyond that, the number of antibiotics to which a pathogen is resistant has expanded world-wide (Cohen, 1992; McCormick, 1998; Andersson & Levin, 1999). Therefore, the evolution of antibiotic resistance is relentless (Davies & Davies, 2010). The sudden rise in the evolution of resistance toward antibiotics might result in a simple infection turning into a fatal epidemic (Andersson & Hughes, 2010; Davies & Davies, 2010).

Despite admitting antibiotic resistance as a growing public health problem, physicians prescribe antibiotics extensively in medical treatment, even worse broad-spectrum antibiotics (Wester et al., 2002). The World Health Organization (WHO) recommended tackling several issues to reduce antibiotic resistance. These include: scaling down antimicrobial use in infection control of animal livestock and encouraging the development and production of newer antimicrobial agents (WHO, 2012). More than 70% of the world produced antibiotics are used in animal industry (Van Boeckel et al., 2017), which is associated with drug-resistant infections in both animals (Aarestrup et al., 2000) and humans (O'Neill et al., 2016). Egypt, Iran, eastern Turkey, northern India and Northern China are some of the hotspots of resistance in the world (Van Boeckel et al., 2019).

One of the most promising suggested therapeutic agents is antimicrobial peptides (Zasloff, 2002). To weigh the pros and cons of development of AMPs-based therapeutics, the AMPs characteristics that favour their pharmaceutical application will be reviewed herein. Since the significant obstacle of clinical administration of AMPs is the doubt of selecting for cross-resistance to the host's own immune antimicrobials. Therefore, from a host-pathogen relationship perspective, the fitness cost of the evolution of AMP resistance in an insect model will be discussed. Some dominant mutations that are assumed to be responsible for AMP resistance in *Staphylococcus aureus* will be reviewed. Moreover, the thesis will focus on some critical growth and pharmacodynamic parameters that co-evolve with AMP resistance that should not be simplified or ignored when testing for AMPs resistance. Based on this knowledge, a pipeline that is meant to assess the risks of utilizing AMPs in medical remedies could be developed. Immunological response of a host to infection with AMP-resistant pathogen could be evaluated. Pharmacodynamic parameters that might be convenient in the evaluation of AMP resistance could be highlighted.

2.2. Antimicrobial peptides (AMPs): cosmopolitan class of antimicrobials

Being evolutionarily conserved amongst all forms of life, the gene-encoded AMPs, are assumed to play a fundamental role in the successful evolution of complex multicellular organisms (Zasloff, 2002). AMPs are exploited by simple prokaryotes, such as bacteria, to provide a competitive advantage against close residing species in particular ecological niches (Hassan et al., 2012). In multicellular eukaryotes, however, AMPs represents a central component of the innate immune system (Lehrer & Ganz, 1999; Zasloff, 2002). AMPs can be either constitutively expressed or induced to resist invading pathogens (Hancock & Diamond, 2000; Boman, 2003; Johnston et al., 2014).

In vertebrates, AMPs play an indispensable immunogenic role by directly killing invading microbes in the early stage and augmenting the adaptive immune response in a later stage (Boman, 2003; Bulet et al., 2004). For example, amphibians synthesize antimicrobial peptides and store them as larger inactive proteins with an acidic pro-piece in skin granular glands. Upon injury, pro-AMPs are cleaved producing mature active peptides (Amiche et al., 1999; Bowie et al., 1999), and the granular glands are emptied releasing a layer of active AMP-cocktails on their skin to protect against bacterial and fungal infections (Rollins-Smith et al., 2005; Sheafor et al., 2008).

Moreover, AMPs also protect humans from pathogens (Bulet et al., 2004). The clonal expansion of slow-growing B and T cells can take up to seven days, while one bacterial cell with 50 min doubling time can produce up to $5*10^8$ CFU in 24h (Hancock & Diamond, 2000). Therefore, our body depends on the innate immune system to control the onset of infection. Several AMPs have been identified in human tissues that more often come into contact with microbes such as skin, eyes, ears, mouth, airways, lung, intestines, and the urinary tract (Wang, 2014).

As a consequence of close coexistence with microbes, insects have evolved efficient mechanisms to recognize and defend themselves against pathogenic attacks by viruses, bacteria, fungi, and macro-parasites (Lemaitre & Hoffmann, 2007). Invertebrates, such as crustaceans and insects, have no equivalent of the vertebrate adaptive immune system, instead rely entirely on the innate immune system (Boman, 1998; Siva-Jothy et al., 2005). Upon challenging, the innate system produces AMPs that are capable of eradicating an invading pathogen (Bulet et al., 2004) or more explicitly mopping up persistent pathogens that failed to be cleared out by cellular immune effectors (Haine et al., 2008).

Upon septic injury, AMPs are rapidly synthesized in the insect fat body, a functional equivalent of the mammalian liver (Lehrer & Ganz, 1999). AMPs are, then, secreted into the hemolymph and might reach a concentration of 100 μM (Meister et al., 1997). When challenged by bacteria, insects exploit AMP-cocktail rather than a single compound (De Gregorio et al., 2001; De Gregorio et al., 2002; Vogel et al., 2011; Johnston et al., 2014; Marxer et al., 2016). The antimicrobial effect of combinations of AMPs are not additive but rather synergistic (Pöppel et al., 2015; Yu et al., 2016; Zanchi et al., 2017). A strategy that is believed to diminish the risk of evolution of resistance towards AMPs (Lazzaro, 2008; Chernysh et al., 2015).

2.2.1. AMPs structure

AMPs usually have a molecular mass below 25–30 kDa, mostly displaying hydrophobic, cationic properties and have an amphipathic structure: alpha-helix or beta-sheet (e.g. mammalian defensins) (Bulet et al., 2004; Fjell et al., 2011). For example, human antimicrobial peptides are more than 100 amino acids with a high positive charge (on average positive 10) (Wang, 2014). AMPs are, generally, classified according to their secondary confirmation into (i) linear peptides with alpha-helix (e.g. insect cecropin, magainins), (ii) cyclic peptides with pairs of cysteine residues that are stabilized by disulfide bridges (e.g. defensins.), (iii) peptides which are rich in particular amino acids (e.g. proline, tryptophan and histidine-rich AMPs) (Lehrer & Ganz, 1999; Fjell et al., 2011; Wang, 2014). Nuclear magnetic resonance NMR spectroscopy studies have pointed out that appealing characteristics of AMPs for chemical synthesis, that ensure their pharmaceutical efficacy and activity, depend on several points: (i) the ability to form amphipathic structures, (ii) the hydrophobicity, (iii) the net charge, (iv) the size, sequence, and overall structure complexity (Bulet et al., 2004).

2.2.2. Magnitude and mechanism of action

AMPs have a broad-spectrum of activity, being efficient scavenger of Gram-positive and Gram-negative bacteria, fungi and virus (Wimley & Hristova, 2011). The structure and the physical properties of AMPs drive their success and conservation among the animal kingdom.

One distinct feature of cationic AMPs is their positive net charge at physiological pH (Bulet et al., 2004). Being cationic, AMPs, are attracted to the negatively charged lipid head groups of bacterial membrane over zwitterionic mammalian cytoplasmic membranes (Nguyen et al., 2011). Exceptionally, there are few anionic AMPs, for example, dermcidin, the human skin AMP (Lai et al., 2007; Harris et al., 2011).

AMPs could be categorized generally into membrane disruptive and non-membrane disruptive, which target intracellular sites (Toke, 2005). The AMPs are then inserted into the outer aspect of the cytoplasmic membrane lipid bilayer, leading to the displacement of lipids and alteration of the membrane structure such as membrane thinning, pore formation, deformed curvature, altered electrostatics, localized perturbations and finally a risk of translocation through the membrane into the cytoplasm to reach intracellular targets (Fjell et al., 2011). Such disruptions might lead to oxidative stress (Choi et al., 2017), disrupted energy production machinery (Wenzel et al., 2014) or graded leakage and thus, bacterial cell lysis (Fjell et al., 2011).

AMPs are capable of rapid and efficient elimination of bacteria. Cationic AMPs kill bacteria within one to few minutes (Boman, 2003; Barns & Weisshaar, 2016), in contrary with antibiotics which might require one to few hours (Brauner et al., 2017) as membrane perturbations were captured by Atomic Force Microscope (AFM) after 50-200 seconds of incubation with AMPs (Fantner et al., 2010). Growth inhibition of *Escherichia coli* occurs within 30 minutes of incubation with LL-37, a human AMP, and by far no colonies growth were observed after one hour of incubation (Choi et al., 2017).

2.2.3. Mechanisms of AMP resistance

Electrostatic attractions seemed to be a universal mechanism by which most described cationic AMPs damage the bacterial plasma membrane (Anaya-Lopez et al., 2013; Andersson et al., 2016; Joo et al., 2016a). Since the current thesis focus on *Staphylococcus aureus* as a pathogen, only, mechanisms of resistance of Gram-positive bacteria are further reviewed. Bacteria evolved several resistance mechanisms towards AMPs, including passive and adaptive resistance (Andersson et al., 2016). The earlier refers to bacteria tendency to decrease affinity to cationic AMPs by increasing the net positive charge through modification wall teichoic acids (Ernst et al., 2009; Dorling et al., 2015).

Genetic mutation plays an essential role in the evolution of AMP resistance. Different mutations were shown to result in AMP resistance in *S. aureus* (Dobson et al., 2013; Makarova et al., 2018). These mutations evolved despite selection against different AMPs, so one mutation could result in muti-AMPs resistance (Johnston et al., 2016). These genetic mutations vary in their impact on bacterial fitness.

Altered expression of some transporters might generally lead to multidrug resistance via active pumping of the drug out of the bacterial cell (Poole, 2001). Our laboratory showed previously that experimental selection of *S. aureus* against AMPs resulted in evolved strains harbouring a mutation in either the *pmt*or *nsa* operons (Makarova et al., 2018). Pmt is an ATP-binding cassette (ABC) transporter that

facilitates the efflux of the cytotoxins, Phenol soluble modulin (PSM) peptides (Chatterjee et al., 2013). Upstream of the *pmt*A-D genes lies *pmt*R which codes for a GntR-type transcriptional regulator. Hooking of PmtR to the binding operator site of the *pmt* promoter represses the *pmt* operon expression (Joo et al., 2016a). The AMP-resistant *pmt* mutants can exploit the Pmt A-D efflux pump to get rid of AMPs along with PSM (Joo et al., 2016b) which is an example of adaptive AMP resistance.

Nisin and bacitracin resistance in *S. aureus* evolved through mutations in the nisin susceptibility-associated two-component system. The NsaR binding site is located upstream of two ABC transporters BraDE and VraDE. In *S. aureus*, the *vra*FG encodes an efflux pump (Coates-Brown et al., 2018). A reason why *nsa*SR mutants constitutively capable of nisin A extracellular efflux (Arii et al., 2019). Active pumping might be a common adaptive mechanism by which *S. aureus* harbouring a mutation in *nsa* operon evolved AMP resistance (Makarova et al., 2018).

2.2.4. Pharmacodynamics of AMP

Based on pharmacodynamic characteristics of AMPs, the evolution of resistance towards AMPs has a much lower probability compared to antibiotics (Yu et al., 2018). Pharmacodynamics studies are based on time-kill curves. Regoes et al. (2004) analyzed time-kill curves using a four-parameter pharmacodynamic function that correlates drug concentration and bacterial growth or death rates (Regoes et al., 2004).

The Hill coefficient, as described by the steepness of pharmacodynamic curves, is much higher for AMPs than for antibiotics (Yu et al., 2016). AMPs kill faster than antibiotics; thus, the maximum killing effect of AMPs is much stronger than that of antibiotics (Fantner et al., 2010; Yu et al., 2018). As a result, AMPs exhibit a narrower mutation-selection window if compared to antibiotics; therefore, the evolution of resistance towards AMPs is less frequent (Yu et al., 2018).

Inspired by innate defence cocktails in nature, a combination of AMPs in a cocktail is advantageous as it was shown that it has higher kappa values than single AMPs (Yu et al., 2016). “The more AMPs combined, the better antimicrobial performance they have” concept (Yu et al., 2016) is vital for combinational therapy, a proposed antibiotic replacement regimes (Walkenhorst, 2016). Minimum inhibition concentration (MIC) is, commonly, used to assess cross-resistance (Brauner et al., 2016; Wen et al., 2016), albeit, the proved importance of pharmacodynamic parameters in predicting drug resistance evolution (Chevereau et al., 2015; Lukacisinova & Bollenbach, 2017; Yu et al., 2018). We

explored whether the pharmacodynamic parameters co-evolve with AMP resistance beyond the overlooked MIC in **chapter 3** (El Shazely et al., 2020).

2.2.5. Pros and Cons of therapeutic application of AMP

Generally, the attractive features of AMPs include their broad-spectrum activity and fast killing, which defines their unique pharmacodynamic curves and consequently complicates the evolution of resistance (Yu et al., 2018). Moreover, additional effects such as immunomodulation (Mansour et al., 2014) and wound healing promotion (Ramanathan et al., 2002; Carretero et al., 2008; Pfalzgraff et al., 2018) are demonstrated for specific peptides (Zharkova et al., 2019).

Despite being proposed as a promising replacement for antibiotics, AMPs still have some clinical limitation, such as (i) high cytotoxicity (ii) short half-lives because of their low resistance to proteolytic degradation (iii) high production cost (iv) coevolution of resistance to host own AMPs, which has been dubbed "Arming the enemy" (Bell & Gouyon, 2003).

Many attempts have been made to alleviate these limitations. Cytotoxicity is type and concentration-dependent. Screening for less cytotoxic AMPs and adjusting their pharmaceutical properties might be useful to develop new therapeutics (Mookherjee et al., 2020). Short half lifetime limits AMPs systemic application but not topical and medical device-related application for example AMPs might be used to overcome bone cement- and catheter-related infections (Giuliani et al., 2007; Volejnikova et al., 2019).

Synthesizing AMP-analogues with optimized characteristics (Mookherjee et al., 2020) is a promising field, such as peptide mimics. Peptide mimics resemble the activity spectrum, mechanism of action, and amphiphilic structure, however, their backbone composed of non-proteogenic amino acid linked in a chain rather than the regular amino acids (Rotem & Mor, 2009; Findlay et al., 2010).

2.2.5.1. Arming the enemy

In contrast to its low incidence in natural populations, bacterial diseases have commonly evolved devastating resistance to many antibiotics within a few years of its commercial adoption (Palumbi, 2001). For example, currently, the majority of *Staphylococcus* infections are multidrug-resistant; however, in 1940, almost all Gram-positive infections, including *Staphylococcus* infections, were susceptible to penicillin (Palumbi, 2001). It was, initially, assumed that it would be difficult for bacteria

to evolve resistance to AMPs (Hancock & Chapple, 1999; Schröder, 1999; Zasloff, 2002), a reason why AMPs were studied and developed as novel therapeutics against multidrug-resistant microbial infections and biofilms (Ge et al., 1999; Zasloff, 2002; Giuliani et al., 2007; Afacan et al., 2012; Mylonakis et al., 2016; Pfalzgraff et al., 2018). Based on *in vitro* bacterial selection experiments, it is now well demonstrated that bacteria can readily evolve resistance towards AMPs (Perron et al., 2006; Habets & Brockhurst, 2012; Dobson et al., 2013; Makarova et al., 2018), albeit less frequently if compared to antibiotics (Anaya-Lopez et al., 2013; Yu et al., 2018).

Considering that mechanisms by which bacteria evolve resistance are conserved (Anaya-Lopez et al., 2013; Joo et al., 2016a), cross-resistance to human AMPs might be a standing risk (Bell & Gouyon, 2003; Hancock, 2003; Habets & Brockhurst, 2012; Dobson et al., 2014; Fleitas & Franco, 2016). Bell and Guoyon (2003) highlighted that the evolution of resistance to human antimicrobial peptides would have much more tremendous consequences than the evolution of resistance to conventional antibiotics. Evolution of resistance to therapeutic AMPs might lead to cross-resistance to host immune antimicrobials, and this consequently would directly limit our natural immunity ability to resist infection (Bell & Gouyon, 2003).

Moreover, most multicellular organisms use AMPs not only to combat infection but also to regulate bacterial symbionts in the intestine and other tissues (Bevins & Salzman, 2011). For example, In the mammalian gut, specialized cells of the mucosal epithelium, Paneth cells, are the primary source of AMPs secretion (Wehkamp et al., 2005), which set immune boundaries of the gut microbiota. Paneth cells dysfunction leads to chronic inflammatory bowel disease (Bevins & Salzman, 2011). Co-evolution between host and symbionts is a homeostatic relationship (Bevins & Salzman, 2011). Given this knowledge, the risk of evolution of resistance toward self AMPs might unleash microbiota over the borderline, causing pathogenesis (Lazzaro et al., 2020).

The only test of this hypothesis has been carried out previously in our laboratory using the model insect, *Tenebrio molitor*, as a host (Dobson et al., 2014), where iseganan, melittin and pexiganan resistant *S. aureus* were introduced in the beetle. Infection load and bacterial virulence were quantified. Dobson et al. (2014) found that only iseganan-resistant *S. aureus* survived better than the AMP-sensitive strains in the beetle while melittin- and pexiganan-selected strains did not. The results suggested that AMP-resistant pathogen might be able to establish more potent infection and hence pathogenesis. Tests with pathogens selected for resistance against the host's own AMPs was lacking by then. We explicitly tested this concept in **chapter 1** (El Shazely et al., 2019) using *Tenebrio molitor* as a model and

Staphylococcus aureus as a pathogen. Tests in a mammalian host or with human AMPs are also still lacking (Lazzaro et al., 2020). It is necessary to understand the fitness cost of AMP resistance in natural

context before AMPs are rushed into a medical application. Such a knowledge lacking might lead to a rapid evolution of resistance to AMP, rendering them as ineffective therapeutics as antibiotics and magnifying the resistant infections crisis (Lazzaro et al., 2020).

2.3. Evolution of host-pathogen interactions

Host and pathogen inflict selection power on one another that shapes their interaction outcome (Siva-Jothy et al., 2005). Hosts evolve sophisticated immune cascades in response to selection by pathogens (Rolff & Schmid-Hempel, 2016). To establish colonization, persistent pathogens or even symbionts evolve mechanisms to either stand for the host immune effectors (Cheung et al., 2018) or seek protected compartment hiding beyond their reach (Bevins & Salzman, 2011; McGonigle et al., 2016).

2.4. The natural history of insect immunity: Tenebrio molitor as a model

In nature, the mealworm *T. molitor* are exposed to a wide range of pathogens and parasites, which might reduce the mealworm survival or reproductive success. Accordingly, *T. molitor* exploits an arsenal of behavioural, physical, and physiological mechanisms to combat infection (Vigneron et al., 2019). *T. molitor* had been used as a model to study host-pathogen interactions (Haine et al., 2008; Fabrick et al., 2009; Chae et al., 2012; Dobson et al., 2014; Johnston et al., 2014; Dorling et al., 2015; Makarova et al., 2016; McGonigle et al., 2016; Khan et al., 2017; Kim et al., 2017; Zanchi et al., 2017; Maistrou et al., 2018; Urbanski et al., 2018; de Carvalho et al., 2019; El Shazely et al., 2019).

Generally, the immune mediators in arthropods could be classified into either cellular or humoral components. Those effectors might be either constitutively expressed or induced upon pathogen recognition (Gillespie et al., 1997). “Cellular immunity” refers to hemocytes, dependent immune responses (Strand, 2008). “Humoral” immunity includes soluble proteins dependent reactions such as antimicrobial peptides, lysozymes and cytotoxins (Bulet & Stocklin, 2005; Siva-Jothy et al., 2005).

Once a pathogen has breached the physical cuticular barrier, the insect attempts to localize and neutralizes the microbe. Resembling other insects, *T. molitor* relies on innate immune effectors. Initially, insects use recognition receptors (PRRs) to detect conserved non-self-microbe associated

molecular patterns (MAMPs), such as peptidoglycans (PGN), lipopolysaccharides (LPS) and β-1,3 glucans, and other sugar moieties (Hoffmann, 1995; Vigneron et al., 2019). PRRs set off the stream of signalling pathways to activate: (i) Cellular defence such as phagocytosis (Kim et al., 2017). (ii) Melanization processes regulated by prophenoloxidase activation cascade (Park et al., 2007). (iii) AMP synthesis regulated by the Toll and immune deficiency (IMD) pathways (Kan et al., 2008; Roh et al., 2009; Rolff & Reynolds, 2009; Johnston et al., 2014).

2.4.1. Cellular immunity

Hemocytes are responsible for cellular defences, primarily phagocytosis, nodulation, or encapsulation of invading pathogen. Phagocytosis refers to a pathogen engulfment (Strand, 2008), nodulation involves the aggregation of hemocytes around microorganisms (Ratcliffe & Gagen, 1977; Satyavathi et al., 2014), while encapsulation refers to the binding of hemocytes to more giant invaders such as parasitoids or nematodes (Strand, 2008).

Various hemocytes differ in shape and function, and prevalence percentage in the hemolymph. *Tenebio* beetles have four main types of hemocytes: granulocytes, plasmatocytes, oenocytes, and prohemocytes accounts for 50-60%, 23-28 %, 1-2% and 10-15 % of total hemocytes number respectively (Urbanski et al., 2018; Vigneron et al., 2019). Granulocytes are 10 μm phagocytes, harbouring dense cytoplasmic dense granules (hence nomenclature). Plasmatocytes are elongated shaped hemocytes that are likely involved in encapsulation. Oenocytoids are large oval hemocytes with a centrally located nucleus and responsible for producing enzymes of the melanization cascades. Prohemocytes are small oval undifferentiated progenitor hemocytes (less than 10 μm) (Chapman, 1998; Strand, 2008; Vigneron et al., 2019).

The recognition of microbes and hence phagocytosis occurs directly or after pathogen opsonization by thioester proteins. Scavenger, Nimrod receptors and spliced Dscam facilitate opsonization (Cherry & Silverman, 2006). It is worth a notion that *T. molitor* Scavenger Receptor class C (SR-C) is essential for phagocytosis of fungi and bacteria (Kim et al., 2017). Whether AMP-resistant pathogen is less or more recognized by the host phagocytes, to the current knowledge, has not been investigated before. Although antibiotic-(chlortetracycline) resistant *E. coli* were shown to be more susceptible to phagocytosis (Macfadden et al., 1960), vancomycin resistance was shown to impair opsonization by the human serum and hence trigger resistance to phagocytosis of *S. aureus* (Gemmell, 2004). Resistance to antimicrobials that necessitate cell surface variations, such as AMPs, might impair pathogen

recognition by the phagocytes and consequently phagocytosis. We investigated the susceptibility of AMP-resistant *S. aureus* to recognition by phagocytes in **chapter 1** (El Shazely et al., 2019).

2.4.2. Cytotoxins and melanization

Melanization refers to production and deposition of melanin around nonself objects including bacteria, protozoan, or parasitoid eggs (Lemaitre & Hoffmann, 2007; Zhu et al., 2013; Vigneron et al., 2019). Prophenoloxidase (PPO), is an inactive precursor of an enzyme (zymogen) present in some hemocytes, plasma, hindgut, wing disc (in *Bombix mori*), and hind wing in *Tribolium castaneum* (Diao et al., 2012; Dittmer et al., 2012; Shao et al., 2012).

Prophenoloxidase is sensitive to wounding or recognition of a foreign object by pattern recognition receptors such as Gram-negative binding proteins (GNBPs) and peptidoglycan recognition proteins (PGRPs). It is cleaved through a cascade of serine proteases to liberate the active phenoloxidase (PO) (Lu et al., 2014; Vigneron et al., 2019).

T. molitor, with a darker cuticle, thus a higher PO activity, are immunocompetent (Armitage & Siva-Jothy, 2005). However, melanization had been reported to be costly (Sadd & Siva-Jothy, 2006; Khan et al., 2017), as enzymatic melanin production cascade is associated by the production of cytotoxic intermediates, such as phenols, quinones, and reactive oxygen species (Nappi & Vass, 1993; Nappi & Ottaviani, 2000). We investigated whether different AMP-resistant pathogens have either cross-sensitivity or cross-resistance toward PO activity in **chapter 2**.

2.4.3. Antimicrobial peptides

Upon microbe recognition, AMP synthesis, in the fat body cells, is induced via the Toll and IMD signal transduction cascades, c-Jun N-terminal kinase (JNK), and Janus kinase/Signal Transducer and Activator (JAK-STAT). Activation of these signal transduction pathways triggers nuclear translocation of some NF-κB transcription factors such as Relish, Dorsal, and Dif. Consequently, the expression of antimicrobial peptides is induced (Vigneron et al., 2019). *T. molitor* has conserved AMP synthesis signal transduction pathways (Johnston et al., 2014). *T. molitor's* AMP synthesis is induced within 24 to 48 h after microbe-associated molecular patterns, MAMP, recognition. AMP synthesis genes are upregulated 14-21 days post-infection (Haine et al., 2008; Johnston et al., 2014; Makarova et al., 2016). When *T. molitor* is injected with an infecting dose of *S. aureus,* several AMPs genes are upregulated including tenecin 1 (T1, anti-Gram-positive defensin), tenecin 2 (T2, anti-Gram-negative coleoptericin),

and tenecin 4 (T4, attacin) (Johnston et al., 2014). They are inducible and regulated by the Toll and IMD pathways (Roh et al., 2009; Chae et al., 2012), while tenecin 3 is a constitutively expressed anti-fungal Thaumatin (Maistrou et al., 2018). *T. molitor* with T2 knockdown treatment show distinctly high mortality rate, showing that although T2 might not have anti-Gram-positive activity on its own, however, it seems to have host protective effect (Zanchi et al., 2017). Whether being resistant to one or two antimicrobial peptides would deliver cross-resistance to the *in vivo* antimicrobial defence cocktail is unclear; thus, we performed a preliminary study in **chapter 2** to investigate possible resistance/sensitivity of AMP-resistant pathogens toward other *in vivo* AMPs.

3. Aim of the thesis

Bacteria evolve resistance at a cost. Generally, our work aimed to examine the costs and benefits of AMP resistance, if found, in a host-pathogen association. We used the mealworm beetle *Tenebrio molitor* as a host and *Staphylococcus aureus* as a pathogen. We, explicitly, aimed to highlight the *in vivo* and *in vitro* pathogen fitness cost implicated by antimicrobial peptide resistance evolution.

3.1. *In vivo* survival of an AMP-resistant pathogen

In the first manuscript (El Shazely et al., 2019), in order to assess the bacterial evolutionary costs of AMP resistance, the infection dynamics of several *S. aureus* strains that were previously selected against, the *T. molitor* AMP, tenecin 1 or a combination of tenecin 1 plus tenecin 2 were monitored. The bacterial colonization was assessed at four-time points over 14 days post-infection. Moreover, the impact of resistance to one or two of the host's own AMPs on pathogen virulence, as inferred by host survival, was studied. The *in vivo* survival of AMP-selected *S. aureus* was reanalyzed based on the underlying resistance mutation.

3.2. Collateral sensitivity of AMP-resistant pathogen to host's immune effectors

Driven by the unexpected compromised infection success of the *S. aureus* selected to one of the most abundant AMPs in *T. molitor*, we investigated reasons that might elucidate such a mismatch between the evolution of AMP resistance and reduced colonization and virulence.

1. Since most of AMP resistance mechanisms require altering cell surface charge or components, it was intriguing to test how this might influence recognition by hemocytes and consequently phagocytosis (El Shazely et al., 2019). In **chapter 1,** we could highlight undermined cellular recognition of AMP-resistant pathogen by an *in vitro* phagocytosis assay.
2. Moreover, in **chapter 2**, the collateral sensitivity of an AMP-resistant pathogen to phenoloxidase as part of the host humoral immunity was investigated. We utilized RNAi based *in vivo* knockdown protocol, that is well established in our laboratory.
3. Additionally, in **chapter 2**, we designed a preliminary RNAi *in vivo* knockdown study to test for collateral sensitivity of *S. aureus* selected against tenecin 1 or a combination of tenecin 1 plus tenecin 2 to the *in vivo* defence cocktail: for example tenecin 1, tenecin 2, and tenecin 4.

3.3. *In vitro* fitness costs of AMP resistance evolution

1. We investigated, in **chapter 1**, the impact of the evolution of AMP resistance on bacterial growth parameters at a temperature resembling the *in vivo* infection scenario.
2. In **chapter 3** (El Shazely et al., 2020), we used a four-parameter pharmacodynamic mathematical model, based on E_{max} models, to assess the coevolution of pharmacodynamics and AMP resistance. Those four parameters are kappa (describing the steepness of the curve), the pharmacodynamic minimum inhibition concentration (zMIC), the maximal bacterial growth rate in the absence of AMP, and the minimal bacterial growth rate at high concentration of AMP.

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4. Chapter 1

In vivo exposure of insect AMP resistant *Staphylococcus aureus* to an insect immune system

Manuscript 1

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Appendix (Chapter 1)

Supplementary material

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5. Chapter 2

Collateral sensitivity of experimentally evolved AMP-resistant *Staphylococcus aureus* to host immune effectors

Manuscript 2

In preparation

Abstract:

Antimicrobial peptides (AMP) are essential immune effectors in insects. Bacteria can evolve resistance against AMPs. Surprisingly, *Staphylococcus aureus* resistant against an abundant beetle AMP did not increase host mortality or bacterial load in the same host species. A possible explanation for this mismatch is that antimicrobial resistance evolution is costly due to collateral sensitivity of AMP-resistant strains to other immune effectors. Here, we study the susceptibility of AMP-resistant *S. aureus* to other immune effectors such as phenoloxidase (PO) and other AMPs of the defence cocktail, using *Tenebrio molitor* as a host. Exploiting RNAi-based knockdown (KD), we studied *S. aureus in vivo* survival in host lacking other immune effectors. We found that all except one AMP-resistant strain (*nsa-rpo* mutant) displayed collateral sensitivity toward phenoloxidase. Moreover, *pmt* and *nsa-rpo* mutant strains showed cross-sensitivity to one or more component of the *in vivo* AMP defence cocktail. Thus, one reason why the differences in resistance against AMPs and phagocytosis did not translate into changes in virulence is that it is balanced by collateral sensitivity to the host's other immune effectors. AMP resistance, while a prerequisite for an infection in vertebrates, does not provide a survival advantage to *S. aureus* in a host environment that is dominated by AMPs because of collateral sensitivity to other immune effectors.

1. Introduction:

Antimicrobial peptides are essential immune effectors of multicellular organisms (Zasloff, 2002). Upon infections, organisms exploit non-specific AMP defence cocktail with broad antibacterial and antifungal activity (Johnston et al., 2014; Mylonakis et al., 2016). AMPs are also under development as new antimicrobial drugs, partly because AMP resistance evolves slower than antibiotic resistance (Yu et al., 2018) and AMP resistance is very limited among environmental bacteria (Spohn et al., 2019). AMP resistance is prerequisite for bacterial infection establishment (Cheung et al., 2018). Albeit that, we reported earlier that, mostly, experimentally evolved AMP-resistant pathogens do not perform well in the host (Dobson et al., 2014; El Shazely et al., 2019). Specifically, neither colonization of a pathogen selected against one or two of the host's own AMPs nor mortality rate of the host post-infection was distinguishable from that of the AMP-sensitive strains (El Shazely et al., 2019). Mechanisms of bacterial evolution of resistance against AMPs are conserved (Joo et al., 2016), mostly including altering of bacterial cell surface charge or components (Andersson et al., 2016; Cheung et al., 2018; Cheung & Otto, 2018; Arii et al., 2019; Ahmad et al., 2020). The cell surface conformational changes hampered bacterial recognition (Mehes et al., 2012). Consequently, phagocytosis resistance coevolved mostly along with AMP resistance (El Shazely et al., 2019). Resistant to phagocytosis did not reflect in higher bacterial load of infected beetles.

One possible explanation for this mismatch between *bona fide* AMP resistance (Makarova et al., 2018) and low virulence and survivorship in the host (El Shazely et al., 2019) might be collateral sensitivity of AMP-resistant strains to other immune effectors. Collateral sensitivity was first described in the 1950s (Gocke & Finland, 1951; Bryson & Szybalski, 1952). Mostly, it is used to refer to the probability of evolution of hypersensitivity to drug A, upon acquiring resistance to drug B (Nichol et al., 2019). Henceforth, several studies pointed out that such a concept could be exploited clinically (Pal et al., 2015; Udekwu & Weiss, 2018). In sharp contrast, it has remained unclear how frequently evolution of resistance to AMPs as part of the innate immune system would increases sensitivity to other immune effectors, which might be designated as 'collateral sensitivity to an armed enemy'. We hypothesize that AMP-resistant pathogen might co-evolve sensitivity to other AMPs and/or phenoloxidase. Using RNA interference (RNAi) based knockdown (Khan et al., 2017; Zanchi et al., 2017), we aim to investigate AMP-resistant *S. aureus in vivo* survival in host lacking those other immune effectors.

The degree of melanization cascade activation is informed by the type of infection (Ayres & Schneider, 2008). Whether it is sensitive to the resistance level of the pathogen to the host antimicrobial peptides has not been studied before. Our lab previously showed that the iseganan-selected *S. aureus* survived better in the beetles compared to tenecin-, melittin-, and pexiganan-selected strains (Dobson et al., 2014; El Shazely et al., 2019). Such a diverse host perception of different pathogens infers that they are

perhaps recognized and offered varying degrees of *in vivo* collateral sensitivity to the host's immune modulators, which is deterministic for infection outcomes.

An earlier RNAseq study from our laboratory showed that an array of AMPs are upregulated in *Tenebrio molitor* upon *Staphylococcus aureus* infection, including one defensin, three coleoptercin, and four attacin (Johnston et al., 2014). We designed an RNAi knockdown study to test whether *S. aureus* strains that were, experimentally, evolved against tenecin 1 or a combination of tenecin 1 plus tenecin 2 (Makarova et al., 2018) are sensitive to the *in vivo* AMP mixture. Thus, we knockdown three main AMPs that were, previously (Johnston et al., 2014), confirmed by relative quantitative PCR to be upregulated in response to *S. aureus* infection. These AMPs are a defensin (tenecin 1), a coleoptercin (tenecin 2), and an attacin (tenecin 4). The earlier exhibited, primarily, anti-Gram-positive activity (Moon et al., 1994), while the others displayed little to no activity toward *S. aureus in vitro* (Roh et al., 2009; Chae et al., 2012).

In the current study, we assessed the infection dynamics of AMP-selected *S. aureus* over seven days in a manipulated host immune environment. These strains were, previously, selected for resistance to either the *Tenebrio molitor* AMP tenecin 1 or both tenecin 1 & tenecin 2 (Makarova et al., 2018). We performed RNAi-based knockdown of either prophenoloxidase or three leading players of *T. molitor* AMP defence cocktail (tenecin 1, tenecin 2 and tenecin 4) expression. We quantified bacterial load and host survival. We previously reported that resistance was associated with mutations in either the *pmt*RS or *nsa*RS operons (Makarova et al., 2018). Whether cross-sensitivity of AMP-selected *S. aureus* to other immune effectors, if found, is mutation dependent or not is further interesting. Thus, we analyzed the effects of particular mutations on the survival of *S. aureus* within hosts lacking either phenoloxidase or main representative of the AMP defence mixture. Such an experiment will shed some light on the *in vivo* collateral sensitivity of AMP-resistant pathogen to other host immune effectors.

2. Materials and methods:

2.1. Rearing of *Tenebrio molitor*

The mealworm beetles, *Tenebrio molitor,* were reared as previously described in (El Shazely et al., 2019). Briefly, *T. molitor* larvae were purchased from a commercial supplier. Then individualized at a density of 500 individuals per container and kept 25℃ in the dark. Cultures were kept with *ad libitum* access to wheat bran supplemented with fresh peeled apple pieces 2 to 3 times per week as a source of moisture. The rearing boxes were checked for pupae which were examined and sexed under a binocular dissecting microscope. Newly emerged female adults were maintained individually in grid boxes. We provided each with bran, piece of filter paper, and 1 mm^3 piece of apple. Experiments were performed on 7–9 days old females with a weight ranging between 0.120 g and 0.190 g.

2.2. Gene knockdown by RNA interference

Double-stranded RNA injection was used to knockdown gene expression by RNA interference as described by (Zanchi et al., 2017) and (Khan et al., 2017). Briefly, we used synthetic constructs as templates for tenecin 1, tenecin 2 and tenecin 4 dsRNA synthesis. At least two prophenoloxidase (PPO) genes were reported, previously, in a *T. molitor* RNAseq study (Johnston et al., 2014), which encode two subunits of a heterodimer enzyme. We synthesized prophenoloxidase RNAi using the internal region of *T. molitor* cDNA as a template for PO1 and PO2 dsRNA as described previously by (Khan et al., 2017). The PO1 is based on a previously sequenced *Tenebrio* PPO gene transcript (NCBI accession AB020738.1), and PO2 is based on an orthologue to *Tribolium castaneum* PPO subunit 1. We used RNAi based on *Galleria mellonella* lysozyme (Lys) as procedural control because it has no proven homology of sequence with any known gene of *T. molitor* (Johnston & Rolff, 2015; Zanchi et al., 2017).

Templates were amplified by PCR (KAPA2G Fast ReadyMix, KAPA Biosystems) using gene-specific primers (Table S1) tailed with the T7 polymerase promoter sequence (Sigma Aldrich). Integrity and length of the amplicon were checked by running it on a 2 % agarose gel and cleaned-up using a kit (PCR DNA Clean-Up Kit, Roboklon). The resulting amplicon was used as a template for RNA synthesis (T7 RiboMAX™ Express Large Scale RNA Production System, Promega) according to the manufacturer's recommendations. We then purified the RNA with a phenol-chloroform extraction. Finally, the RNA pellet was resuspended in a nuclease-free insect Ringer solution (128 mM NaCl, 18 mM CaCl2, 1.3 mM KCl, 2.3 mM) and kept in -80 °C freezer. In order to obtain dsRNA, annealing was performed by warming up to 65 °C for 30 min then allowing gradual cool down and incubate at least 15 min at 22 °C.

2.3. Experimental design

We performed two knockdown experiments, either of prophenoloxidase (PO1 & PO2) or three-component of AMP defence cocktail (T1T2T4) as follows:

2.3.1. Experiment 1: Collateral sensitivity of AMP-resistant pathogen toward phenoloxidase (one of the host's pro-inflammatory responses)

We aimed to investigate whether the evolution of AMP resistance minimizes or maximizes the survival costs of early inflammation. We experimentally manipulated the degree of inflammation during early-life immune response using RNA interference (RNAi) of prophenoloxidase (PPO) and quantified survival of both pathogen and host. To perform PO1&PO2 knockdown (PO KD) of *T. molitor* prophenoloxidase, we injected 2100±100 ng absolute concentration of dsRNA at a concentration of ca. 350 ng/µl in 6 µl insect Ringer solution. The 6 µl is a mixture of dsRNA, 3 µl based on PO1 gene sequence and 3 µl based on PO2 gene sequence. The procedural control (Lys) received the same dsRNA concentration and total injection volume. Since the prophenoloxidase gene is constitutively expressed. Therefore, we injected each beetle with 5 µl peptidoglycan (Sigma-Aldrich, Cat # 77140, concentration: 100 ng/ml suspended in Ringer solution) two days after RNAi manipulation to deplete the basal amount of PPO in the hemolymph. Two days later, *T. molitor* was challenged with either the AMP-sensitive or -resistant *S. aureus* strain.

2.3.2. Experiment 2: Collateral sensitivity of T1- and T1T2-resistant *S. aureus* toward T2 and/or T4 (other AMP of the defence cocktail).

To investigate whether resistance evolution toward one or two AMP minimizes or maximizes the sensitivity toward other AMPs, we experimentally manipulated the degree of AMP-based immune response using RNA interference (RNAi) of tenecin 1, tenecin 2 and tenecin 4 expression and quantified survival of both pathogen and host. In the case of T1T2T4 knockdowns, we injected the 3000±100 ng absolute quantity of dsRNA in 6 µl total volume of Ringer circa 1000 ng for each gene, resulting in a concentration of 500 ng/ µl of dsRNA in 2 µl of Ringer solution for each gene. The procedural control (Lys) received the same dsRNA concentration and total injection volume. Females were infected with one of AMP-sensitive or -resistant *S. aureus* strains 2 days later.

2.4. Bacteria

2.4.1. Bacteria strains

We picked five AMP-resistant *Staphylococcus aureus* strains, their three respective sensitive controls, and the ancestor strain (SH1000) (listed in Table S2). The bacterial isolates were evolved and described previously (Makarova et al., 2018). The AMP-resistant strains are either resistant to tenecin 1 or a combination of tenecin 1 plus tenecin 2. AMP-resistant strains are representative of the five genotypes studied previously in chapter 1 (El Shazely et al., 2019). *S. aureus* might harbour a mutation in the *pmt*, *nsa*, *rpo*, *pmt-rpo* or *nsa-rpo* operons (listed in Table S2). All strains were kept as glycerol stocks in −80℃.

2.4.2. Bacterial culture and injection

S. aureus was streaked over Müller Hinton (MH) agar from the glycerol stocks and incubated at 30℃ for 48 h. A liquid culture was obtained by scratching three separate colonies with culture needle and transferring to MH broth (PanReac AppliChem, Cat # 413788.1210), then incubated overnight at 25 °C. The optical density of the 16-h culture was adjusted to 0.98 at a wavelength of 595 nm. The bacterial culture was washed three times by centrifuging for 10 min at 7500 g. The supernatant was discarded, and the pellet was resuspended in Ringer solution (Sigma-Aldrich, Cat # 96724-100TAB). Each female beetle received 5 µl of the prepared inoculum; a bacterial load which is equivalent to $6*10^6$ CFU.

A manual injector was used to perform the injections. It was attached to a sterile disposable capillary needle. In the intersegmental membrane, between the fourth and fifth abdominal sternites, parallel to the anterior-posterior axis of the body, the bacterial inoculum was injected. Injections were alternated between left, and right sides of the beetles for dsRNA, peptidoglycan (if found) and bacterial injections to avoid several wounding at the same spot. To prevent perforating the internal organs, the needle was inserted parallel to the peripheral lateral aspect of the haemocoel. The specimen was discarded and replaced if leakage was noticed. The negative control group (sham infected) was injected with 5 µl Ringer solution.

2.5. Quantification of infection

Initially, the CFU count was monitored at 1, 7 and 14 days post-infection. However, there was always a substantial mortality rate after 10 days of infection. Therefore, we excluded the 14 days time point as the cohort was biased for individuals that missed the high risk of death. Only day 1 and 7 time points

were considered for analysis. *Staphylococcus aureus* was recovered by a perfusion bleed technique as previously described by (Haine et al., 2008; El Shazely et al., 2019). Briefly, the beetles were placed over ice for 10-15 min to be temporary sedated. They were washed with 70% ethyl alcohol and carefully dried. After making a small incision in the abdominal lateral periphery, 500 µl of phosphate buffer saline (PBS, ChemSolute, Cat # 8435.0100) was injected into the haemocoel using a 22-gauge needle (Henke Sass Wolf FINE-JECT, Cat # 4710004020) that was inserted between the head and thorax. Then, from the abdominal incision, the outflow was collected into a sterile 1.5 ml tube. The collected hemolymph was vortexed, and then 100 µl was plated on MH agar. We performed serial dilution of the hemolymph. Colony-forming units (CFU) were counted manually two days after the plates were incubated at 30 °C.

2.6. Survival experiment

We also checked whether the virulence of AMP-resistant strains as inferred from the survival of *Tenebrio molitor* is controlled by the host phenoloxidase and other components of the AMP defence cocktail. Each group was injected with one of 9 *Staphylococcus aureus* strains. Each group consisted of 30 beetles. Negative control was injected with Ringer solution (sham infected). The study only included female beetles. Mortality was monitored for 30 days post-injection.

2.7. Data analysis

All the data were analyzed in R version 3.6.0 (RCoreTeam, 2013).

2.7.1. Quantification of infection in *T. molitor* with different knockdown treatment

The sham infected group showed no recovered *S. aureus* CFU. Thus, it was excluded from further analysis. First, we analyzed whether the sensitivity of particular AMP-resistant strains toward knockdown of prophenoloxidase and a cocktail of AMPs T1T2T4 could explain the variations (if found) in the number of CFU of the recovered *S. aureus* strains at two-time points of infection (1 and 7 days post-infection). To account for the nested ontogeny of the nine *S. aureus* strains, a mixed model was fitted to the data set, and "lines" was implemented as a random factor. The CFU data were best fitted when Box-Cox transformed (power transform).

We used the function "lme" from "nlme" package to run a linear mixed model on the Box-Cox transformed data. The heteroscedasticity of the variances between each knockdown treatment was modelled using function "varIdent" which was implemented in the weight of the lmm model. *Post hoc* comparisons were performed using "lsmeans" function with a "Tukey"

adjustment from package “lsmeans”. Models were assessed and compared using “Anova” function from package “car”.

2.7.2. Survival analysis

We tested whether the AMP resistance of the infecting strains and knockdown treatment of the beetles as well as the interaction between them, explained the mortality of the beetles over 30 days. Based on the assumption of non-constant hazard using Weibull errors, we analyzed the data using the “survreg” function from the “survival” package. Post hoc comparisons were performed using the function “pairwise_survdiff” from package “survminer” with “Benjamini-Hochberg” adjustment.

2.7.3. Gene expression analysis

To test the efficiency of dsRNA-based knockdown treatment, the relative expression was calculated using the “pcr_analyze” function from the “pcr” package. The Ct values of target sequences were normalized to Ct values of the housekeeping gene (RPL27 gene that codes for ribosomal protein). The relative expression was compared across control and knockdown treatments by using a linear model on a log (log_{10}) transformed data (see appendix (Chapter 2), Fig. S1, S2, for results of gene expression analysis). The model was assessed, and F statistics were extracted using “anova” function.

2.8. Checking knockdown efficiency

2.8.1. RNA extraction

A quantitative real-time polymerase chain reaction (qRTPCR) was performed to test for the effectiveness of target genes knockdown on the mRNA extracted from the beetles’ fat bodies (an equivalent to the human liver).

Five random corpses of frozen beetles per treatment, stored at -80°C, were selected to check for dsRNA-based knockdown effectiveness. Over dry ice, 800-1000 µls of RNALater -ICE Frozen Transition Solution (Ambion life technologies, Cat. # AM7030, 4427575) were added to the frozen samples and stored in -20 °C for 24 h. Frozen corpses were then dissected under a dissecting microscope using sterile, RNAse-free tools. The fat bodies were isolated and stored in a 2 ml tube containing 1 ml TRIzol Reagent (Sigma-Aldrich, Cat # T9424-200ML). To grind the tissues, two sterile PCR-clean 3mm beads (Qiagen, Cat # 69997) were added to the TRIzol-

fat bodies mixture. In a Retsch MM 400 homogenizer, the samples were run twice for 3 min at 30 Hz.

The homogenized samples were then incubated at room temperature for 5 min, then 400 µl Chloroform (Sigma-Aldrich, Cat # 472476) were added. The samples were vortexed vigorously and incubated at room temperature for 10 min. The samples were centrifuged at 14 000 g for 15 min. The upper aqueous phase was transferred into a new tube. Then, 1000 µl isopropanol (Carl Roth, Cat # 6752.4) were added and stored at -20°C overnight to precipitate nucleic acids. The samples were centrifuged at 20 000 g at 4 °C for 20 min. The supernatant was removed, and the pellet was washed by adding 1000 µl 75% ethanol (prepared from Carl Roth, Cat # 9065.3). The samples were centrifuged at 7 500 g for 5 min. For each sample, the supernatant was discarded, and the pellet was shortly air-dried. The palette was then solubilized in 88 µl nuclease-free water (Carl Roth, Cat # T143.1).

One µl of DNase I and 1 µl of 10X reaction buffer with MgCl2 (Thermo Fischer Scientific, Cat # ENO521) were added to remove any contaminating traces of genomic DNA. The samples were incubated at 37 °C for 30 min. Then, phenol-chloroform extraction was performed.

One hundred and ten µl of nuclease-free water and 200 µl of Phenol/Chloroform/Isoamyl alcohol (Carl Roth, Cat # A156.1) were added to each sample. The samples were vigorously vortexed for 1 min and centrifuged for 10 min at 12 000 g. The upper aqueous phase was removed and transferred to a PCR-clean 1.5 ml tube, then 200 µl of Chloroform were added. The samples were vortexed for 1 min and incubated at room temperature for 10 min then centrifuged for 10 min at 12 000 g. Five hundred microliters of isopropanol and 15 µl of ammonium acetate solution (Fluka, Cat # 09691-100ML) were added to the upper aqueous phase after transferring into a new tube. The samples were stored at -20 °C overnight to precipitate RNA. The samples were then centrifuged at 14 000 g for 15 min, and the pellets were washed with ethanol. Finally, the RNA pellet was resuspended in 50 µl of nuclease-free water. The concentration and quality of RNA were assessed using NanoDrop ND-1000 spectrophotometer (Peqlab).

2.8.2. cDNA synthesis (Reverse transcription)

The RNA was reverse transcribed into library cDNA using High capacity reverse transcription kit (Applied biosystems, Thermo Fischer Scientific, Cat # 4368814) according to manufacturer instruction. For each sample, 1000 ng of RNA were used per 20 µl reaction volume.

2.8.3. Quantitative Real-Time PCR

To test the efficiency of gene knockdown, a quantitative real-time PCR (qRTPCR) on the transcribed cDNA of *Tenebrio molitor* fat bodies. The qRTPCR was carried out using qPCR Mastermix Plus for SYBR Green I – dTTP (Eurogentec, Cat # RT-SN2X-03+WOUN). 6.25 µl of SYBR Green master mix was mixed with 2 µl of cDNA and 0.5 µl of each of forward and reverse primers (1:10 dilution of 100 pmol/ µl, Table S1). The mixture was adjusted with nuclease-free water to a total volume of 12 µl.

The PCR plates were incubated in Eppendorf MasterCycler RealPlex (Eppendorf AG, Hamburg, Germany) for 2 min at 50 °C, followed by 10 min at 95 °C for denaturation. Then 40 PCR cycles were performed, which consisted of 15 sec denaturation at 95 °C, and 1 min annealing at 60 °C. The resulted Ct values (the cycle quantification value), which is the number of PCR cycle at which a reaction curve intersects the threshold line) were analyzed at a threshold of 200.

3. Results:

Bacterial loads of *S. aureus* segregated by mutation, and knockdown treatment (Anova (type I error) PO KD treatment*infection*time point: $F_{7, 430} = 2.678$, p= 0.01).

3.1. Knockdown of phenoloxidase decreases the survival of *Staphylococcus aureus* (ancestor strain) infected individuals

The *in vivo* survival of ancestral strain *Staphylococcus aureus* (SH1000) in *Tenebrio molitor* was not affected by phenoloxidase knockdown. As similar bacterial loads of phenoloxidase knockdown (PO KD) and the procedural control groups (C) were observed through different infection time points (PO KD treatment: $F_{1, 54} = 0.1694$, p= 0.6823; time point: $F_{1, 54} = 27.7511$, p<.0001, Fig. 1A).

The PO KD *Staphylococcus aureus* infected beetles showed a decreased survival compared to the control, with a mean age at death of 12.9 days (PO KD treatment*infection: X^2= 14.6, df =3, p =0.002, Fig. 1B). However, the survival of PO KD sham infected procedural control was not affected. Therefore, the observed effect is a result of both bacterial infection and knockdown treatment, not the later only.

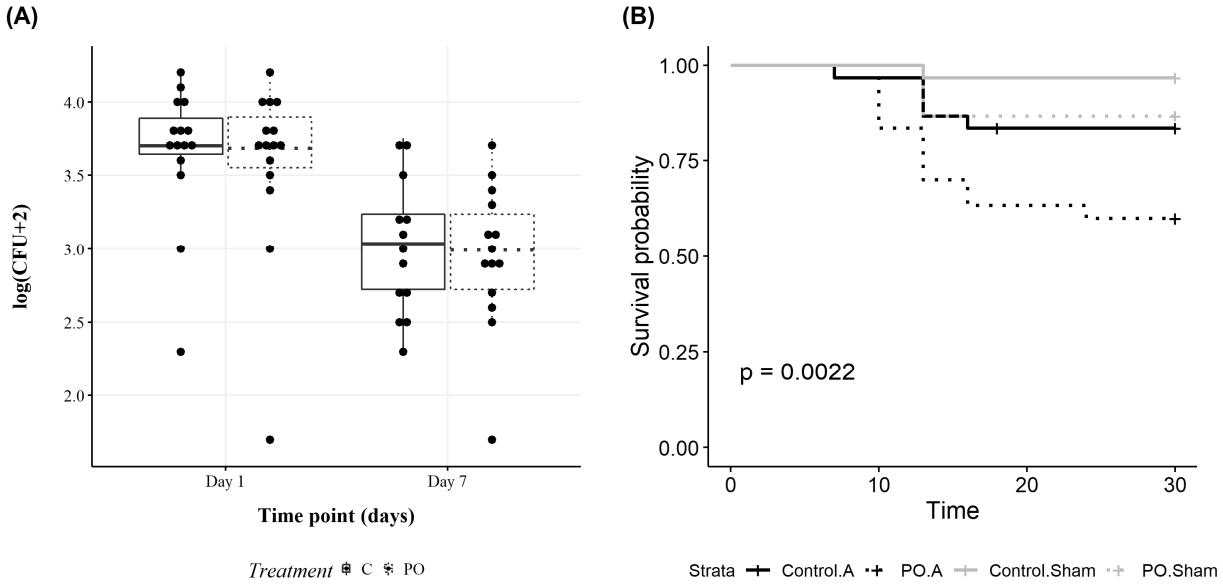

Figure 1. (**A**) Bacterial load of the ancestor *Staphylococcus aureus* strain (SH1000). The PO knockdown (**dotted**) did not affect *in vivo* colonization (**black**) at any time point. The CFU recovered from 100 µl of hemolymph are represented on a log scale by box plots showing quartiles and medians. The bars represent the 1.5 interquartile. (**B**) Survival of *T. molitor* infected with ancestor strain (**black**) decreased by PO knockdown, while the sham infected (**grey**) did not vary with knockdown treatment.

3.2. The *rpo Staphylococcus aureus* mutant is cross-sensitive to the phenoloxidase dependent immune response

The bacterial load of *S. aureus* harbouring a mutation in *rpo* operon did not differ from their respective unselected control strain (C1) 1-day post-infection. Moreover, at this time point, the knockdown treatment had no effect either (*rpo* $_{C}$ vs. C1$_{C}$: T = 1.446, df = 430, p = 0.148; *rpo* $_{PO}$ vs. C1$_{PO}$: T = -1.141, df = 430, p = 0.254; *rpo* $_{PO}$ vs. *rpo* $_{C}$: T = 1.758, df = 430, p = 0.079, Fig. 2A). At 7-days post-infection, the bacterial load of *rpo* mutant decreased compared to the unselected control (*rpo* $_{C}$ vs. C1 $_{C}$: T = 2.056, df = 430, p = 0.0316, Fig. 2A). Knocking-down the prophenoloxidase genes increased the bacterial load of *rpo* mutant to that at normal prophenoloxidase expression levels (*rpo* $_{PO}$ vs. *rpo* $_{C}$: T = 4.480, df = 430, p <.0001, Fig. 2A). This result suggested cross-sensitivity of the tenecin resistant *rpo* mutant to phenoloxidase, unlike their respective unselected control (C1).

The survival of *T. molitor* with *S. aureus* infection segregated neither by strain nor by PO knockdown treatment (PO KD treatment*infection: X^2 = 5.5, df =3, p =0.1, Fig. 2B).

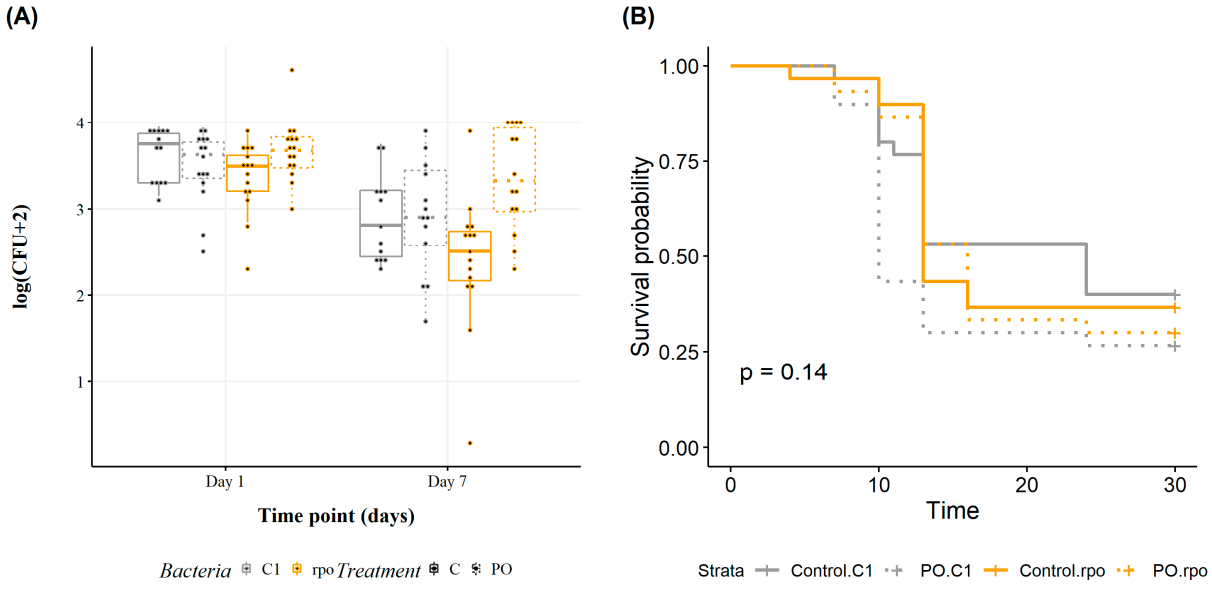

Figure 2. (**A**) Bacterial load of the tenecin 1- (defensin) resistant *Staphylococcus aureus* harbouring a mutation in *rpo* operon. The PO knockdown (**dotted**) increased colonization of *rpo* mutant (**yellow**) by day 7, unlike the unselected control (**grey**). The CFU recovered from 100 µl of hemolymph are represented on a $\log_{10}$ scale by box plots showing quartiles and medians. The bars represent the 1.5 interquartile. (**B**) Survival of *T. molitor* was affected neither by PO knockdown nor by bacterial AMP sensitivity.

3.3. AMP-resistant *Staphylococcus aureus* harbouring a mutation in *pmt, pmt-rpo* or *nsa* operons have cross-sensitivity towards phenoloxidase

One day post-infection, the bacterial load of *S. aureus* harbouring a mutation in *pmt* operon was significantly lower than that of the unselected control (C2) regardless of knockdown (*pmt* $_{C}$ vs. C2 $_{C}$: T = 5.451 , df = 433, p <.0001; *pmt* $_{PO}$ vs. C2 $_{PO}$: T = 4.629, df = 433, p <.0001, Fig. 3(A)). The recovered CFU of *pmt* mutant and the unselected control strain (C2) were equal by day 7 post-infection (*pmt* $_{C}$ vs. C2 $_{C}$: T = 1.759, df = 433, p =0.0792; *pmt* $_{PO}$ vs. C2 $_{PO}$: T = -1.015, df = 433, p =0.3105, Fig. 3(A)). The bacterial load of *S. aureus* harbouring a mutation in both *pmt* and *rpo* operons was slightly lower than that of the unselected control 1-day post-infection (*pmt-rpo* $_{C}$ vs. C2 $_{C}$: T = 2.491, df = 433, p =0.013, Fig. 3(A)), however was not significantly different 7-days post-infection (*pmt-rpo* $_{C}$ vs. C2 $_{C}$: T = 1.399, df = 433, p =0.1626; *pmt-rpo* $_{PO}$ vs. C2 $_{PO}$: T = -1.103 , df = 433, p =0.2707 ,Fig. 3(A)).

The knockdown of phenoloxidase did not affect any of the mutants nor the unselected control 1-day post-infection. However, 7-days post-infection, unlike the unselected control, the bacterial load of tenecin 1 resistant *pmt* and *pmt-rpo* and tenecin 1-tenecin 2 resistant *nsa S. aureus* increase significantly with knockdown treatment (C2 $_{C}$ vs. C2 $_{PO}$: T = -0.878, df = 433, p =0.3802; *pmt* $_{PO}$ vs. *pmt* $_{C}$: T = -2.868, df = 433, p =0.004; *pmt-rpo* $_{PO}$ vs. *pmt-rpo* $_{C}$: T = -2.872, df = 433, p =0.004; ; *nsa* $_{PO}$ vs. *nsa* $_{C}$: T = -2.334, df = 433, p =0.02). Therefore, AMP-resistant *S. aureus* harboring mutation in *pmt*, *pmt-rpo* or *nsa* operon are cross-sensitive to phenoloxidase.

Knocking-down the expression of prophenoloxidase affected the survival of the *S. aureus* (line 2) infected *T. molitor* (PO KD treatment*infection: X^2 = 37.5, df =7, p <.0001, Fig. 3B). Upon PO knockdown, the mortality of *T. molitor* infected with *S. aureus* with a mutation in *pmt* operon increased significantly (p = 0.002). The unselected control *S. aureus* infected beetles showed reduced survival with PO knockdown treatment (p = 0.0001). This was not proved for *pmt-rpo* (p = 0.233) and *nsa* (p = 0.224) mutants. Therefore, phenoloxidase limits the virulence of *pmt* mutant and unselected control (C2).

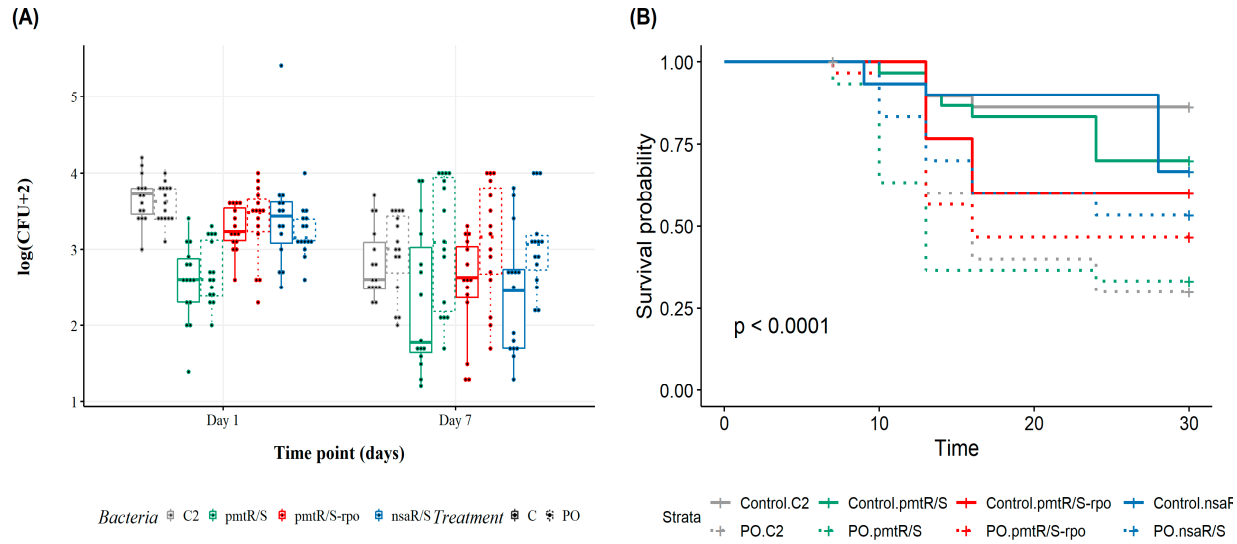

Figure 3. (**A**) Bacterial load of *Staphylococcus aureus* harbouring a mutation in *pmt* (**green**), *pmt&rpo* (**red**) or *nsa* (**blue**) operon. The PO knockdown (**dotted**) increased colonization of AMP-resistant *S. aureus* by day 7 for all mutants, unlike the unselected control C2 (**grey**). The CFU recovered from 100 µl of hemolymph are represented on a log scale by box plots showing quartiles and medians. The bars represent the 1.5 interquartile. (**B**) PO treatment (**dotted**) decreased the survival rate of *T. molitor* infected with *pmt* mutant (**green**) as well as those infected with the unselected control strain, C2 (**grey**). The mortality rates of *T. molitor* infected with *pmt-rpo* (**red**) and *nsa* (**blue**) mutants were not affected by PO knockdown.

3.4. The tenecin 1 and tenecin 2 resistant *nsa-rpo Staphylococcus aureus* mutant is cross-resistant to phenoloxidase.

The T1T2-sensitive unselected *S. aureus* (C3) was not affected by the knockdown treatment 1-day post-infection (T = -0.212, df = 433, p =0.8322, Fig. 4(A)). However, their bacterial load slightly increased 7-days post-infection (T = -2.241, df = 433, p =0.02, Fig. 4(A)).

At normal levels of PO expression, the bacterial load of T1T2-selected *S. aureus* harbouring mutations in both *nsa* and *rpo* operons was significantly higher than knockdown treatment at 7-days post-infection (*nsa-rpo* $_{C}$ vs. $_{PO}$: T = 3.441, df = 344, p =0.0006, Fig. 4(A)). However, it had no effect at earlier infection time point (*nsa-rpo* $_{C}$ vs. $_{PO}$: T = -0.872, df = 344, p = 0.3838, Fig. 4(A)).

In Figure 4(B), The Kaplan-Meier curve of *T. molitor* infected with *S. aureus* descended from line-3, showed that the survival was affected by knocking-down the expression of prophenoloxidase gene (PO KD treatment*infection: X^2 = 37, df =3, p <.0001, Fig. 4B). The mortality of T1T2-sensitive unselected control (C3) increased by approximately 33% with PO knockdown treatment (p = 0.001). However, the mortality of T1T2-resistant *nsa-rpo* mutant decreased by 53.3% with PO knockdown treatment (p< .0001). Therefore, *nsa-rpo* mutant established a better infection and caused higher mortality in the presence of phenoloxidase, in sharp contrast to their unselected control (C3).

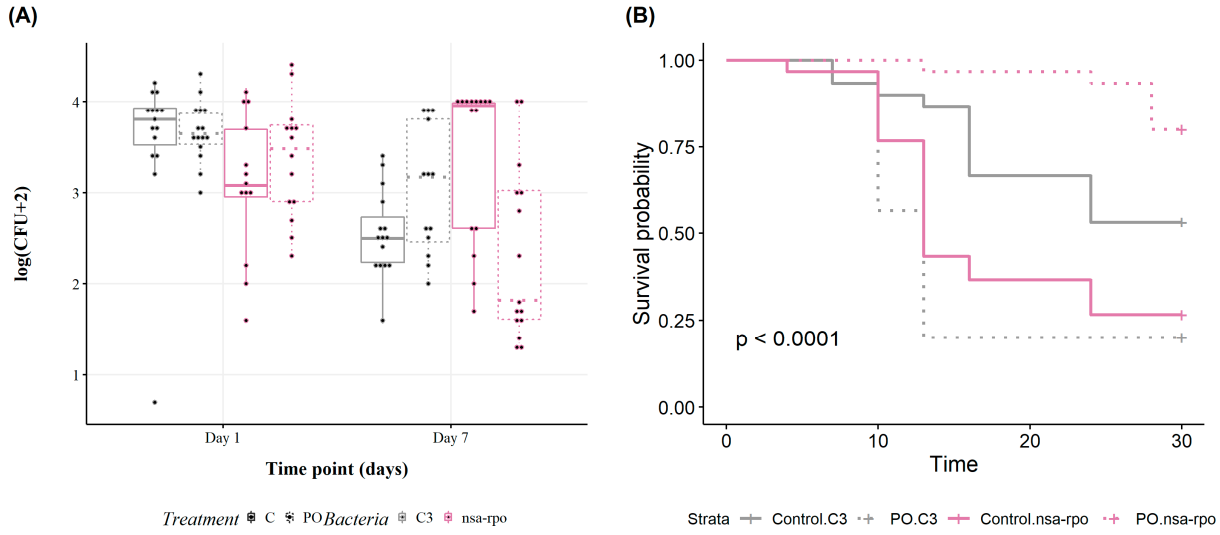

Figure 4. (**A**) Bacterial load of *Staphylococcus aureus* harbouring mutation in *nsa&rpo* (**purple**) operon. The PO knockdown (**dotted**) decreased colonization of AMP-resistant *S. aureus* by day 7, unlike the unselected control C3 (**grey**). The CFU recovered from 100 µl of hemolymph are represented on a $\log_{10}$ scale by box plots showing quartiles and medians. The bars represent the 1.5 interquartile. (**B**) PO treatment (**dotted**) increased the survival rate of *T. molitor* infected with *nsa-rpo* mutant (**purple**) while those infected with the unselected control strain, C3 (**grey**).

3.5. Knockdown of AMPs decreases the survival of *Staphylococcus aureus* (ancestor strain) infected individuals.

When we down-regulated the expression of tenecin 1 (defensin), tenecin 2 (coleoptericin) and tenecin 4 (attacin) in *Tenebrio molitor* before *S. aureus* infection, the bacterial load showed an increased tendency (Anova: T1T2T4 KD treatment: $F_{1,57} = 4.7655$, p= 0.033; time point: $F_{1,57} = 12.02$, p=0.001, Fig. 5A) but not significant if compared to individuals with normal expression levels 7-days (T =-2.183, df = 57, p = 0.1) or 1-day (T =-2.183, df = 57, p = 0.1) post-infection.

The mortality of *Tenebrio molitor* infected with ancestral *Staphylococcus aureus* strain (SH1000) with the knockdown treatment of T1T2T4 genes increased significantly compared to the control, with a mean age at death of 16.3 days (T1T2T4 KD treatment*infection: X^2= 35.4, df =3, p <.0001, Fig. 5B). The observed result is due to both bacterial infection and knockdown treatment because the survival of T1T2T4 knockdown sham infected procedural control was not affected.

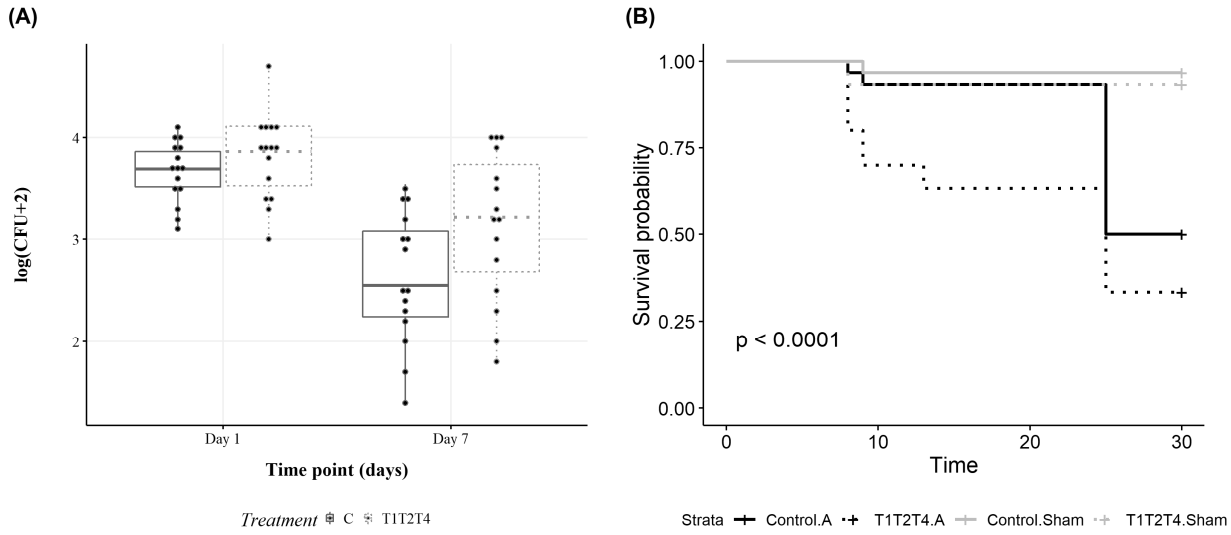

Figure 5. (**A**) Bacterial load of the ancestor *Staphylococcus aureus* strain (SH1000). The T1T2T4 knockdown (**dotted**) did not affect *in vivo* colonization (**black**) at any time point. The CFU recovered from 100 µl of hemolymph are represented on a $\log_{10}$ scale by box plots showing quartiles and medians. The bars represent the 1.5 interquartile. (**B**) Survival of *T. molitor* infected with ancestor strain (**black**) decreased by T1T2T4 knockdown (**dotted**), while the sham infected (**grey**) did not vary significantly by knockdown treatment.

3.6. The tenecin 1-resistant *rpo Staphylococcus aureus* mutant is cross-resistant to the T1T2T4 defence cocktail and more virulent to the host.

The bacterial load of the unselected control increased significantly with T1T2T4 knockdown treatment 7-days post-infection, emphasizing its expected cross-sensitivity (T = -2.724, df = 455, p = 0.006). This was not the case for *rpo* mutant, as their bacterial load did not fluctuate with knockdown treatment (day 7: T = 1.265, df = 455, p = 0.206). Therefore, being resistant to a defensin (tenecin 1) of the defence cocktail offered *rpo* mutant cross-resistance to the rest of the mixture.

The survival of the unselected control infected beetles followed the same pattern as those infected with the ancestral strain (T1T2T4 KD treatment*infection: X^2= 53.5, df =3, p <.0001, Fig. 6B). The T1T2T4 knockdown treatment increased the mortality of C1(p <0.0001). *T. molitor* infected with *rpo* mutant had a high mortality rate in normal expression levels of T1T2T4 with a mean death age of 19.3 days. However, the *rpo*-infection induced-mortality decreased significantly with T1T2T4 knockdown treatment (p <0.0001). It seems that *S. aureus* harbouring a mutation in *rpo* operon are more virulent in the presence of a host's AMPs (T1T2T4).

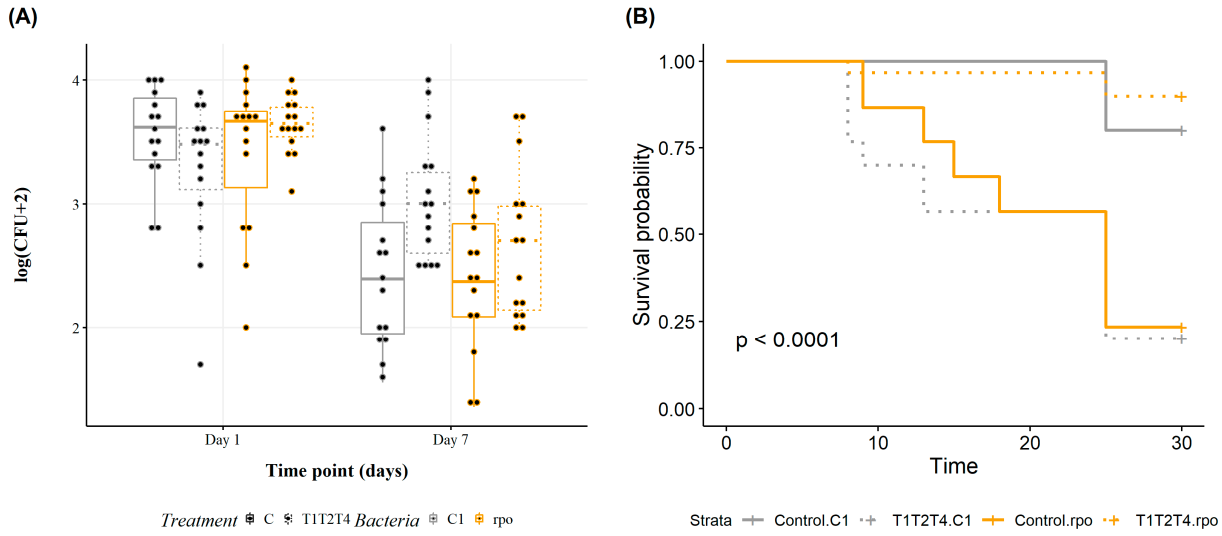

Figure 6. (**A**) Bacterial load of *Staphylococcus aureus* harbouring a mutation in *rpo* (**yellow**) operon. The T1T2T4 knockdown (**dotted**) did not affect colonization of AMP-resistant *S. aureus* by day 7, unlike the unselected control C1 (**grey**) which increased significantly. The CFU recovered from 100 µl of hemolymph are represented on a $\log_{10}$ scale by box plots showing quartiles and medians. The bars represent the 1.5 interquartile. (**B**) T1T2T4 knockdown treatment (**dotted**) increased the survival rate of *T. molitor* infected with *rpo* mutant (**yellow**) in contrary to those infected with the unselected control strain, C1 (**grey**).

3.7. The tenecin 1-resistant *pmt Staphylococcus aureus* mutant is cross-sensitive to one or more components of the defence cocktail T1T2T4.

The recovered CFU of *S. aureus* harbouring a mutation in the *pmt* operon increased significantly with the T1T2T4 knockdown treatment 7-days post-infection (T = -2.210, df = 445, p = 0.027). However, the infection load of *S. aureus* harbouring an additional mutation in the *rpo* operon (*pmt-rpo*) was irresponsive to the absence of AMPs (T = 0.967, df = 445, p = 0.3339). Therefore, *pmt* mutant is cross-sensitive, while *pmt-rpo* mutant is cross-resistant to coleoptericin (tenecin 2), attacin (tenecin 4) or both. *S. aureus* harbouring a mutation in *nsa* operon showed a similar pattern as *pmt* mutant with almost-significant increased bacterial load with T1T2T4 knockdown treatment (T = -1.819, df = 445, p = 0.0696).

In consistence to the ancestral strain, the survival of the unselected control infected beetles decreased significantly with T1T2T4 knockdown treatment (T1T2T4 KD treatment*infection: X^2= 20.4, df =7, p = 0.005, Fig. 6B). However, the mortality rate of line 2 descended T1- and T1T2-selected *S. aureus* was not affected by the knockdown treatment.

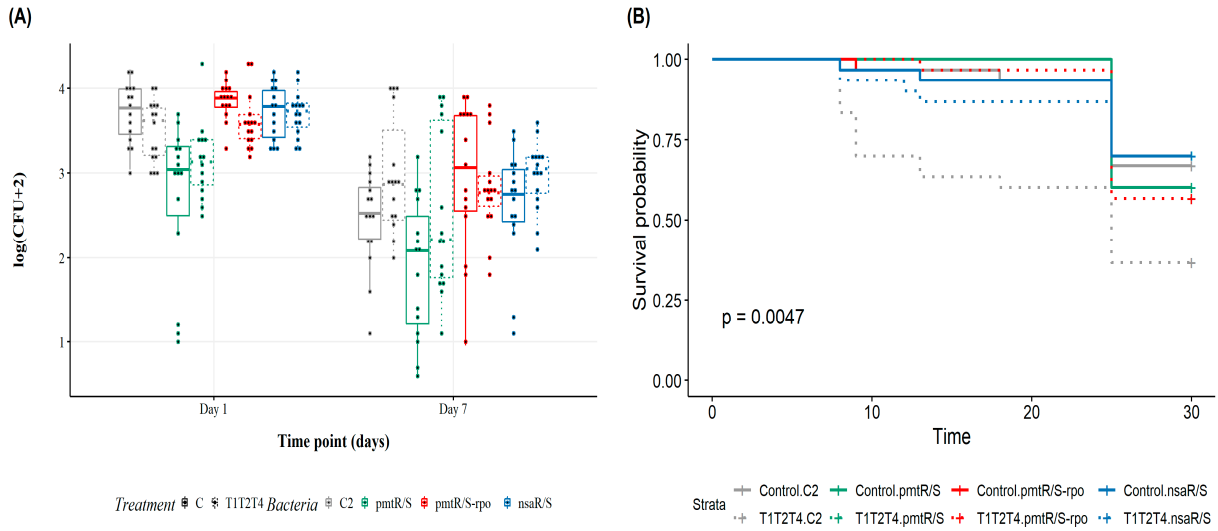

Figure 7. (**A**) Bacterial load of *Staphylococcus aureus* harbouring a mutation in the *pmt* (**green**), *pmt&rpo* (**red**) or *nsa* (**blue**) operon. The T1T2T4 knockdown (**dotted**) increased colonization of AMP-resistant *S. aureus* by day 7 for the *pmt* mutant and the unselected control C2 (**grey**). The CFU recovered from 100 µl of hemolymph are represented on a log scale by box plots showing quartiles and medians. The bars represent the 1.5 interquartile. (**B**) T1T2T4 knockdown treatment (**dotted**) decreased the survival rate of *T. molitor* infected with the unselected control strain, C2 (**grey**). The mortality rate of *T. molitor* infected with *pmt* (**green**), *pmt-rpo* (**red**) and *nsa* (**blue**) mutants was not by T1T2T4 knockdown.

3.8. The tenecin 1, tenecin 2-resistant *nsa-rpo Staphylococcus aureus* mutant is cross-sensitive to an attacin (tenecin 4).

The infection load of *S. aureus* (T1T2-3) harbouring a mutation in both *nsa* and *rpo* operons increased significantly with the T1T2T4 knockdown treatment 7-days post-infection (T = -2.851, df = 445, p = 0.0046). Since being *in vitro* selected against tenecin 1 and tenecin 2, the *nsa-rpo* mutant might be cross-sensitive to the attacin, tenecin 4.

Similar to the ancestor SH1000, the survival of beetles infected with the unselected control strain C3 decreased significantly with T1T2T4 knockdown treatment (T1T2T4 KD treatment*infection: X^2= 14.8, df =3, p = 0.002, Fig. 6B). The mortality rate of the *nsa-rpo* mutant was high regardless of knockdown treatment (p = 0.93) with age at death of 16.5 (C) and 18.4 (T1T2T4 KD) days. Consequently, knockdown of the defence mixture T1T2T4 did not affect the virulence of *S. aureus* with mutations in *nsa* and *rpo* gene loci.

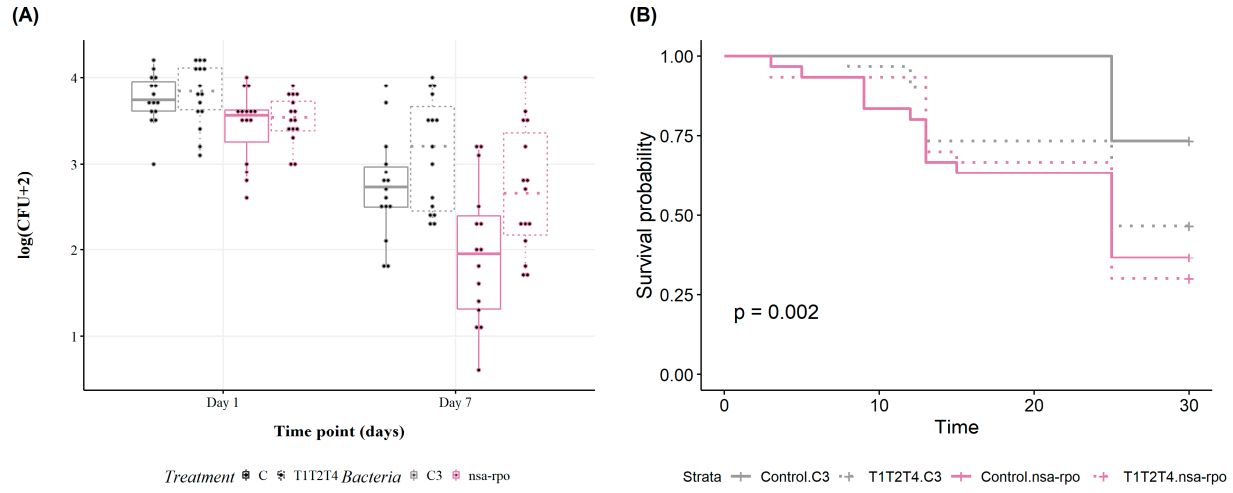

Figure 8. (**A**) Bacterial load of *Staphylococcus aureus* harbouring a mutation in both *nsa & rpo* (**purple**) operons. The T1T2T4 knockdown (**dotted**) increased colonization of both AMP-resistant *S. aureus* and the unselected control C3 by day 7 (**grey**). The CFU recovered from 100 µl of hemolymph are represented on a log scale by box plots showing quartiles and medians. The bars represent the 1.5 interquartile. (**B**) T1T2T4 knockdown treatment (**dotted**) decreased the survival rate of *T. molitor* infected with the unselected control strain, C3 (**grey**). However, it did not affect those infected with *nsa-rpo* mutant (**purple**).

4. Discussion:

Despite being doubly armed, the phagocytosis resistant *S. aureus* with evolved resistance against the host's own AMPs did not show either increased bioburden or host's mortality (El Shazely et al., 2019). A probable explanation might be that antimicrobial resistance evolution is costly (Andersson & Hughes, 2010; Melnyk et al., 2015). The bacterial load in the host is an essential feature of bacterial fitness (Howick & Lazzaro, 2014). Unexpectedly (Bell & Gouyon, 2003; Habets & Brockhurst, 2012), we showed previously that AMP-resistant *S. aureus* has limited fitness both *in vivo* (El Shazely et al., 2019) and *in vitro* (Makarova et al., 2018). In an attempt to illustrate such findings versus expectations contradictions, we shed some light on the possible collateral sensitivity of AMP-resistant *S. aureus* to other immune effectors such as phenoloxidase and/or other AMPs of the defence cocktail, using *Tenebrio molitor* as a host. All AMP-resistant *S. aureus strains* except the *nsa-rpo* mutant showed cross-sensitivity to the phenoloxidase dependent immune response. Moreover, *pmt* and *nsa-rpo S. aureus* mutants showed cross-sensitivity to one or more components of the *in vivo* AMP defence cocktail.

Phenoloxidase knockdown increased the mortality rate significantly of ancestor strain. RNAi-based inhibition of the prophenoloxidase activating enzyme (CG3066) (Leclerc et al., 2006) blocked the induction of phenoloxidase activity in *Drosophila* fly extracts (Tang et al., 2006). In agreement with our Ancestor strain survival pattern, *S. aureus* infected CG3066 mutant showed a reduction in the mean-time to death (Ayres & Schneider, 2008). However, the Schneider lab did not measure bioburden in *S. aureus*–infected flies to avoid over-dispersed counts due to aggregation of *S. aureus* when grown in flies. Leclerc et al. (2006) found no difference in survival between *S. aureus*–infected CG3066 mutants and wild-type flies.

In contrast to our findings, the *S. aureus*-infected PO knockdown *T. molitor* showed decreased mean-time to death (Khan et al., 2017), this study focused on the overall host median longevity in a host with reduced proinflammatory responses. We have modified the dsRNA dose and composition from Khan et al. (2017) to better reach a host with silenced prophenoloxidase expression. We could speculate, for wild type *S. aureus* strains, that reducing the PPO expression to certain threshold might be beneficial to the host, increasing its longevity (Khan et al., 2017). However, further PPO down-regulation hinders containment of pathogenesis and subsequently increases mortality as the results shown herein. Further analysis is required to explore the relationship of different PO knockdown levels to host survival outcome. It is worth noting that, Lemaitre lab showed that mutant flies with PPO gene deletion recorded higher mortality upon Gram-positive bacterial infection, in agreement with our results, particularly those with Lysine-type peptidoglycan, for example, *S. aureus* infection(Binggeli et al., 2014).

We found that except for *S. aureus* harbouring mutations in both *nsa* and *rpo* operons, the knockdown of phenoloxidase increased the bacterial load. Disrupting the melanization pathways for *D. melanogaster* did not influence the bacterial load of *Pseudomonas rettgeri* (Duneau et al., 2017) and *E. coli* (Ayres & Schneider, 2008), however, increased those of *Salmonella typhimurium* and *Listeria monocytogenes.* Therefore, different bacterial strains trigger distinctive perception by the host immune system and subsequently, a unique response. In a beetle with no/highly-diminished PO activity, *nsa-rpo S. aureus* mutants reached reduced bacterial loads, triggering lower host mortality rate. In contrast, *pmt* mutants caused increased host mortality and a slight increase in bacterial load at least 7 days post-infection.

Ayres and Schneider (2008) showed that proinflammatory responses are dependent on pathogen species. Herein, we showed that it is strain-dependent, and the mutation driving the AMP resistance seemed to be a significant effector. The PPO response is activated *via* recognition of membrane structural components of bacterial and fungal cells (Soderhall & Cerenius, 1998). It is possible that the common AMP resistance mechanism *via* altering bacterial membrane cause AMP-resistant pathogens to be more prone to recognition by PPO-activating system and thus mainly cross-sensitive to PO dependent immune response. It is worth noting that the T1T2 resistant *nsa-rpo* mutants, which are uniquely sensitive to phagocytosis (El Shazely et al., 2019) seem to benefit more from the proinflammatory immune response modulated by phenoloxidase. Blocking such responses decreased both multiplication and virulence in the host. This might be due to shown collateral damage to the Malpighian tubules (an equivalent of the human kidney) to proinflammatory responses (Khan et al., 2017). The success of AMP-resistant pathogens is limited via balanced sensitivity to host immune effectors. A mutant that is resistant to one immune effector is sensitive to another. Thus, eradication or diminishing of AMP-resistant pathogen infection is achieved through different contribution levels of host's immune effectors.

There are few bacterial AMP resistance mechanisms, for example, active efflux or electrostatic repulsion of cationic AMPs by increasing the positive net charge (Andersson et al., 2016). Consequently, we expect a high frequency of cross-resistance of bacteria evolved toward one AMP to another AMP, especially for AMPs with related structure. We previously reported that the tenecin resistant *S. aureus* showed cross-resistance towards melittin and colistin but cross-sensitivity toward pexiganan and vancomycin (Makarova et al., 2018; El Shazely et al., 2020). The tenecin 1- selected *S. aureus* harbouring a mutation in *pmt* operon showed cross-sensitivity toward either tenecin 2 or tenecin 4 or both. While the tenecin 1 tenecin 2- selected *nsa-rpo S. aureus* strain showed cross-sensitivity to tenecin 4, such a result explains the observed *in vivo* lower bacterial load those particular two mutants had than other AMP-resistant mutants at 14 days post-infection (El Shazely et al., 2019). Here, we

showed that AMP resistance might confer either cross-resistance or collateral sensitivity the host's other AMPs, and again this was informed by the mutation driving resistance.

The T1T2T4 knockdown did not influence the virulence of all AMP-resistant strains except the tenecin 1- selected *S. aureus* harbouring a mutation in *rpo* operon, where it decreased unlike that of the unselected control. It is unclear why the *rpo* mutant caused lower host mortality rate in case of T1T2T4 KD treatment.

Given the notion that a single mutation can cause various phenotypic changes that regulate the evolution of resistance and susceptibility to various drugs simultaneously (Lazar et al., 2013), evolution mutation trajectories to adapt one immune modulator (for example, AMPs) might result in cross-sensitivity to another immune modulator defining the outcomes of host-pathogen interaction. This might explain why AMP-resistant bacteria are not abundant in nature (Spohn et al., 2019). Through the last decade, *in vitro* selection experiments have been performed to match drug pairs exhibiting collateral sensitivity for bacterial strains (Imamovic & Sommer, 2013; Lazar et al., 2013; Rodriguez de Evgrafov et al., 2015). However, our study is the first to investigate the collateral sensitivity of an armed enemy to the host immune effector. Based on our findings in *T. molitor*, we wonder if pathogens do evolve resistance to human AMPs during AMP-based therapy, would collateral sensitivity to other immune effectors limit its fitness and success?

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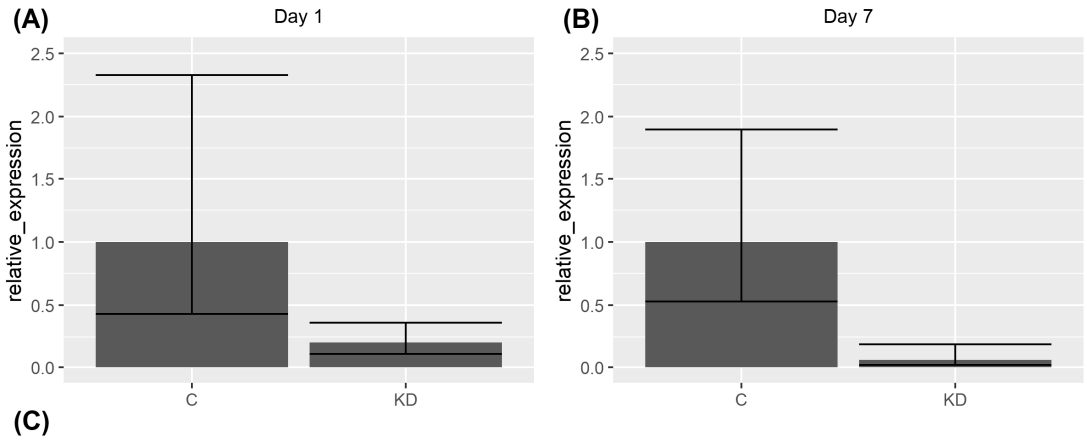
Appendix (Chapter 2)

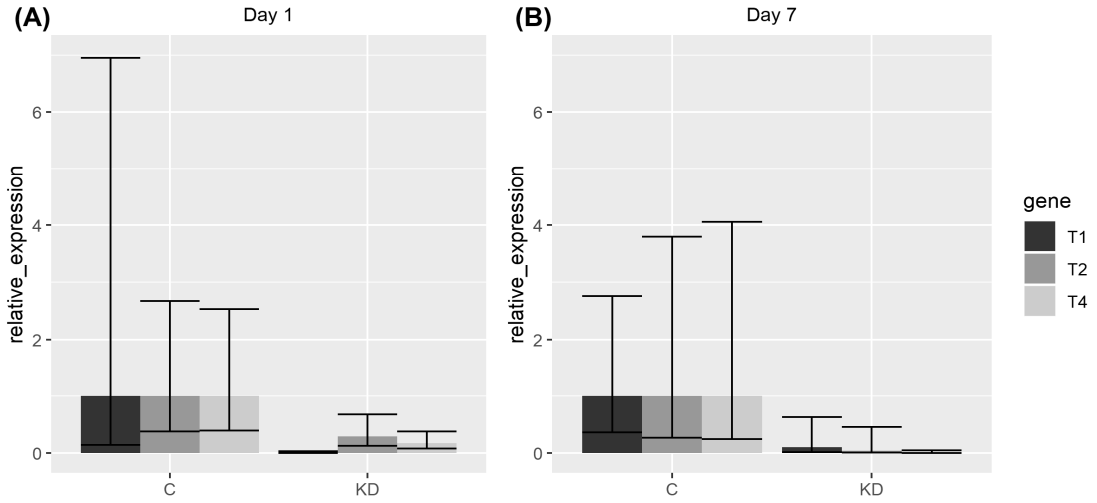
RNAi- based knockdown efficiency assessment



(C)

Group	Gene	Time.point	Normalized	Calibrated	Relative.expression	error	lower	upper
C	PO	Day 1	12.208	0.000	1.000	1.219	0.429	2.329
KD	PO	Day 1	14.562	2.354	0.196	0.887	0.106	0.362
C	PO	Day 7	10.380	0.000	1.000	0.923	0.527	1.896
KD	PO	Day 7	14.468	4.088	0.059	1.634	0.019	0.182

Figure S1: Relative expression ($2^{-\Delta\Delta Ct}$) of the prophenoloxidase gene compared to house-keeping gene/internal control (ribosomal protein RPL27A gene) in both control and PO knockdown treatment at: **(A)** 1 day post-infection with different *S. aureus* strains (there is a significant average 80.44% reduction of relative expression of the prophenoloxidase gene in PO.KD beetles compared to control PO.C($\log_{10}$ (ΔCt_{PO}) 0.06 vs $\log_{10}$ (ΔCt_{C}) 1.06; $F_{1,88} = 24.99$; $p<0.0001$)). **(B)** 7 days post-*S. aureus* injection (there is a significant average 94.1% reduction of expression of the prophenoloxidase gene in PO.KD beetles compared to control PO.C ($\log_{10}$ (ΔCt_{PO}) 0.1223 vs $\log_{10}$ (ΔCt_{C}) 0.999; $F_{1,88} = 49.54$; $p<0.0001$)). (**C**) The table summarizes the normalized (ΔCt), calibrated (ΔΔCt) and relative prophenoloxidase gene expression ($2^{-\Delta\Delta Ct}$) ± standard error at different time points post-infection as calculated by the "pcr_analyze" function from "pcr" package in R.



(C)

Group	Gene	Time.point	Normalized	Calibrated	Relative_Expression	error	lower	upper
C	T1	Day 1	5.402	0.000	1.000	2.797	0.144	6.948
KD	T1	Day 1	12.648	7.246	0.007	2.132	0.002	0.029
C	T2	Day 1	-3.056	0.000	1.000	1.418	0.374	2.672
KD	T2	Day 1	-1.264	1.792	0.289	1.218	0.124	0.672
C	T4	Day 1	-3.390	0.000	1.000	1.340	0.395	2.532
KD	T4	Day 1	-0.860	2.530	0.173	1.116	0.080	0.375
C	T1	Day 7	3.570	0.000	1.000	1.465	0.362	2.761
KD	T1	Day 7	6.922	3.352	0.098	2.679	0.015	0.627
C	T2	Day 7	-3.594	0.000	1.000	1.925	0.263	3.797
KD	T2	Day 7	1.108	4.702	0.038	3.566	0.003	0.455
C	T4	Day 7	-1.716	0.000	1.000	2.022	0.246	4.060
KD	T4	Day 7	5.852	7.568	0.005	3.233	0.001	0.050

Figure S2: Relative expression "$2^{-\Delta\Delta Ct}$" of tenecin 1, tenecin 2 and tenecin 4 genes, respectively, compared to house-keeping gene/internal control (ribosomal protein RPL27A gene) in both control "C" and AMP knockdown "KD" treatment at: **(A)** 1 day post-infection with different *S. aureus* strains (there is a significant average 99.3% reduction of relative expression of the tenecin 1 gene ($\log_{10}(\Delta Ct_{AMP})$ 0.411 vs $\log_{10}(\Delta Ct_C)$ 0.557; $F_{1,88} = 37.13$; $p<0.0001$), 80% reduction of relative expression of the tenecin 2 gene ($\log_{10}(\Delta Ct_{AMP})$ 0.064 vs $\log_{10}(\Delta Ct_C)$ 1.129; $F_{1,77} = 74.06$; $p<0.0001$), and 82.7% reduction of relative expression of the tenecin 4 ($\log_{10}(\Delta Ct_{AMP})$ 0.064 vs $\log_{10}(\Delta Ct_C)$ 1.135; $F_{1,88} = 57.85$; $p<0.0001$) in AMP.KD beetles compared to control individuals. **(B)** 7 days post-*S. aureus* injection (there is a significant average 90.2% reduction of relative expression of the tenecin 1 gene ($\log_{10}(\Delta Ct_{AMP}+6)$ 0.245 vs $\log_{10}(\Delta Ct_C+6)$ 0.949; $F_{1,88} = 26.92$; $p<0.0001$), 96.2% reduction of relative expression of the tenecin 2 gene ($\log_{10}(\Delta Ct_{AMP}+6)$ 0.501 vs $\log_{10}(\Delta Ct_C+6)$ 0.315; $F_{1,88} = 28.78$; $p<0.0001$), and 99.5% reduction of relative expression of the tenecin 4 ($\log_{10}(\Delta Ct_{AMP}+6)$ 0.309 vs $\log_{10}(\Delta Ct_C+6)$ 0.535; $F_{1,88} = 24.98$; $p<0.0001$) in AMP.KD beetles compared to control individuals. (**C**) The table summarizes the normalized (ΔCt), calibrated ($\Delta\Delta Ct$) and comparative relative tenecin 1, tenecin 2, and tenecin 4 gene expression ($2^{-\Delta\Delta Ct}$) ± standard error at different time points post-infection as calculated by the "pcr_analyze" function from "pcr" package in R.

Table S1: list of primers used in the current study

Primer	Type	Sequence	Reference
Lysozyme (*Galleria mellonella)-fw*	T7	TAA TAC GAC TCA CTA TAG GGA GAG CAA GCC GAA TAA AAA TGG A	(Khan et al., 2017; Zanchi et al., 2017)
Lysozyme (*Galleria mellonella)-rv*	T7	TAA TAC GAC TCA CTA TAG GGA GAT ATC TGG CAG CGG CTT ATT T	
Prophenoloxidase 1 (*T. molitor*) PO1-fw	T7	TAA TAC GAC TCA CTA TAG GGA GAA GAG GCG TAT TTC CCC AAG	(Khan et al., 2017)
Prophenoloxidase 1 (*T. molitor*) PO1-rv	T7	TAA TAC GAC TCA CTA TAG GGA GAG ATT CCT TCG TTC TCG GTC	
Prophenoloxidase 2 (*T. molitor*) PO2-fw	T7	TAA TAC GAC TCA CTA TAG GGA GAA ATT CTT GAT TCT GTA GAT	
Prophenoloxidase 2 (*T. molitor*) PO2-rv	T7	TAA TAC GAC TCA CTA TAG GGA GAG AGA GAT CCT GTG TTC TT	
Tenecin 1 (*T. molitor*)-fw	T7	TAA TAC GAC TCA CTA TAG GGA GAC ACG AGA TCA CGA TGA AGC	(Zanchi et al., 2017)
Tenecin 1 (*T. molitor*)-rv	T7	TAA TAC GAC TCA CTA TAG GGA GAA AAT CA G TTT TTA TTT ATC GTC ATG TT	
Tenecin 2 (*T. molitor*)-fw	T7	TAA TAC GAC TCA CTA TAG GGA GAA TCA GTT CGC TTT CGA ACA GTC T	
Tenecin 2 (*T. molitor*)-rv	T7	TAA TAC GAC TCA CTA TAG GGA GAA ATT AGT TTC CAT TGA AAT GGT TTG	
Tenecin 4 (*T. molitor*)-fw	T7	TAA TAC GAC TCA CTA TAG GGA GAA TGT TAA AAG CGG TTC AAT TCG	
Tenecin 4 (*T. molitor*)-rv	T7	TAA TAC GAC TCA CTA TAG GGA GAA AAA ATC TAC TGT TCC TGT GAG GTG	
RPL27A housekeeping (*T. molitor*)-fw	qPCR	TCG ACT CAT AAG AAA AAG ACA AGA AA	(Khan et al., 2017; Zanchi et al., 2017)
RPL27A housekeeping (*T. molitor*)-rv	qPCR	CAT TAC CGC GAC CTC CTG	
Prophenoloxidase (*T. molitor*)-fw	qPCR	GCA CGA GCT GGA ATT GTG T	(Khan et al., 2017)
Prophenoloxidase (*T. molitor*)-rv	qPCR	GGT CGA ACA AAC AGG AGG ATG	

Primer	Type	Sequence	Reference
Tenecin 1 (*T. molitor*)-fw	qPCR	GGA AGC GGC AAC AGC TGA AGA AAT	(Zanchi et al., 2017)
Tenecin 1 (*T. molitor*)-rv	qPCR	AAC GCA GAC CCT CTT TCC GTT ACA	
Tenecin 2 (*T. molitor*)-fw	qPCR	GAA TGG AGG GTG GTC CGT CAA C	
Tenecin 2 (*T. molitor*)-rv	qPCR	TTG TGC TGC ACC TCA ACG TTG GTC	
Tenecin 4 (*T. molitor*)-fw	qPCR	CAA CAA CGG CGG CCA CAA ATT AGA	
Tenecin 4 (*T. molitor*)-rv	qPCR	TGT AAT CCA GCT TCC CAC CGA AGA	

Table S2: List of the AMP-selected *Staphylococcous aureus* strains and their corresponding procedural controls (nested per line) used in this study. All the strains were originally experimentally evolved either towards tenecin 1 or a combination of tenecin 1 plus tenecin 2 in our laboratory, in a former study (Makarova et al., 2018).

Strain	Major mutation group	Line
Ancestor	None	-
C1	None	1
T1-1L	*rpo*	1
C2	None	2
T1-2L	*pmt*	2
T1-2S	*pmt/rpo*	2
T1T2-2	*nsa*	2
C3	None	3
T1T2-3	*nsa/rpo*	3

6. Chapter 3

Resistance evolution against antimicrobial peptides in Staphylococcus aureus alters pharmacodynamics beyond the MIC

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Resistance Evolution Against Antimicrobial Peptides in *Staphylococcus aureus* Alters Pharmacodynamics Beyond the MIC

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Antimicrobial peptides (AMPs) have been proposed as a promising class of new antimicrobials partly because they are less susceptible to bacterial resistance evolution. This is possibly caused by their mode of action but also by their pharmacodynamic characteristics, which differ significantly from conventional antibiotics. Although pharmacodynamics of antibiotic resistant strains have been studied, such data are lacking for AMP resistant strains. Here, we investigated if the pharmacodynamics of the Gram-positive human pathogen *Staphylococcous aureus* evolve under antimicrobial peptide selection. Interestingly, the Hill coefficient (kappa κ) evolves together with the minimum inhibition concentration (MIC). Except for one genotype, strains harboring mutations in *men*F and *atl*, all mutants had higher kappa than the non-selected sensitive controls. Higher κ results in steeper pharmacodynamic curve and, importantly, in a narrower mutant selection window. *S. aureus* selected for resistance to melittin displayed cross resistant against pexiganan and had as steep pharmacodynamic curves (high κ) as pexiganan-selected lines. By contrast, the pexiganan-sensitive tenecin-selected lines displayed lower κ. Taken together, our data demonstrate that pharmacodynamic parameters are not fixed traits of particular drug/strain interactions but actually evolve under drug treatment. The contribution of factors such as κ and the maximum and minimum growth rates on the dynamics and probability of resistance evolution are open questions that require urgent attention.

Keywords: resistance evolution, Hill coefficent, pharmacodynamics, pexiganan, melittin

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INTRODUCTION

Bacterial drug resistance is a growing problem (Davies and Davies, 2010). Under conventional antibiotic treatment resistance evolves frequently (Levy and Marshall, 2004; Davies and Davies, 2010). Solving this problem requires new approaches including prudent use, understanding evolutionary dynamics (zur Wiesch et al., 2014) and the identification of new drug candidates (WHO, 2012) that are likely to avoid evolution of resistance (Czaplewski et al., 2016). Antimicrobial peptides (AMPs) have been proposed as promising new drug candidates

(Zasloff, 2002; Fjell et al., 2012; Mylonakis et al., 2016; Pfalzgraff et al., 2018). Though resistance against AMPs evolves readily in *in vitro* systems (Perron et al., 2006; Habets et al., 2012; Dobson et al., 2013; Makarova et al., 2018), this does not seem to be the case *in vivo*. Based on pharmacodynamic studies of AMPs, one of their alleged advantage is that evolution of resistance has a much lower probability compared to antibiotics (Yu et al., 2018).

Pharmacodynamics are based on time-kill curves. Regoes et al. (2004) analyzed time-kill curves using a pharmacodynamic model that is closely related to Emax models (Mueller et al., 2004). Pharmacodynamic functions link drug dosage and bacterial growth or death rates. Four parameters are important for this model (Regoes et al., 2004): the Hill coefficient (κ), i.e., the slope, the maximal bacterial growth rate in the absence of antimicrobial (Ψ_{max}), the minimal bacterial growth rate at high concentrations of antimicrobial (Ψ_{min}), and the pharmacodynamic minimum inhibition concentration (zMIC) (Regoes et al., 2004).

The steepness of pharmacodynamic curves, as described by κ, is much greater for AMPs than for antibiotics (Yu et al., 2016). The maximum killing effect of AMPs is much stronger than that of antibiotics, as measured via the speed of killing (Fantner et al., 2010; Yu et al., 2018). Consequently, AMPs display a narrower mutation selection window compared to antibiotics, thus resistance toward AMPs is less likely to evolve (Yu et al., 2018). Moreover, AMPs cocktail have higher kappa values than single AMPs (Yu et al., 2016); a crucial information for combinational therapy, a proposed antibiotic replacement regimes (Walkenhorst, 2016).

Despite short comings, the minimum inhibition concentration (MIC) is still the most common bioassay to explore cross resistance (Brauner et al., 2016; Wen et al., 2016). The importance of pharmacodynamic parameters in predicting drug resistance evolution has been reported in several studies (Chevereau et al., 2015; Lukačišinová and Bollenbach, 2017). In addition to *in vivo* infection dynamics studies (Dobson et al., 2014; McGonigle et al., 2016; Zanchi et al., 2017; El Shazely et al., 2019), pharmacodynamic approaches are useful to understand how antibiotics and antimicrobial peptides eradicate bacteria in physiological systems (Yu et al., 2016). It is assumed that the shape of pharmacodynamic curve does not change (Craig, 1998).

Here, we use a pharmacodynamic approach, that has been previously described to generate a sigmoid dose–response relationship (Bonapace et al., 2002; Regoes et al., 2004; Sampah et al., 2011; Yu et al., 2016), to study the evolution of AMP resistance. We explored whether the steepness of the pharmacodynamic curve (described by the Hill coefficient, κ), can evolve. It is the first time that pharmacodynamic parameters (kappa, Ψ_{min}, Ψ_{max}) of AMP resistant strains have been investigated. In this study, a standardized *in vitro* time-kill curve assay for the human pathogen, *Staphylococcous aureus,* which has been selected against either pexiganan, melittin (this study), tenecin 1 or tenecin 1 + 2 (strains from our previous study (Makarova et al., 2018)) was performed. We address two questions. (i) Do kappa, Ψ_{min}, Ψ_{max} evolve? (ii) Does cross resistance or cross sensitivity influence the pharmacodynamic parameters: kappa, Ψ_{min}, Ψ_{max} and zMIC?

MATERIALS AND METHODS

Bacterial Strains and Culturing Conditions

Staphylococcus aureus SH1000 was used in this study. Non-cation adjusted Mueller Hinton Broth (MHB) (PanReac AppliChem, Cat #413788-1210) was used for bacterial cultures. Bacterial cultures were incubated at 37°C with shaking at 180 rpm and plated on Mueller Hinton Agar (MHA). The later was prepared by adding 15 g/l bacteriological Agar Agar (Carl Roth, Cat #2266.2) to MHB.

Antimicrobial Peptides

We used two different AMPs: pexiganan and melittin. Pexiganan is a synthetic AMP, an analog of Magainin II that was originally isolated from the epidermis of the African clawed frog, *Xenopus laevis* (Zasloff, 1987) (Pexiganan was a kind gift from Michael Zasloff). Melittin (purity > 95%, GenScript, Cat #RP10290) is a synthetic AMP known to be an analog of the main toxin of bee venom (Habermann, 1972). To avoid multiple freeze-thaw cycles, peptides were re-suspended in (1:1 v/v) sterile distilled water and glycerol (Sigma life science, Cat #G5516) to the final concentration of 5 mg/ml and stored at −20°C in sterile vials.

Selection Experiment

The selection experiment was done according to Makarova et al. (2018). Briefly, preadapted *S. aureus* SH1000 glycerol stocks were stored at −80°C from the above-mentioned study. Five preadapted strains were inoculated into 10 ml MHB and incubated overnight with shaking at 37°C. The cultures were then diluted 1:1000 and incubated at 37°C in 50 ml polypropylene Falcon tubes (Th.Geyer, Cat #7696724) containing 3.7 ml MHB. Short pre-adaptation was carried out by serial passage every 24 h for 4 days, with daily measurements of optical density at 600 nm, contamination checks by plating out on MHA and cryopreservation of culture aliquots at −80°C in 12% glycerol solution.

For the selection protocol, the experiment was performed at 37°C in a microplate reader (Synergy 2, Biotek). To avoid attachment of the peptides to the plastic surfaces, flat bottom polypropylene non-binding 96-well plates were used (Greiner Bio-One, Cat #655261). To minimize evaporation, the 96-well plates were covered with clear polystyrene lids with condensation rings (Greiner Bio-One, Cat #656171). The plates were filled with 200 μl of MHB per well. Growth dynamics were monitored by optical density measurement at a wavelength of 600 nm every 10 min. Measurements were preceded by a moderate shaking for 10 s and continued for 24 h. For each of the five replicate lines there were two experimental conditions: pexiganan or melittin, as well as two controls: negative control, and non-selected control.

The serial passage started at 1 μg/ml for pexiganan (MIC of the preadapted strains toward pexiganan was 2–4 μg/ml) and 2 μg/ml melittin (MIC of the preadapted strains toward melittin was 4–8 μg/ml). Overnight cultures of the five replicate lines were diluted 1:100 and sub-cultured until OD_{600} of 0.5. Ten μl of these cultures were inoculated into each treatment and control

wells resulting in final total volume of 200 μl. Four μl (2%) of the culture were transferred every 24 h to a fresh 96 well plate. The concentration of AMPs was doubled each week. Plates were regularly checked for contamination. Glycerol was added to the rest of the cultures to the final concentration of 12% and the plates were stored at −80°C. During the selection experiment, a strain required 5–7 days to evolve resistance such that the culture could survive a two-fold increase in the AMP concentration. The selection experiment continued for 8 weeks where MIC was duplicated 64 folds for both pexiganan and melittin (64 × *MIC*).

Antimicrobial Susceptibility Testing

Minimal inhibitory concentration (MIC) was determined using a broth micro-dilution method. Briefly, 5 μl (1×10^5 CFU/ml) of 1:100 dilution of the mid-exponential phase bacterial culture (OD_{600} = 0.5) were inoculated into the wells of polypropylene V-bottom 96-well plates (Greiner Bio-One,Cat #651261) containing two-fold dilution series of the stressor in a total volume of 100 μl MHB per well. The assay was performed in triplicate. The plates were incubated at 37°C in a humidity chamber. The lowest concentration that inhibited visible bacterial growth after 24 h of incubation is the MIC. Visual observations were confirmed by heat maps generated by Gen 5 software (Biotik) of OD_{600} measurements on a microtiter plate reader (Synergy 2, Biotek).

Growth Curves

Growth curve assays were performed using a microtiter plate reader (Synergy 2, Biotek). The changes in turbidity at OD_{600} of the selected mutant lines and the non-selected controls were monitored in un-supplemented MHB. For this, 20 μl of 1:10 dilution of mid exponential phase of bacterial culture were inoculated into 180 μl MHB. Each assay had three replicates. The measurements were taken at 10 min intervals during 38 h of incubation at 37°C, with 5 s shaking before each reading. Growth parameters such as final OD, the maximum growth rate (Vmax) and lag time were calculated with Gen5 software (Biotek).

DNA Isolation

Genomic DNA for whole genome sequencing was isolated using Quick-DNA Fungal/Bacterial Microprep kit (Zymo Research, Cat #D6007) following manufacturer's instructions. Briefly, 2×10^8 log phase bacteria were resuspended in 200 μL of phosphate buffer saline (PBS) (pH = 7.4, Chem solute, Cat #8035.0100) solution. Then, 750 μL of bashing beads buffer were added and the mixture was transferred into bashing beads lysis tubes. The tubes were placed in a homogenizer (Retsch MM 400) at maximum speed for 5 min. The mixture was centrifuged shortly at 10,000 g for 1 min. Then, 400 μL of supernatant were transferred to a spin filter. The filtrate was chemically lysed by adding 1200 μL genomic lysis buffer. Then the mixture was passed into a zymo-spin IC column, centrifuged and washed twice, first, with DNA Pre-wash buffer, then, with DNA wash buffer. Finally, the DNA was eluted using 20 μL of DNA elution buffer. The DNA quantity and quality were estimated by measuring the optical density at A260/280 using the Nanodrop spectrophotometer (Thermo Scientific) and agarose gel electrophoresis.

Sequencing

To identify mutations in experimentally evolved populations and strains, the haploid variant calling pipeline snippy v3.2 (Seemann, 2015) was used with default parameters [minimum read mapping quality (–mapqual) 60, minimum base quality (–basequal) 20, minimum coverage (–mincov) 10, and minimum proportion of variant coverage (minfrac) 0.9] as previously described in Makarova et al. (2018). Briefly, quality-filtered adaptor-trimmed reads were aligned to the SH1000 reference genome using bwa (Li, 2013). The Bayesian genetic variant detector freebayes (Garrison and Marth, 2012) was used to detect single-nucleotide polymorphisms, insertions, deletions, multi-nucleotide polymorphisms, as well as composite insertion and substitution events.

Killing Curves

For pexiganan- and melittin-selected strains, pexiganan was serially diluted (two-fold concentration gradient), starting with 256 × MIC (1024 μg), in 96-well plate. Approximately, $2–3 \times 10^6$ log-phase bacteria were added to a total volume of 100 μl. The plates were incubated at 37°C. Killing by AMPs is rapid (Sochacki et al., 2011; Rangarajan et al., 2013), therefore dose response was monitored within 60 min (Yu et al., 2016). Ten microliters of bacterial suspension were taken out after 30 s and then every 20 min, then immediately diluted in PBS and plated on square solid MHA plates. These solid agar plates were transferred into 30°C incubator. CFU were counted 24 h later. The incubation of plates at 30°C facilitate counting CFU before colonies overgrow and overlap. The limit of detection in our system is 14 CFUs.

Following the same protocol, we determined the killing curves for the tenecin 1 and tenecin 1 + 2-selected strains available in the laboratory from a former study (Makarova et al., 2018). For these 36 genotypically unique strains, pexiganan was serially diluted starting with 16 × MIC (64 μg), to save material as it was known from MIC results that they do not share cross resistance to pexiganan.

Data Analysis

Statistical analysis was done in R (R Core Team, 2013).

MIC Analysis

The best fit was obtained when the MIC values were $\log_2$ transformed. A linear model was fitted to the transformed data. Treatment and mutation were considered as explanatory variables in the model. Several normality checking functions were used to test normality assumptions such as "bptest" (Breusch-Pagan test against heteroskedasticity) and "dwtest" (Durbin-Watson test for autocorrelation of disturbances) from "lmtest" package (Hothorn et al., 2019), "gvlma" (Global Validation of Linear Models Assumptions) from "gvlma" package (Pena and Slate, 2012) and "durbinWatsonTest" from "car" package (Fox et al., 2012). The function "anova" was used to analyze the model and extract *F*-statistics and degrees of freedom. The "mean", "sd" and "var" functions were used to calculate the mean, standard

deviation, standard error and variance. For analysis of contrasts, *post hoc* comparisons were performed using "lsmeans" function with a "tukey" adjustment (Lenth and Lenth, 2018). We used the function "visreg" from package "visreg" (Breheny et al., 2019) to visualize the contrast plot of the treatment effect as extracted from the model.

Growth Curve Analysis

Growth parameters (Vmax, duration of lag phase and final OD $_{600}$) were analyzed by using the "lm" function for linear models. For contrasts, *post hoc* tests were performed using "lsmeans" and "visreg" functions as described before.

Modeling Killing Curves

A Hill function was used to model the killing curve as previously described (Regoes et al., 2004; Yu et al., 2016). Briefly, generalized linear regression was used to determine growth and killing rates of bacteria from the time-kill curves as the change of CFUs over time. The CFU data were log-transformed ($\log_{10}$). Using the rjags package (Plummer et al., 2016), the growth and killing rates were fitted and extracted based on Markov Chain Monte Carlo (MCMC) method and the pharmacodynamic curves were generated.

Analysis of Pharmacodynamic Parameters

The pharmacodynamic parameters were extracted from the MCMC output. We tested whether the pharmacodynamic MIC of various *S. aureus* strains selected against AMPs segregated by selection treatment and/or by mutation. The zMIC was $\log_2$ transformed and a linear model was fitted as described above. A generalized linear model with gamma distribution was fitted to analyze Ψ_{max} variances across *S. aureus* strains with different selection treatment and different mutation. The Ψ_{min} variances were analyzed using a linear model. The Hill coefficient κ data set was normalized by log-transformation ($\log_{10}$) then a linear model was fitted. *Post hoc* analysis was performed as described above.

RESULTS

Resistance Evolved at a Cost

After 8 weeks of selection, all lines were able to grow in the presence of 256 μg/ml pexiganan or 512 μg/ml melittin, which is equivalent to 64-fold of MIC of non-selected preadapted strains for both stressors. According to minimum inhibition sensitivity test, MIC $_{pexiganan}$ segregated by treatment ($F_{(2,40)}$ = 143.2300, $p < 0.0001$, **Figure 1A**), but not by mutation ($F_{(2,40)}$ = 1.8769, $p = 0.166$, **Figure 1B**). *S. aureus* evolved pexiganan resistance both when selected against pexiganan ($T = 16.554$, df = 42, $p < 0.0001$, **Figure 1**) and melittin ($T = 9.121$, df = 42, $p < 0.0001$, **Figure 1**). Moreover, MIC $_{pexiganan}$ differed significantly between pexiganan- and melittin-selected lines ($T = 7.432$, df = 42, $p < 0.0001$, **Figure 1A**). Pexiganan-selected lines showed cross resistance to melittin ($T = 8.457$, df = 42, $p < 0.0001$, **Supplementary Table S1**).

Antimicrobial peptides-selected strains had consistently slower growth rates in the exponential phase for both, pexiganan (Vmax: $T = 2.821$, df = 42, $p = 0.01$, **Figure 2A**) and melittin-selected strains (Vmax: $T = 3.146$, df = 42, $p = 0.008$, **Figure 2A**) compared to non-selected controls (**Supplementary Figure S1**). However, lag phases (lag $_{Pexiganan}$: $T = 0.356$, df = 42, $p = 0.932$, lag $_{Melittin}$: $T = 1.234$, df = 42, $p = 0.440$, **Figure 2B**) and final population sizes measured as OD did not differ (Final $OD_{Pexiganan}$: $T = -1.313$, df = 42, $p = 0.396$, Final $OD_{Melittin}$: $T = 0.592$, df = 42, $p = 0.825$, **Figure 2C**).

Genome Sequencing Reveals Mutations in a Number of Loci Related to Selection Treatment

Whole genome sequencing of the selected mutants and the non-selected controls (at the single colony level) showed differences between treatments. In each melittin-resistant strain at least four mutations in *pmt*R (also known as *ytr*A), *vra*G (also known as *bce*B), *atl,* and *nam*A genes were identified. All those mutations are known to be involved in cell wall stress tolerance and detoxification (see also **Supplementary Table S2** for a full list of mutations). The mutations included stop gain for *pmt*R, missense (c.1727C > T p.Ala576Val for 8 strains and c.924T > A p.Ser308Arg for nine strains) for *vra*G, frameshift for *atl* (c.2705_2706dupAT p.Ala903fs) and synonymous (c.477G > A p.Ala159Ala) for *nam*A. Interestingly, we found the same *pmt*R stop-gain mutation (c.77T > A p.Leu26*) in all melittin-selected strains in this study, which has previously been described for melittin-selected *S. aureus* lines (Johnston et al., 2016) and for tenecin-selected *S. aureus* (Makarova et al., 2018). For all pexiganan-selected strains, a missense mutation (c.571C > G p.Arg191Gly) for *men*F gene and stop gains and disruptive in-frame deletion mutations (c.2331_2342delTACTGTTACTAA p.Tyr777_Lys781delinsTer) for *atl* gene were consistent. Two strains only harbored these two mutations, while the other pexiganan-selected strains carried additional mutations either conservative in-frame insertion (c.178_192dupTCACAAGGTTCTATT p.Ser60_Ile64dup) in *vra*F gene (also known as *bce*A), or missense (c.884C > T p.Ser295Phe) in *rpo*A gene or both. Six pexiganan mutants had a missense in *rpo*A, one had conservative in-frame insertion in *bce*A gene while six strains had mutations in both loci.

Killing (Dose-Response) and Pharmacodynamic Curves

We tested *in vitro* killing of pexiganan using different AMP resistant *S. aureus* strains (pexiganan, melittin, tenecin 1 and tenecin 1 + 2-selected strains) and their respective controls. Time-kill curves were obtained by counting viable CFUs after treatment with various concentrations of pexiganan (**Supplementary Figure S2**). The CFUs of surviving bacteria strongly decreased as a function of time at higher pexiganan concentrations, however slight increases were noticed at lower concentrations. There are four measurements of the bacterial density during the first 60 min after exposure to pexiganan. This time interval was appropriate to statistically estimate the bacterial net growth rate at a given concentration and construct the pharmacodynamic curves (**Supplementary Figure S3**).

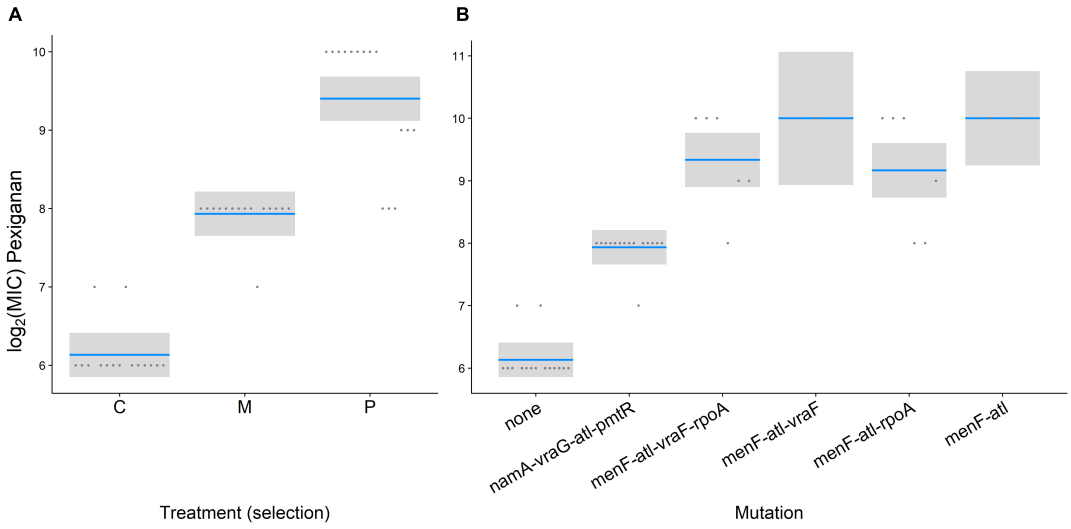

FIGURE 1 | Log_2 MIC of pexiganan and melittin resistant *S. aureus* strains compared to procedural controls tested against pexiganan separated by treatment **(A)** or by mutation **(B)** (C and none: unselected control, M: melittin-selected strains with mutation in *nam*A, *vra*G, *atl*, and *pmt*R gene loci, P: pexiganan-selected strains segregated into four genotype variants with mutation in *men*F, *atl,* and *vra*F, *rpo*A or both, blue line: mean, gray box: 95% confidence intervals). According to minimum inhibition sensitivity test, MIC $_{pexiganan}$ segregated by treatment ($F_{(2,40)} = 143.2300$, $p < 0.0001$, **Figure 1A**) but not by mutation ($F_{(2,40)} = 1.8769$, $p = 0.166$, **Figure 1B**).

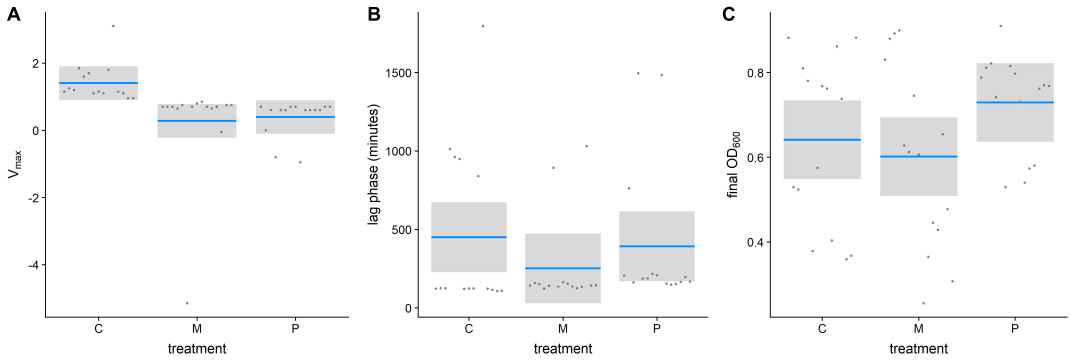

FIGURE 2 | Growth parameters of evolved AMP-resistant *Staphylococcus aureus* in relation to treatment [Vmax **(A)**, lag phase **(B)**, final OD **(C)**]. (Con: unselected control, M: melittin-selected strains, P: pexiganan-selected strains, blue line: mean, gray box: 95% confidence intervals).

TABLE 1 | Parameter estimates and their standard errors.

S. aureus	MIC (μg/ml)	zMIC (μg/ml)	Ψ_{min} (h^{-1})	Ψ_{max} (h^{-1})	κ
Con1	72.53 ± 5.81	66.76 ± 7.42	−9.90 ± 0.22	0.23 ± 0.06	2.86 ± 0.13
P	768.00 ± 86.54	300.32 ± 23.33	−7.60 ± 0.30	0.26 ± 0.06	**15.32 ± 1.32**
M	247.46 ± 8.53	283.19 ± 30.16	−8.20 ± 0.21	0.11 ± 0.03	**15.10 ± 1.42**
Con2	48.00 ± 7.16	22.94 ± 3.77	−9.04 ± 0.16	0.46 ± 0.07	4.84 ± 0.53
T1	49.78 ± 3.86	21.02 ± 2.06	−8.86 ± 0.13	0.41 ± 0.03	4.02 ± 0.34
T1T2	53.33 ± 4.54	19.29 ± 2.86	−7.97 ± 0.35	0.27 ± 0.03	4.18 ± 0.69

MIC, minimum inhibition concentration determined by a two-fold dilution protocol; zMIC, pharmacodynamic MIC; Ψ_{min}, the minimum bacterial net growth rate at high pexiganan concentrations; Ψ_{max}, the maximum bacterial net growth rate; κ, the Hill coefficient; Con, passaged non-selected control for pexiganan- and melittin-selected lines (Con1) and for tenecin-selected lines (Con2); P, pexiganan-selected S. aureus; M, melittin-selected S. aureus;T1, tenecin 1-selected S. aureus; T1T2, tenecin1 + 2-selected S. aureus. Pexiganan (P) and Melittin (M) selected S. aureus lines evolved higher kappa values (Bold) compared to their unselected controls (Con1).

The average values of pharmacodynamic MICs (zMIC) and those determined by a two-fold dilution protocol (MIC) are listed in **Table 1**. The estimated pharmacodynamic zMICs differs slightly from the MIC measurements.

The pharmacodynamic MIC (zMIC) segregated by treatment ($F_{(5,283)}$ = 127.67, $p < 0.0001$, **Figure 3A**). The pexiganan resistant strains had a zMIC higher than its respective control (T = 9.451, df = 238, $p < 0.0001$, **Figure 3A**). Melittin resistant strains had a cross resistance to pexiganan (T = 7.911, df = 238, $p < 0.0001$, **Figure 3A**). The zMIC values of pexiganan- and melittin-selected *S. aureus* did not differ significantly (T = 0.542, df = 238, p = 0.9944, **Figure 3A**). Tenecin 1 and tenecin 1 + 2 strains were as sensitive to pexiganan as their respective non-selected controls (tenecin1-control: T = –0.782, df = 238, p = 0.9703; tenecin1 + 2 -control: T = –1.522, df = 238, p = 0.6503, **Figure 3A**). zMIC did not segregate by mutation within each treatment, for example *vra*F (*bce*A) mutants are not significantly different from *men*F-*atl* mutants (T = 1.569, df = 232, p = 0.9184, **Figure 3B**).

Ψ_{max} and Ψ_{min}

Ψ_{max} values were found to be almost identical across treatments (**Figure 4**). This suggests that growth rates of bacteria in low concentration of AMP(s) were presumably close to the natural growth rate (Yu et al., 2016). It is interesting that differences in Vmax, referred to earlier, were not reflected by differences in Ψ_{max} values.

Ψ_{min} values segregated by treatment ($F_{(5,232)}$ = 10.285, $p < 0.0001$, **Figure 5A**). Pexiganan- and melittin-selected *S. aureus* had higher Ψ_{min} values than the non-selected controls (P-Con: T = 6.130, df = 238, $p < 0.0001$; M-Con: T = 4.049, df = 238, p = 0.001, **Figure 5**). The Ψ_{min} values for tenecin 1- and tenecin 1 + 2-selected strains were not different from non-selected controls (T1-C: T = 0.511, df = 238, p = 0.995; T1T2-C: T = 2.759, df = 238, p = 0.0678, **Figure 5A**). However, tenecin 1-selected *S. aureus* had a slightly lower Ψ_{min} values than tenecin1 + 2-selected strains (T = –3.037, df = 238, p = 0.0315, **Figure 5A**). Pexiganan resistant strains had almost equal Ψ_{min}. Tenecin-selected *S. aureus* with mutation in the *nsa* operon had uniquely higher Ψ_{min} than the non-selected control (T = 5.303, df = 232, $p < 0.0001$, **Figure 5B**), having an additional mutation in the *rpo* operon at C or B loci would decrease the Ψ_{min} values significantly (T = –5.494, df = 232, $p < 0.0001$, **Figure 5B**).

Does Kappa Evolve?

Pexiganan-selected *S. aureus* had significantly higher kappa values than non-selected controls (T = 11.191, df = 238, $p < 0.0001$, **Table 1** and **Figure 6A**), resulting in very steep pharmacodynamic curves (**Supplementary Figure S3**). Pexiganan-selected strains lacking mutations in both *vra* (*bce)* and *rpo* operons showed however a kappa value as low as the non-selected controls (T = 0.570, df = 232, p = 1.00, **Figure 6B**); therefore, a shallower pharmacodynamic curve compared to pexiganan resistant mutants (**Supplementary Figure S3**). The cross resistance of melittin-selected strains toward pexiganan seemed to result in kappa values greater than in the control strains (T = 10.353, df = 238, $p < 0.0001$, **Table 1** and **Figure 6A**). Tenecin-selected strains were as sensitive as their non-selected control and for that kappa was consistent (T1-Con: T = –1.521, df = 238, p = 0.651; T1T2-Con: T = –2.147, df = 238, p = 0.267, **Table 1** and **Figure 6A**). However, tenecin-selected strains with mutations in the *pmt* operon had a kappa value lower than non-selected controls (T = –3.617, df = 232, p = 0.0185, **Figure 6B**).

DISCUSSION

Our study was probably the first to explore the co-evolution of pharmacodynamic parameters (κ, Ψ_{min}, Ψ_{max}) and bacterial AMP resistance. We found that AMP resistance evolution in *S. aureus* resulted not only in increasing MICs, but, importantly in some strains also in changes in the Hill coefficient (κ), resulting in steeper pharmacodynamic curves. Kappa evolved in a mutation dependent manner. Despite the slower AMP selected bacterial growth rate, as captured by Vmax extracted from growth curve analysis, the maximum bacterial growth rate in absence of pexiganan, Ψ_{max}, did not evolve. The maximum killing rate at high concentration of pexiganan, Ψ_{min}, co-evolved with *in vitro* AMP selection. Cross resistance (melittin-selected strains) and cross sensitivity (tenecin 1- and tenecin 1 + 2-selected strains) affected both zMIC and kappa but not Ψ_{min} and Ψ_{max}.

Our selection protocol covered several weeks. This is consistent with treatment regimens for complicated *S. aureus* infections, where 4–6 weeks of intravenous therapy has been the standard practice for over half a century and continues to be recommended (Tong et al., 2015). Although *in vitro* selection of *S. aureus* (SH1000) against tenecin 1 and a combination of tenecin 1 + 2 lasted for 8 days (Makarova et al., 2018), following the same protocol herein, to evolve pexiganan and melittin resistance required 8 weeks. Moreover, in one of our former studies *S. aureus* (JLA513) extinction was observed after 2 weeks of pexiganan selection (Dobson et al., 2013), which was not repeated in the current study. The explanation of differences in pathogen/drug evolutionary dynamics remains poorly understood (Chevereau et al., 2015).

The fitness cost of a pathogen can be inferred from a reduced growth rate *in vivo* (Majcherczyk et al., 2008) or *in vitro* (zur Wiesch et al., 2010). Here, we find clear evidence for costly resistance as measured in slower growth rate (Vmax). However, unlike tenecin-selected lines (Makarova et al., 2018), lag phase for both pexiganan and melittin AMP-selected lines was not prolonged.

Selecting *S. aureus* against melittin resulted in consistent patterns of mutations. All melittin-resistant strains had the following four mutations: stop gains for *pmt*R, missense for *bce*B, frameshift for *atl* and synonymous for *nam*A gene. Mutation in *pmt*R was identical to the stop-gain mutation described in melittin-selected *S. aureus* JLA513 (Dobson et al., 2013) and in tenecin-selected *S. aureus* SH1000 (Makarova et al., 2018). Bacteria harboring a mutation in the gene encoding GntR-type transcriptional repressor, PmtR (Joo et al., 2016b), can continuously efflux AMPs (Cheung and Otto, 2018; Cheung et al., 2018) along with Phenol-soluble modulins (PSMs), a bacterial secreted cytotoxin (Joo et al., 2016a). Mutation in *nam*A, a gene encoding flavin oxidoreductases, was previously reported for antibiotic stress response (Loi et al., 2019). All

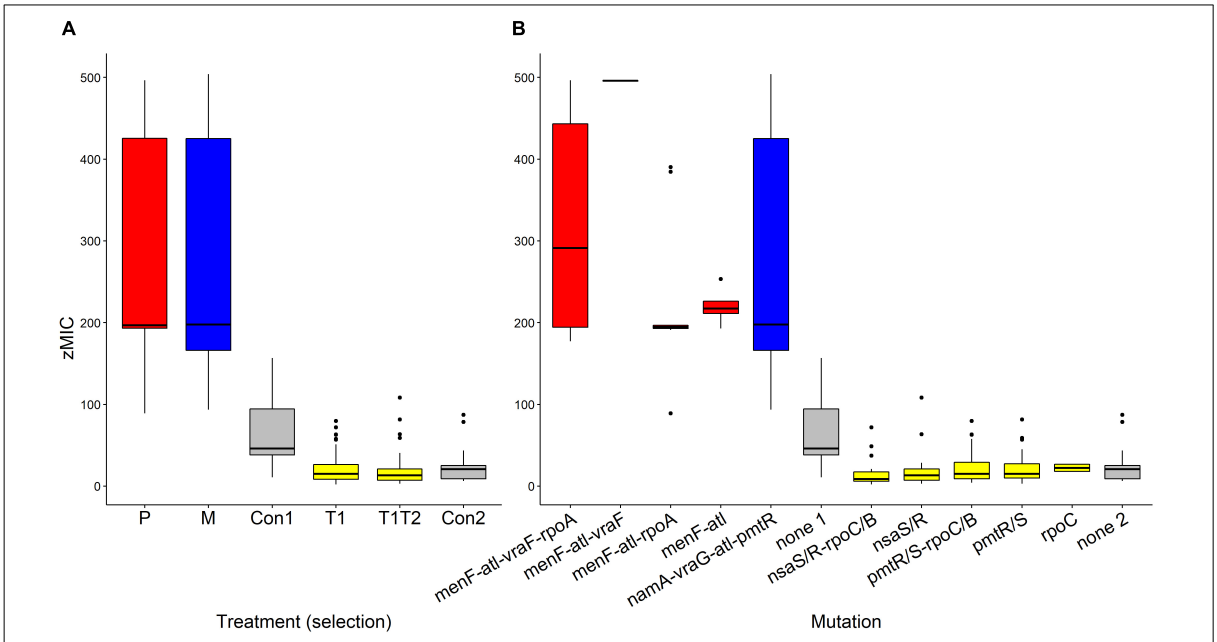

FIGURE 3 | Estimated pharmacodynamic MIC (zMIC) of AMP-selected versus AMP-sensitive *S. aureus* segregated by treatment **(A)** or by mutation **(B)**. (Con: passaged non-selected control for pexiganan- and melittin-selected lines (Con1, none1, gray) and for tenecin-selected lines (Con2, none2, gray), P: pexiganan-selected *S. aureus* (red) segregated into 4 genotype variants with mutation in *men*F, *atl,* and *vra*F, *rpo*A or both, M: melittin-selected *S. aureus* (blue) with mutation in *nam*A, *vra*G, *atl*, and *pmt*R gene loci,T1: tenecin 1-selected *S. aureus* (yellow) and T1T2: tenecin1 + 2-selected *S. aureus* (yellow) harboring a mutation in either *pmt*R/S or *nsa*S/R gene loci which might be accompanied by a mutation in *rpo*C/B). The boxplots show the first to the third quartiles and the median. The bars indicate the 1.5 interquartile of the lower and upper quartiles. The dots represent outliers.

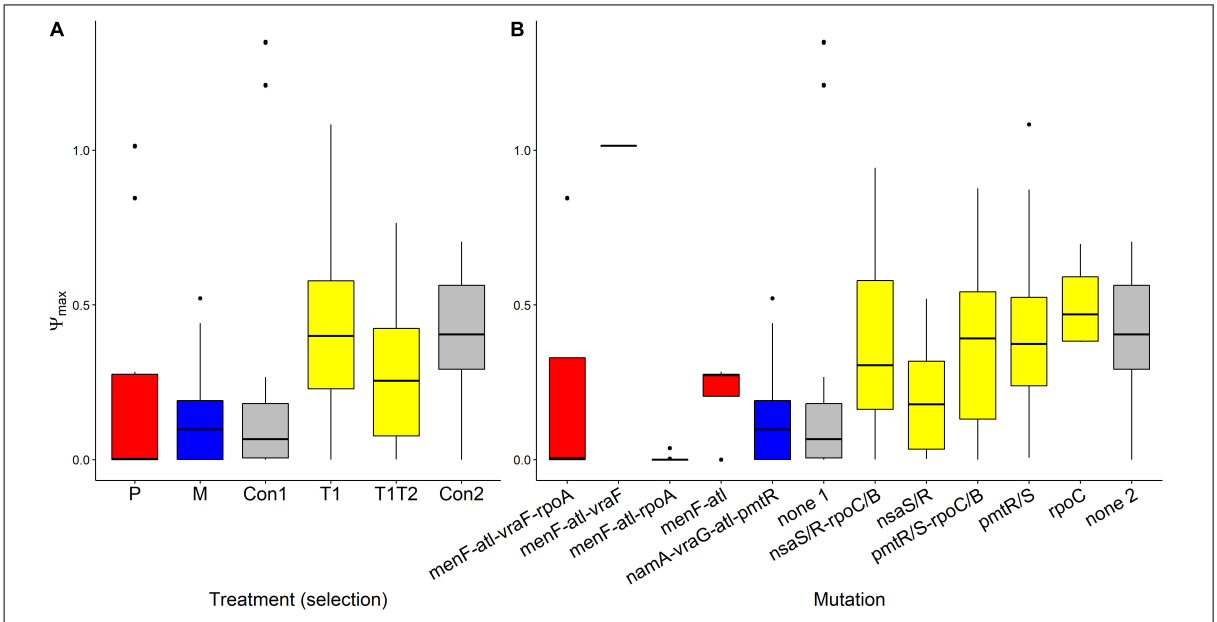

FIGURE 4 | Variation of Ψ_{max} values of AMP-selected versus AMP-sensitive *S. aureus* predicted by MCMC segregated by treatment **(A)** or by mutation **(B)**. Ψ_{min} refers to the maximal growth rate of bacteria at low pexiganan concentration. The Boxplot description, abbreviations and color reference are previously described in **Figure 3**.

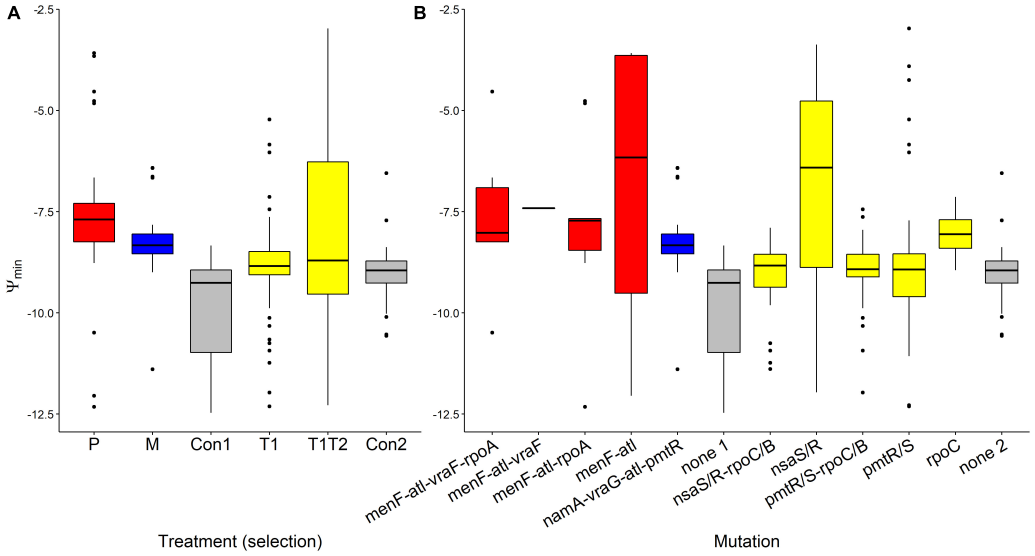

FIGURE 5 | Variation of Ψ_{min} of AMP-selected versus AMP-sensitive *S. aureus* predicted by MCMC segregated by treatment **(A)** or by mutation **(B)**. Ψ_{min} describes minimal growth rate of bacteria under at high pexiganan concentration. Ψ_{min} values segregated by treatment ($F_{(5,232)} = 10.285$, $p < 0.0001$). The Boxplot description, abbreviations and color reference are described in **Figure 3**.

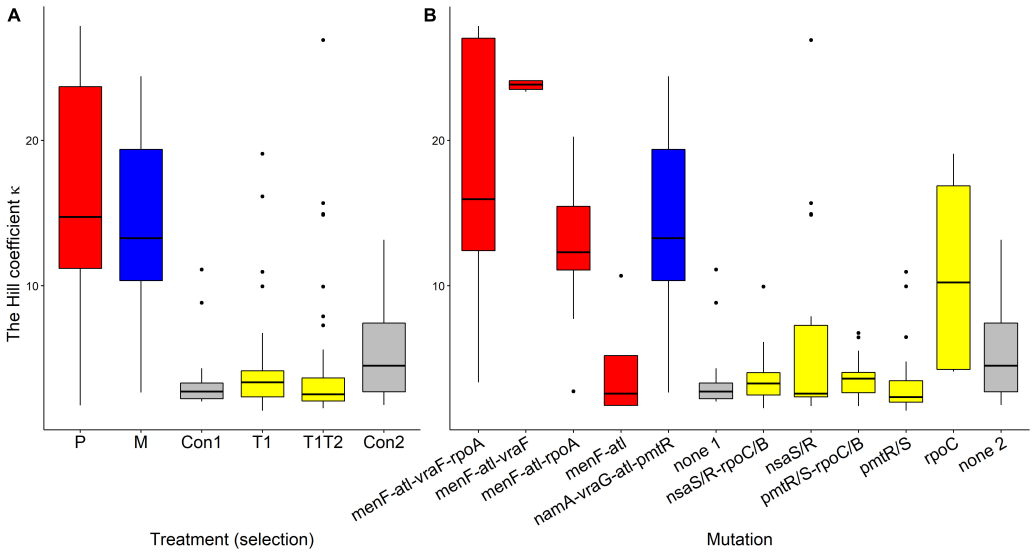

FIGURE 6 | Variations of Hill coefficients κ of AMP-selected versus AMP-sensitive *S. aureus* predicted by MCMC segregated by treatment **(A)** or by mutation **(B)**. Kappa predicts the shape slope of the pharmacodynamic curve, the larger the κ value, the steeper is the pharmacodynamic curve. The Boxplot description, abbreviations and color reference are described in **Figure 3**.

pexiganan-selected lines had mutations in *atl* and *men*F genes. Autolysis decreased in an *atl* (Bi functional autolysin gene) *S. aureus* mutant (Schlag et al., 2010). Some pexiganan-selected lines had a mutation in *vra*F (*bce*A) gene, *rpo*A gene or both. The *vra*FG gene (also designated as *bce*AB gene) codes for bacitracin export permease protein VraFG (BceAB), an ABC transporter controlling BceSR, a bacterial two-component systems (TCSs) associated with antimicrobial susceptibility (Yoshida et al., 2011). VraFG transporter sense the presence of cationic AMPs and transmit signaling through BceS (also known as NsaS and

GraS) to activate BceR (also known as NsaR and GraR)-dependent transcription (Falord et al., 2012). Increased levels of phosphorylation due to point mutations in the *bce*SR (also known as *nsa*SR) operon leads to constitutive expression of BceAB/VraGF which facilitates detoxification by efflux (Coates-Brown et al., 2018). A mechanism by which *S. aureus* can evolve resistance to nisin (Arii et al., 2019), bacitracin (Hiron et al., 2011), and likely to other AMPs (Johnston et al., 2016; Makarova et al., 2018) such as human host defense peptides. Such resistance is a prerequisite for establishing chronic infection (Chaili et al., 2015). Many of the mutations identified herein were described previously in *S. aureus* clinical isolates from patients (Hafer et al., 2012). In summary, such mutations facilitate acquisition of drug resistance, contribute to immune evasion or alter host immune function (DeLeo et al., 2009). It is noteworthy that a recent large-scale whole-genome comparison in *Pseudomonas aeruginosa* showed that experimental antimicrobial resistance evolution reflects and predicts changes in naturally evolved clinical isolates (Wardell et al., 2019).

By analyzing the data with a four-parameter pharmacodynamic model (Regoes et al., 2004), we found that the Hill coefficient, kappa, evolves. Despite its potential importance (Yu et al., 2018; Firsov et al., 2003), kappa is often missed in many pharmacodynamic models, where it is set to 1 or another fixed value for all drugs and referred to as the sigmoidal constant (Craig, 1998; Thomas et al., 1998; Lenhard et al., 2015), even though kappa differs distinctly for different drugs (Yu et al., 2016). Using a different modeling approach, Chevereau et al. (2015) tested a dose-response curves of 78 genome-wide *Escherichia coli* gene deletion strains for a number of antibiotics. They found that the steepness of the dose-response curve varied with drug, but dose-sensitivity (the Hill coefficient k or n as denoted in Chevereau et al. (2015) of the mutants was the same as that of the wild types. Their strain collection did not contain strains which were specifically selected for resistance against the tested drugs. Moreover, in contrast to our approach, they modeled the pharmacodynamics for positive growth only and continuously adjusted the drug concentration. Pharmacodynamic parameters, including the Hill coefficient, play an important role in determining the population size of persistent *S. aureus* (Johnson and Levin, 2013) and *P. aeruginosa* (Hengzhuang et al., 2012) and therefore the probability of infection. In previous work it has been shown that the Hill coefficient relates to the probability of resistance evolution against AMPs (Yu et al., 2018). Whether or not a change in the Hill coefficient, driven by bacterial mutations, contributes to the speed and probability of resistance evolution will require additional work.

Kappa of pexiganan resistant strains, with mutations in *vra*F (*bce*A) and/or *rpo*A genes, were markedly high resulting in a very steep pharmacodynamic curve. Additionally, killing curves of melittin-selected strains, which showed cross resistance toward pexiganan, were as steep as pexiganan resistant strains. Interestingly, pexiganan-selected *S. aureus* which harbor mutations in *men*F and *atl* genes only, uniquely recorded extremely low kappa values. Linking variations in kappa values among AMP resistant strains and whether resistance mutations *per se* are costly or mitigated by compensatory mutations needs farther investigation. The steepness of the pharmacodynamic curve (kappa) determines the width of the mutation selection window MSW (Yu et al., 2018). Firsov et al. (2003, 2006) showed that the size of the (MSW) in *S. aureus* clinical isolates correlates positively with selection for resistance in fluoroquinolones, vancomycin, and daptomycin. We can conclude that MSW as inferred by kappa relies on both tested drug type and bacterial genetic background.

The current study is a proof of principle that shows that pharmacodynamics do evolve beyond MIC with AMP resistance. Applying this finding to all kind of host pathogen interactions and investigating co-evolution to host defense antimicrobial peptides or other antimicrobials will be useful to understand the dynamics of drug resistance evolution. For example, *S. aureus* on the skin are continuously under selection pressure from human AMPs. Along with *in vivo* infection dynamics studies (El Shazely et al., 2019), our study might shed some light on understanding host pathogen relationship during persistent infection. Understanding how antimicrobial peptides eradicate bacteria in physiological system, necessitates studying pharmacodynamics of AMPs *in vitro* (Yu et al., 2016) and *in vivo* (Zanchi et al., 2017).

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the NCBI – bioproject accession number is PRJNA399645.

AUTHOR CONTRIBUTIONS

BE, GY, and JR conceived the study. BE carried out the experiments and led the statistical analysis. PJ led the genomic sequencing. PJ and BE analyzed the genomic data. GY and BE carried out modeling of killing curves (MCMC predictions). BE and JR wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.00103/full#supplementary-material

FIGURE S1 | Fitness costs inferred by growth parameters over mutation/operon in relation to the selective pressure treatment. [Vmax **(A)**, lag phase **(B)**, final OD **(C)**].

FIGURE S2 | Time-kill curves of AMP selected *S. aureus* (SH1000) versus non-selected controls exposed to various concentrations of pexiganan. See **Supplementary Table S1** for a full list of tested strains.

FIGURE S3 | The pharmacodynamic curves of AMP resistant versus AMP sensitive *S. aureus* segregated by treatment **(A)** or by mutation **(B)**. The curves illustrate the effects (reflected as net bacteria growth rate) of increasing the concentration of pexiganan. The ribbon represents 95% of confidence interval.

TABLE S1 | List of the MIC values of pexiganan and melittin resistant strains and their procedural controls tested against pexiganan and melittin. The MIC values of tenecin 1- and tenecin 1 + 2-evolved strains as well as their unselected controls tested against pexiganan only are listed below. The results were determined by a two-fold dilution protocol.

TABLE S2 | Mutation/operon list of pexiganan and melittin resistant *S. aureus* strains and their procedural controls. (M: melittin-evolved strains, P: pexiganan-evolved strains, C: procedural control unselected strains, 1: present, 0: absent).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Appendix (Chapter 3)

Supplementary material

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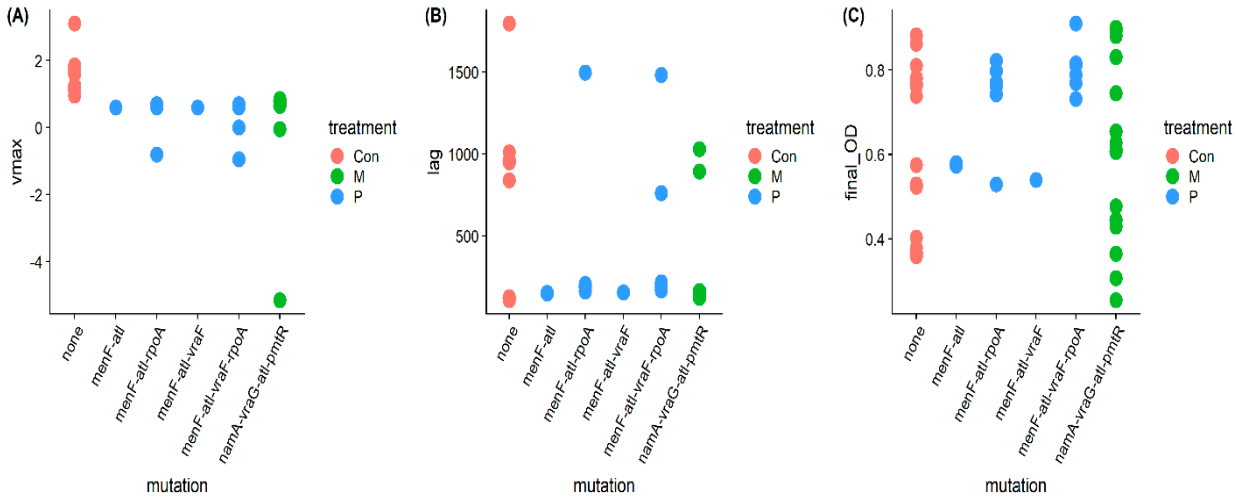

Figure S1. Fitness costs inferred by growth parameters over mutation/operon in relation to the selective pressure treatment. [Vmax (**A**), lag phase (**B**), final OD (**C**)].

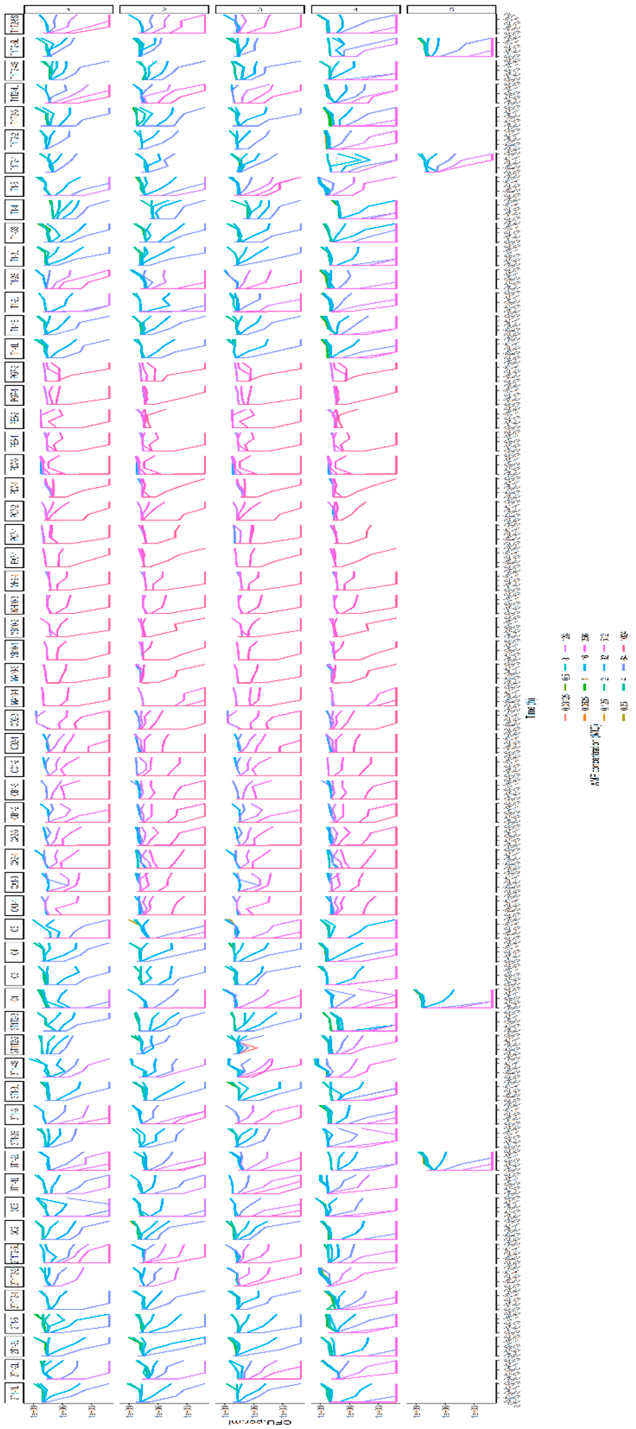

Figure S2. Time-kill curves of AMP-selected S. aureus (SH1000) versus unselected controls exposed to various concentrations of pexiganan. See Supplementary Table S1 for a full list of tested strains, for enlarged figure visit https://www.frontiersin.org/articles/10.3389/fmicb.2020.00103/full#supplementary-material

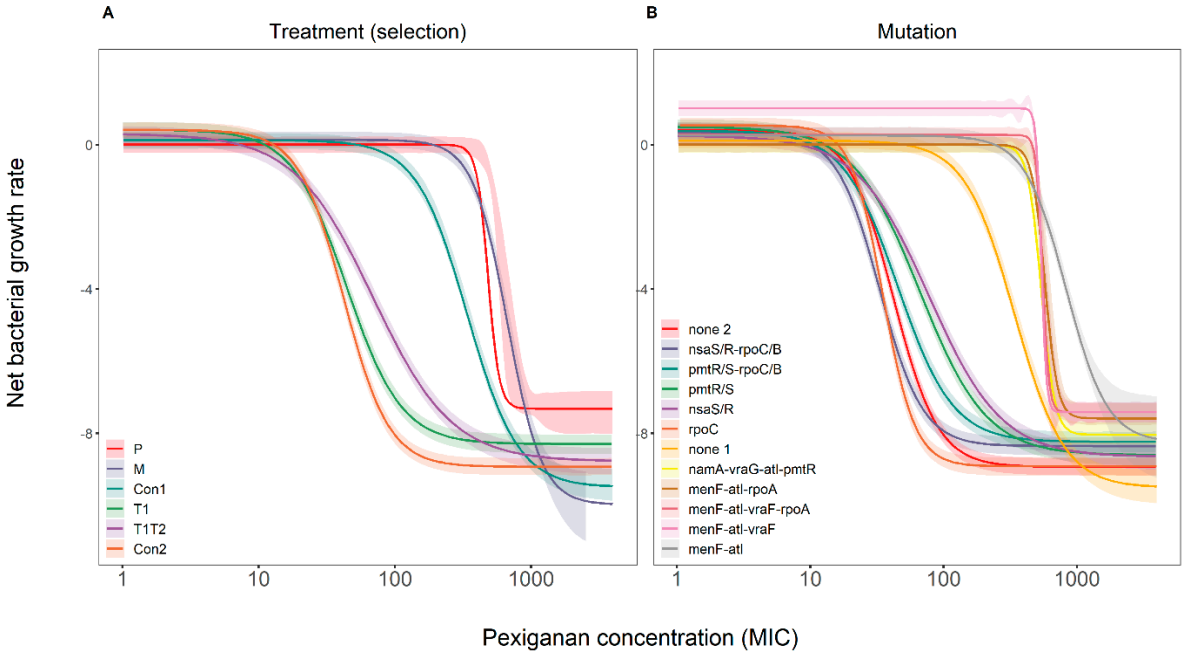

Figure S3. The pharmacodynamic curves of AMP-resistant versus AMP-sensitive S. aureus segregated by treatment (**A**) or by mutation (**B**). The curves illustrate the effects (reflected as net bacteria growth rate) of increasing the concentration of pexiganan. The ribbon represents the 95% confidence interval.

Table S1. List of the MIC values of pexiganan- and melittin-resistant strains and their procedural controls tested against pexiganan and melittin. The MIC values of tenecin 1- and tenecin 1 + 2-evolved strains, as well as their unselected controls tested against pexiganan only, are listed below. A two-fold dilution protocol was used for the analysis.

Strain	Treatment	MIC (Pexiganan)	MIC (Melittin)	mutation
Mel A10-R1	M	256	1024	*nam*A-*atl*-*vra*G-*pmt*R
Mel A10-R2	M	256	1024	*nam*A-*atl*-*vra*G-*pmt*R
Mel A10-R3	M	256	1024	*nam*A-*atl*-*vra*G-*pmt*R
Me1 B10-R1	M	256	1024	*nam*A-*atl*-*vra*G-*pmt*R
Mel B10-R2	M	256	1024	*nam*A-*atl*-*vra*G-*pmt*R
Mel B10-R3	M	256	1024	*nam*A-*atl*-*vra*G-*pmt*R
Mel H9-R1	M	256	512	*nam*A-*atl*-*vra*G-*pmt*R
Mel H9-R2	M	256	512	*nam*A-*atl*-*vra*G-*pmt*R
Mel H9-R3	M	256	512	*nam*A-*atl*-*vra*G-*pmt*R
Mel H10-R1	M	128	512	*nam*A-atl-*vra*G-*pmt*R
Mel H10-R2	M	256	512	*nam*A-*atl*-*vra*G-*pmt*R
Mel H10-R3	M	256	512	*nam*A-*atl*-*vra*G-*pmt*R
Mel G10-R1	M	256	512	*nam*A-*atl*-*vra*G-*pmt*R
Mel G10-R2	M	256	512	*nam*A-*atl*-*vra*G-*pmt*R
Mel G10-R3	M	256	1024	*nam*A-*atl*-*vra*G-*pmt*R
Pex C7-R1	P	1024	256	*men*F-*atl*-*rpo*A
Pex C7-R2	P	1024	256	*men*F-*atl*-*rpo*A-*vra*F
Pex C7-R3	P	1024	256	*men*F-*atl*-*rpo*A
Pex E5-R1	P	1024	256	*men*F-*atl*-*vra*F
Pex E5-R2	P	1024	256	*men*F-*atl*
Pex E5-R3	P	1024	512	*men*F-*atl*
Pex D7-R1	P	1024	256	*men*F-*atl*-*rpo*A-*vra*F
Pex D7-R2	P	1024	256	*men*F-*atl*-*rpo*A
Pex D7-R3	P	1024	256	*men*F-*atl*-*rpo*A-*vra*F
Pex G7-R1	P	256	256	*men*F-*atl*-*rpo*A
Pex G7-R2	P	256	256	*men*F-*atl*-*rpo*A
Pex G7-R3	P	256	256	*men*F-*atl*-*rpo*A-*vra*F
Pex A7-R1	P	512	256	*men*F-*atl*-*rpo*A-*vra*F
Pex A7-R2	P	512	256	*men*F-*atl*-*rpo*A-*vra*F
Pex A7-R3	P	512	256	*men*F-*atl*-*rpo*A
CA2-R1	C1	64	128	none1
CA2-R2	C1	64	128	none1
CA2-R3	C1	64	128	none1
CC1-R1	C1	128	128	none1
CC1-R2	C1	64	128	none1
CC1-R3	C1	64	128	none1
CD2-R1	C1	64	128	none1
CD2-R2	C1	64	128	none1
CD2-R3	C1	128	128	none1
CA1-R1	C1	64	128	none1
CA1-R2	C1	64	128	none1

CA1-R3	C1	64	128	none1
CB1-R1	C1	64	64	none1
CB1-R2	C1	64	64	none1
CB1-R3	C1	64	64	none1
C1	C2	64	NA	none1
C4	C2	32	NA	none1
T1-4	T1	64	NA	*pmt*R/S
2T1-5	T1	64	NA	*pmt*R/S-*rpo*C/B
T1-1S	T1	32	NA	*pmt*R/S
3T1-2S	T1	64	NA	*pmt*R/S
T1T2-4S	T1T2	64	NA	*pmt*R/S
T1T2-2	T1T2	32	NA	*nsa*S/R
C5	C2	64	NA	none2
T1T2-5S	T1T2	64	NA	*pmt*R/S
2T1T2-5L	T1T2	32	NA	*pmt*R/S
T1-2S	T1	32	NA	*pmt*R/S-*rpo*C/B
3T1-2L	T1	64	NA	*pmt*R/S-*rpo*C/B
T1T2-4L	T1T2	64	NA	*pmt*R/S
3T1-3	T1	64	NA	*nsa*S/R-*rpo*C/B
3C5	C2	32	NA	none1
3T1-1L	T1	64	NA	*pmt*R/S-*rpo*C/B
3T1-4S	T1	32	NA	*pmt*R/S
T1-5	T1	64	NA	*pmt*R/S-*rpo*C/B
2T1T2-2	T1T2	64	NA	*nsa*S/R
2T1-2L	T1	64	NA	*pmt*R/S
T1-2L	T1	32	NA	*pmt*R/S
C3	C2	32	NA	none1
T1-1L	T1	64	NA	*rpo*C
T1T2-1	T1T2	64	NA	*nsa*S/R
2T1T2-1	T1T2	32	NA	*nsa*S/R
3T1-3L	T1	32	NA	*nsa*S/R-*rpo*C/B
T1T2-3	T1T2	64	NA	*nsa*S/R-*rpo*C/B
T1-3S	T1	32	NA	*nsa*S/R-*rpo*C/B
3C2	C2	64	NA	none1
3T1T2-3	T1T2	32	NA	*nsa*S/R-*rpo*C/B
2T1-1L	T1	32	NA	*pmt*R/S-*rpo*C/B
2T1-3L	T1	64	NA	*pmt*R/S-*rpo*C/B
T1-3L	T1	32	NA	*nsa*S/R-*rpo*C/B
T1T2-5L	T1T2	64	NA	*pmt*R/S
3T1T2-2	T1T2	64	NA	*nsa*S/R

Table S2. Mutation/operon list of pexiganan- and melittin-resistant *S. aureus* strains and their procedural controls. (M: melittin-evolved strains, P: pexiganan-evolved strains, C: procedural control unselected strains, 1: present, 0: absent).

Strain	Treatment	*namA*	*bceB* (*vraG*)	*atl*	*ytrA* (*pmtR*)	*menF*(*pchA*-1)	*bceA*(*vraF*)	*rpoA*	*agrA*	*dpiB*	*pbpX*_1	*cobT*	*mtaB*	*ddl*	*sigB*	*yezG*_2	PROKKA_01858	*tcaA*	*nrnA*_1	PROKKA_01857	*qoxA*	PROKKA_00774	PROKKA_01856
Mel A10-R1	M	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0
Mel A10-R2	M	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0
Mel A10-R3	M	1	1	1	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Me1 B10-R1	M	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Mel B10-R2	M	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mel B10-R3	M	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Mel H9-R1	M	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mel H9-R2	M	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mel H9-R3	M	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mel H10-R1	M	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mel H10-R2	M	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mel H10-R3	M	1	1	1	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Mel G10-R1	M	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mel G10-R2	M	1	1	1	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Mel G10-R3	M	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pex C7-R1	P	0	0	1	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0
Pex C7-R2	P	0	0	1	0	1	1	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0
Pex C7-R3	P	0	0	1	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0
Pex E5-R1	P	0	0	1	0	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0
Pex E5-R2	P	0	0	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0
Pex E5-R3	P	0	0	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0

train	Treatment	*nam*A	*bce*B (*vra*G)	*atl*	*ytr*A (*pmt*R)	*men*F(*pch*A-1)	*bce*A(*vra*F)	*rpo*A	*agr*A	*dpi*B	*pbp*X_1	*cob*T	*mta*B	*ddl*	*sig*B	*yez*G_2	PROKKA_01858	*tca*A	*nrn*A_1	PROKKA_01857	*qox*A	PROKKA_00774	PROKKA_01856
Pex D7-R1	P	0	0	1	0	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0
Pex D7-R2	P	0	0	1	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0
Pex D7-R3	P	0	0	1	0	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Pex G7-R1	P	0	0	1	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1
Pex G7-R2	P	0	0	1	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Pex G7-R3	P	0	0	1	0	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Pex A7-R1	P	0	0	1	0	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1
Pex A7-R2	P	0	0	1	0	1	1	1	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0
Pex A7-R3	P	0	0	1	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0
CA2-R1	C	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
CA2-R2	C	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0
CA2-R3	C	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0
CC1-R1	C	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0
CC1-R2	C	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0
CC1-R3	C	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0
CD2-R1	C	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0
CD2-R2	C	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0
CD2-R3	C	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CA1-R1	C	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0
CA1-R2	C	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0
CA1-R3	C	0	0	0	0	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0
CB1-R1	C	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
CB1-R2	C	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
CB1-R3	C	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0

7. General discussion

7.1. Summary of findings

Many studies suggested antimicrobial peptides (AMPs) as attractive therapeutics to solve the fast-growing antibiotic resistance crisis. AMPs are primary components of innate immunity in multicellular organisms (Zasloff, 2002). AMP-based therapy might trigger the evolution of undesirable cross-resistance to the host's own AMPs. Coevolution of resistance to endogenous AMPs of the host has been a hampering threat of AMP-based therapeutic success and a significant health concern. This thesis mainly focuses on the implications of the evolution of antimicrobial peptide (AMP) resistance on host-pathogen relationship regarding infection dynamics, virulence inferred by host survival, the fitness cost of resistance evolution, collateral sensitivity/resistance toward host other immune effectors and coevolution of pharmacodynamic parameters.

Chapter 1 revealed that an AMP-resistant pathogen did not perform well in the host, as it did not establish a higher bacterial load than the AMP-sensitive controls within the 14 days post-infection. Thus, for the human pathogen *Staphylococcus aureus*, the costs of genetic trade-offs caused by the evolution of resistance to *Tenebrio molitor*'s antimicrobial peptides lead to survival in the beetle similar to that of unselected AMP-sensitive strains. Moreover, the virulence of *S. aureus* was not influenced by AMP resistance either. As shown in the survival experiment of *T. molitor* infected with *S. aureus*, there was no effect of bacterial antimicrobial peptide resistance on beetle survival. Interestingly, antimicrobial peptide-resistant *S. aureus* strains were mostly (except for *S. aureus* harbouring a mutation in *nsa-rpo* operons) less recognised by the phagocytes (hemocytes) of the mealworm beetle, *Tenebrio molitor*. However, this finding did not translate into a better survival in the beetle in the first twenty-four hours after inoculation, during which cellular immunity contributes most to fighting off the infection.

Chapter 2 showed that AMP resistance regulates pathogen sensitivity to the host's other antibacterial immune responses. For example, AMP-resistant *S. aureus* strains are mostly cross-sensitive to phenoloxidase, except for *S. aureus* harbouring a mutation in *nsa-rpo* operons which is resistant to phenoloxidase antibacterial activity. The bacterial load of the later mutant did not increase in the case of prophenoloxidase (PPO) knockdown (PO KD) expression.

Additionally, *S. aureus* resistance to one or two AMPs of *Tenebrio*'s defence cocktail selectively affected their sensitivity to other components of the host's AMP mixture. We showed that exclusively the resistant *S. aureus* strains harbouring a mutation in *rpo*, *pmt* or *nsa-rpo* operons are cross-sensitive to either tenecin 2, tenecin 4 or both. Additionally, the virulence of tenecin 1 resistant *rpo S. aureus* mutants unprecedently decreased by knockdown of AMP (T1T2T4) expression.

To further investigate the fitness cost of AMP resistance *in vitro*, the study reported in **chapter 3** has investigated the coevolution of pharmacodynamic parameters of AMP-resistant pathogens. Our results showed that, importantly, the shape of the pharmacodynamic curve represented by the Hill coefficient (kappa) evolves differently for different mutants, although all have the same MIC values. Different kappa values imply different mutation-selection windows, hence determine the susceptibility of such pathogen to further evolution.

7.2. Unexpected infection dynamic outcome of an AMP-resistant pathogen: benefits versus fitness cost.

Graham et al. (2011) stated that eco-immunological studies usually focus on the benefits, costs and fitness outcomes of different immune responses. However, assessment of the fitness costs of immune responses is not a simple quest, as the correlation between host fitness and the within-host density of parasites is complex. Robust immune responses do not necessarily translate into maximal host fitness(Schmid-Hempel, 2005; Cotter et al., 2010). As highlighted by Graham et al. (2011), to understand host-pathogen coevolution, eco-immunologists should consider the fitness of host and parasite and relevant immune responses as completely interconnected (Graham et al., 2011). In this thesis, a trial to assess the fitness costs of the evolution of AMP-resistance by monitoring the survival of both the host and the parasite and measuring the host's immune effector performance through different infection phases was carried out.

According to the experiment presented in **Chapter 1**, acquiring AMP-resistance traits was not advantageous for the pathogen, *S. aureus,* in terms of enhanced infection load in the host. In contrast, the AMP-resistant strains were mopped up by the immune effectors of the host as efficiently as the AMP-sensitive strains. Such a result insinuates that the costs of the evolution of antimicrobial peptide (AMP) resistance are relatively high, considering the outcomes of the *in vivo* host-pathogen interaction.

Many pieces of research suggested that AMP-resistant strains might better survive immunity, persist in the host, and thus might be fitter in a host (Bell & Gouyon, 2003; Hancock, 2003; Habets & Brockhurst, 2012; Dobson et al., 2014; Fleitas & Franco, 2016). Unexpectedly, *Staphylococcus aureus* selected against the host's endogenous AMPs were eradicated rapidly by the host and were just as persistent as the unselected AMP-sensitive control strains. Moreover, we showed that bacterial loads of tenecin 1 + 2 resistant *S. aureus* strains were significantly lower than tenecin 1- selected lines. Such a finding suggests a higher fitness cost of evolving resistance to a cocktail of two AMPs (defensin and coleoptericin) than towards a single AMP (a defensin) only. Tenecin 1 exerts its bactericidal action on the cell membrane. Tenecin 2 has no direct anti-Gram positive activity (Johnston et al., 2014; Makarova et al., 2016), however, limit the bacterial virulence and decrease the infection-induced mortality of the host (Zanchi et al., 2017). Resistance to both is likely to involve costly fundamental structural changes. Therefore, the

findings herein supported the hypothesis that the inherent costs of the evolution of AMP resistance might constrain its natural occurrence frequency. At least, the cost of iseganan resistance was mitigated upon inoculation into *T. molitor,* recording an elevated bioburden (Dobson et al., 2014). In contrary, this was not the case for pexiganan, melittin (Dobson et al., 2014) and tenecin (El Shazely et al., 2019) resistant *S. aureus*.

Some knockout mice studies have found that AMPs restrict microbial proliferation in the skin and mucosal tissues and prevent deep tissues infection development (Nizet et al., 2001; Nizet, 2006). The contribution of AMP resistance mechanisms to pathogenesis is confirmed (Pontes et al., 2011; Earl et al., 2015). Resistance to the host's AMPs is prerequisite of establishing an infection (Cheung & Otto, 2018). This knowledge raises the question: what is the magnitude of the fitness cost of bacteria resistant to human AMPs, given that AMPs are a deterministic factor of infection outcome and pathogenesis level? *Staphylococcus aureus* Pmt ABC transporter predisposes bacteria to human AMP resistance and prevents elimination by human neutrophils by active efflux. Pmt adds to virulence during human skin infection in an AMP-dependent manner (Cheung et al., 2018). It is worth noting that some AMP evolved *S. aureus* strains, in this thesis, were found to harbour a mutation in the *pmt* operon (Makarova et al., 2018), suggesting a common mechanism of resistance to that shown for human AMPs.

7.3. Virulence of AMP-resistant *S. aureus*: life history determines infection outcomes.

Pathogen resistance coevolution towards innate host defence peptides is frequent, with the probability of increasing both morbidity and mortality. For example, the virulent health-care-associated methicillin-resistant *Staphylococcus aureus* (MRSA) USA600 showed increased cross-resistance to human cathelicidin LL-37 killing (Sakoulas et al., 2014). For the first time, the pathogens used in this thesis were resistant toward the host's endogenous AMPs which might reflect a better vision of the fitness cost of resistance evolution toward the host’s own innate immune modulators.

Our results showed (**Chapter 1**) that infection with AMP-resistant pathogen did not decrease the survival of the host. Based on this low host mortality, the AMP-resistant *S. aureus* strains in our experiments were not explicitly virulent toward *T. molitor*, as a host. Host resistance directly affects bacterial fitness because it acts to reduce pathogen prevalence (Roy & Kirchner, 2000). While hosts tolerate a pathogen by limiting the adverse fitness effects of pathogen prevalence (Kutzer & Armitage, 2016). Our results suggest that hosts clearly invest into AMP-selected pathogen eradication and thus, resistance.

It is worth noting that the tenecin- and melittin-selected *S. aureus* strains might harbour a mutation in the *pmt*R region (Makarova et al., 2018; El Shazely et al., 2020). The same *pmt*R mutation is common in nisin-resistant *S. aureus* strains (Joo et al., 2016b; Cheung et al., 2018). A mutation at the *pmt*R locus was recently found to drive higher mortality rate in bacteraemia mice models (Kawada-Matsuo et al., 2020), thus *pmt*R mutants, unlike our findings, were found to be more virulent.

The survival of *Tenebrio molitor* infected with AMP-resistant *S. aureus* might be sensitive to early life inflammation/wounding. For example, results in **chapter 2** suggested that *nsa-rpo S. aureus* mutants, uniquely, trigger high mortality rates in *Tenebrio molitor*, if the experimental design included 1 (dsRNA injection) or 2 (dsRNA and peptidoglycan injection) wounding before bacterial injection. AMP-sensitive and other AMP-resistant mutants did not behave similarly. This result hints that the host's life history before infection, such as early life inflammation, might be one more possible deterministic factor of infection outcomes (Minchella, 1985; Khan et al., 2017). However, more experiments are recommended to explore such speculation specifically.

7.4. The sensitivity of AMP-resistant pathogen to other immune effectors

7.4.1. Phagocytosis: host's hemocytes overlook AMP-resistant pathogens

The results in **chapter 1** showed that AMP-resistant lines (except *nsa-rpo* mutants) are phagocytosed at a much lower rate. Our data agrees with findings of human infection biology studies; as *S. aureus* mutants resistant to human defensins failed to be efficiently inactivated by neutrophils (phagocytes) and thus constitute a more virulent human-pathogen (Peschel et al., 2001; Peschel, 2002). Nevertheless, we reported that AMP-selected lines showed attenuated virulence in *T. molitor* (El Shazely et al., 2019).

The ability of *S. aureus* to resist neutrophil killing (Peschel et al., 2001) and survive within blood cells (neutrophils) contributes to infection in humans (Gresham et al., 2000) and insects (McGonigle et al., 2016). AMP resistance mechanisms, including cell surface modifications (Joo et al., 2016a), might help bacterial cells to employ membrane-camouflage concealing themselves from recognition by phagocytic cells and might explain their successful phagocytosis avoidance tactics. The *in vitro* phagocytic assay used in **chapter 1**, was not designed to disentangle whether the observed phagocytic ratio is due to limited recognition or engulfment of the AMP-resistant strains. To resolve such a question, further analysis is required.

Additionally, *S. aureus* harbouring a mutation in *pmt*R showed higher hemolytic activity when tested against sheep blood agar (Kawada-Matsuo et al., 2020). Some of the tenecin (Makarova et al., 2018; El Shazely et al., 2019) and melittin (El Shazely et al., 2020) resistant *S. aureus* strains studied herein harboured a mutation in the same gene locus. Here, we reported that the tenecin-selected *S. aureus pmt* mutants were significantly less phagocytosed than the unselected control (El Shazely et al., 2020). Whether such a result is caused by increased hemolysis of *T. molitor*'s hemocytes or decreased overall bacterial recognition due to cell surface modifications was not investigated in this thesis.

Albeit being resistant to both host's AMP and phagocytes, AMP-selected *S. aureus* strains were easily eradicated non-virulent pathogens (El Shazely et al., 2019). This finding is an apparent mismatch of characters versus the behaviour of tenecin-selected *S. aureus*. Our lab showed, previously, that phagocytosed *S. aureus* in *T. molitor* might relocate into the hemolymph (McGonigle et al., 2016). As we showed in this thesis that AMP-resistant lines are less recognised and probably less phagocytosed by hemocytes. This characteristic might render AMP-resistant *S. aureus* bacterial cells more exposed and perhaps then better controlled by other immune modulators. Considering this scenario, the lower bacterial load and attenuated virulence for most AMP-resistant mutants seemed possible.

7.4.2. Phenoloxidase activity: AMP- & phagocytosis-resistant mutants are more sensitive to phenoloxidase activity

Melanin evolved over 500 million years ago. Despite being functionally different, it is found in both insects and mammals (Sheehan et al., 2018). In invertebrates, melanisation is essential defence reaction through its role in wound healing and pathogen sequestration. Upon pathogen invasion, prophenoloxidase (PPO) system is activated (Gillespie et al., 1997). However, in mammals, it only protects the skin from solar radiation and forms a skin and hair pigmentation (Sheehan et al., 2018).

Results from **chapter 2** showed that AMP-resistant *S. aureus* strains (except *nsa-rpo* mutants) are cross-sensitive towards phenoloxidase. AMP-selected lines showed significantly higher bacterial loads in knockdown beetles. Therefore, all AMP- & phagocytosis-resistant mutants that might be hazardous pathogens, if not cleared, are precisely recognised and efficiently eliminated via the prophenoloxidase system. It is worth noting that *nsa-rpo* mutants that are sensitive toward phagocytosis (El Shazely et al., 2019), and thus might not form such an infection risk, are, surprisingly, not as susceptible to phenoloxidase. The PPO-activating system recognises the structural characteristics of the bacterial and fungal components (Soderhall & Cerenius, 1998). It is possible that the bacterial membrane conformational changes required for acquiring AMP resistance cause them to be more prone to recognition by the PPO-activating system; however, less prone to

recognition by the phagocytes. The *S. aureus* strain harbouring a mutation in *nsa-rpo* seems to be an exception to the previous hypothesis both ways, suggesting a unique cell surface structure. It is worth noting that *S. aureus* strain harbouring a mutation in *nsa-rpo* behave differently than the *nsa* and the *rpo* mutants.

As a PPO defence system is functionally lacking in mammals (Sheehan et al., 2018), an AMP-resistant pathogen might be an outstanding risk if not neutralised by other immune modulators. Lofton et al. (2013) evolved *Salmonella typhimurium* against LL-37 (human-derived peptide), CNY100HL (synthetic peptide) or wheat germ histones (a mixture of different histones and shorter histone peptides). The selected strains harboured mutations in the *pho*P and *waa*Y gene loci (Lofton et al., 2013). The same lab examined the fitness cost of resistance mutations of *Salmonella typhimurium* both under several *in vitro* conditions that resemble the host environment and *in vivo* by inoculation into a mouse host. In contrary to the study herein, the results suggested that the fitness cost of resistance mutation was minimal both *in vitro* and *in vivo* (Lofton et al., 2015).

To avoid any possible interference with the human innate immune system, screening AMP-based therapeutics for candidates, that render bacterial cells more vulnerable to be eradicated by host's immune modulators, is essential (Kintses et al., 2019; Spohn et al., 2019). For example, the nisin-based antibiotic treatment might trigger slight cross-resistance of the human-pathogen *S. aureus* towards two human AMPs, β defensin-3 and LL37 (Kawada-Matsuo et al., 2020).

7.4.3. One or more component of AMP defence cocktail: *pmt, nsa-rpo* mutants showed collateral sensitivity to T2, T4 or both

Collateral sensitivity of antibiotic-resistant bacteria toward the host's AMPs is frequent; on the other hand, cross-resistance is relatively rare (Lazar et al., 2018). Mechanisms of AMP resistance in bacteria are limited, such as increasing the positive net charge of the cell surface, which alter the binding of cationic AMPs or active pumping (Andersson et al., 2016). Inactivation of AMP resistance pathways results in enhanced susceptibility to host AMPs and thus a reduced virulence (Andersson et al., 2016). Further studies in the future should test whether the close structure and mode of action of AMPs are correlated with the chances of the evolution of cross-resistance. We previously showed that the tenecin-resistant *S. aureus* are cross-resistant towards melittin and colistin, on the other hand, are cross-sensitive toward pexiganan and vancomycin (Makarova et al., 2018; El Shazely et al., 2020). Additionally, a Japanese research group reported, recently, that the nisin-resistant *S. aureus* harbouring a mutation in *pmt*R region are less-susceptible to human β defensin-3 (hBD3) and LL37 (Kawada-Matsuo et al., 2020).

A general characteristic of immune gene upregulation in insects is lack of specificity (Johnston et al., 2014). Upon *S. aureus* infection, eight AMP gene expression was upregulated, including a defensin (anti-Gram-positive, tenecin 1), four attacins (anti-Gram-negative, including tenecin 4 with negligible activity toward *S. aureus*) and three coleoptericin (anti-Gram-negative, including tenecin 2) (Moon et al., 1994; Roh et al., 2009; Chae et al., 2012; Dobson, 2012; Johnston et al., 2014). We knocked down one AMP of each type (tenecin 1, tenecin2 and tenecin 4), to test collateral sensitivity concept of AMP-resistant pathogen to other AMPs in the defence mixture or to their collective *in vivo* combinatory effect.

The results in **chapter 2** showed that collateral sensitivity of AMP-resistant *S. aureus* to other AMPs in the defence cocktail segregate by mutation. For example, the tenecin 1 -resistant *pmt S. aureus* mutants showed cross-sensitivity toward either tenecin 2, tenecin 4 or both. While the tenecin 1 tenecin 2 (T1T2)- selected *nsa-rpo S. aureus* mutants showed cross-sensitivity to tenecin 4. This result coincides nicely with the observed *in vivo* lower bacterial load (**chapter 1**) those particular two mutants had through persistent infection, precisely 14 days post-infection (El Shazely et al., 2019).

The T1T2T4 knockdown experiment only meant to answer whether collateral sensitivity to one or more component of the defence cocktail exists or not. Although it sounds interesting, it was not designed to define to which AMP the collateral sensitivity augment specifically. Indeed, for that different experimental design with all possible single knockdowns should be approached.

7.5. *In vitro* assessment of AMP resistance implications on pathogen fitness cost as inferred by growth and pharmacodynamic parameters

The fitness cost of the evolution of resistance is the rate of pathogen replication under prevailing environmental conditions. Fitness could be measured by competitive fitness trials or inferred by the growth rate (Melnyk et al., 2015). Antimicrobial-induced bactericidal (killing) or bacteriostatic (growth inhibition) does not necessarily increase linearly with drug concentration. The growth rate at therapeutic concentrations is considered as a proxy for pathogen fitness in the presence of treatment (zur Wiesch et al., 2010), which could be studied under pharmacodynamic models (Regoes et al., 2004; Johnson & Levin, 2013).

7.5.1. Growth parameters

The fitness cost of resistance could be reviewed as pathogen reduced growth rate either *in vivo* (Majcherczyk et al., 2008; El Shazely et al., 2019), *in vitro* (zur Wiesch et al., 2010) or both. Our lab previously showed that, at 37℃, that T1- and T1T2-selected *S. aureus* lines had slower growth rates

and thus steeper exponential phase (smaller Vmax values than the unselected controls) and longer lag phases (Makarova et al., 2018).

However, the results in **chapter 1** showed that, at 25℃, a temperature at which the beetles were incubated, T1-selected *S. aureus* only recorded a more prolonged lag phase compared to the sensitive unselected control. Moreover, the V_{max} did not segregate either by treatment or mutation. The *nsa-rpo* mutants showed the most extended lag phase (El Shazely et al., 2019).

Perron et al. (2006) showed that evolution of pexiganan resistance in *Pseudomonas* did not affect V_{max} or the maximal population density, but a prolonged lag phase in absence of stressor (Perron et al., 2006). The extended lag phase might provide bacteria with survival advantages and the chance of regrowth upon removal of antimicrobial stressor. Several bacteria of relevance to endemic nosocomial infections are most likely to evolve antibiotic resistance via extended lag phase (Li et al., 2016).

7.5.2. Pharmacodynamic parameters

The study in **chapter 3** used a four-parameter pharmacodynamic model (Regoes et al., 2004) to analyse time-kill curves of AMP-resistant *S. aureus* strains (El Shazely et al., 2020). The data showed that the Hill coefficient, kappa, representing the shape of the pharmacodynamic curve evolves. Albeit, Hill coefficient highlighted importance (Firsov et al., 2003; Yu et al., 2018), kappa is often neglected in many pharmacodynamic models, where it is set to a constant value for all drugs (Craig, 1998; Thomas et al., 1998; Lenhard et al., 2015). Our lab previously showed that kappa differs distinctly for different drugs (Yu et al., 2016).

Furthermore, outcomes of the pharmacodynamic modelling of time-kill curves proved that kappa differs for different AMP-resistant strains while having the same MIC values. (El Shazely et al., 2020). This finding is particularly important because the Hill coefficient relates to the probability of resistance evolution against AMPs (Yu et al., 2018). The results revealed that pharmacodynamic parameters evolve beyond minimum inhibition concentration (MIC) with AMP resistance. This finding could be extended to all kind of host-pathogen relationship. Evolution of pharmacodynamic parameters should not be ignored or over-simplified to MIC when studying coevolution to host's AMP defence cocktail or other antimicrobials; this might boost our understanding of drug resistance evolution dynamics.

8. Conclusion

How do different host immune system modulators perceive and act against a pathogen selected against one or more endogenous AMP? The thesis herein investigated this intriguing question. In general, the fitness cost of the evolution of resistance to the host's own AMPs was high, rendering them vulnerable to eradication by the host's immune effectors.

Moreover, the mutation driving AMP resistance modulated the *in vivo* fitness costs of AMP-selected bacteria. For example, uniquely, the *nsa-rpo S. aureus* mutants showed high fitness cost as:

a) their bacterial loads were zero-inflated by two weeks of infection,

b) those mutants did not coevolve the phagocytosis resistance trait as other AMP-resistant mutants,

c) additionally, they had collateral sensitivity to other components of the AMP defence cocktail.

Our *in vitro* AMP dose-response experiments of the AMP-evolved strains suggested that the bacterial growth parameters and the pharmacodynamic parameters coevolve with AMP resistance. Importantly, the steepness of the pharmacodynamic curve, described by kappa or the Hill coefficient, evolved and segregated by resistance mutation, despite being ignored or set to constant value in most pharmacodynamic models.

9. Outlook

Evolution of resistance is costly, both ways in a host-pathogen relationship. Considering the fitness cost of resistance when studying host-pathogen relationships is useful in enriching our understanding of their coevolution and helps to predict precisely such relationship outcomes. For example, to better determine the outcomes of infection, the fitness costs of resistance experienced by both hosts and parasites should be considered. This knowledge will help to select better therapeutic agents to which pathogenic microbes ideally have high resistance fitness cost. Thus, the evolution of resistance to such therapeutic agents would be costly, and then those unfit bacterial strains would be easily mopped up by the help of host's immune effectors. This thesis has discussed costs of bacterial resistance to the host's intrinsic AMP, which is significantly essential in understanding microbiome-host homeostasis and evolution of resistance risk if AMPs are to be tackled as therapeutic drugs.

This thesis showed that host's other immune effectors (e.g. phenoloxidase and different components of AMP defence cocktail) selectively controlled AMP-resistant pathogens. Hypothetically, the infection outcomes might be changed, if a pathogen bears the costs of evolving to multiple immune effectors not only AMPs or at least to the whole defence cocktail. Could multi-immune effector-resistant pathogen evolve? Could multi-AMP-resistant pathogen evolve at a low fitness cost; as a cocktail of AMPs is usually deployed by innate immunity of multicellular organisms? AMP-resistant strains usually share cross-resistance to AMPs with similar structure and mode of action. For example, the nisin-selected *S. aureus* exhibit reduced sensitivity towards two human AMPs, β defensin-3 and LL37 (Kawada-Matsuo et al., 2020). Whether the fitness cost of such a resistance scenario should be a naturally good enough obstacle or not should be experimentally explored to avoid evolving super-bugs during AMP-based therapy.

The bacterial resistance evolution to AMPs in nature augmented by their unique pharmacodynamics characters and synergistic mode of action is limited (Yu et al., 2018; Spohn et al., 2019; El Shazely et al., 2020), thus rendering them attractive antibacterial drugs (Lazzaro et al., 2020). Therapeutic approaches such as combinational therapy and cycling therapy are routinely proposed to conquer the antibiotic-resistant bugs. Some factors should be considered to find ideal antimicrobial with the highest resistance fitness cost and, hence, the least probability of resistance evolution, and to avoid following the footsteps of mistakes that resulted in antibiotic resistance. For example, a) infection dynamic experiments highlighting *in vivo* host-pathogen relationships, b) thorough pharmacodynamic studies covering both different drugs and pathogens.

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10. List of figures

I. Chapter 1

El Shazely, B., Urbanski, A., Johnston, P. R., & Rolff, J. (2019). *In vivo* exposure of insect AMP-resistant *Staphylococcus aureus* to an insect immune system. *Insect Biochem Mol Biol, 110*, 60-68. doi:10.1016/j.ibmb.2019.04.017

Fig. S6	*In vitro* phagocytosis ratio of **male** *Tenebrio molitor* haemocytes incubated with either unselected AMP-sensitive control (A) and tenecin-resistant *S. aureus* (B). strains
Fig. S7	*In vitro* growth parameters extracted from growth curve of AMP-selected and AMP-unselected *S. aureus* strains tested at 25℃ (V_{max}, lag phase, maximal OD).

II. Chapter 2

El Shazely, B. & Rolff, J. Collateral sensitivity of experimentally evolved AMP-resistant *Staphylococcus aureus* to host immune effectors. *In preparation*

Fig. 1	**(A)** Bacterial load of the ancestor *Staphylococcus aureus* strain (SH1000). The PO knockdown (dotted) did not affect *in vivo* colonization (**black**) at any time point. (**B**) Survival of *T. molitor* infected with ancestor strain (**black**) decreased by PO knockdown, while the sham infected (**grey**) did not vary by knockdown treatment.
Fig. 2	(**A**) Bacterial load of the tenecin 1 (defensin) resistant *Staphylococcus aureus* harbouring a mutation in ***rpo*** operon. The PO knockdown (**dotted**) increased colonization of *rpo* mutant (**yellow**) by day 7, unlike the unselected control (**grey**). (**B**) Survival of *T. molitor* was not affected neither by PO knockdown nor by bacterial AMP sensitivity.
Fig. 3	(**A**) Bacterial load of *Staphylococcus aureus* harbouring a mutation in *pmt* (**green**)*, pmt&rpo* (**red**) or *nsa* (**blue**) operon. The PO knockdown (**dotted**) increased colonization of AMP-resistant *S. aureus* by day 7 for all mutants, unlike the unselected control C2 (**grey**). (**B**) PO treatment (**dotted**) decreased the survival rate of *T. molitor* infected with *pmt* mutant (**green**) as well as those infected with the unselected control strain, C2 (**grey**). The mortality rate of *T. molitor* infected with *pmt-rpo* (**red**) and *nsa* (**blue**) mutants was not affected by PO knockdown.
Fig. 4	(**A**) Bacterial load of *Staphylococcus aureus* harbouring mutation in *nsa&rpo* (**purple**) operon. The PO knockdown (**dotted**) decreased colonization of AMP-resistant *S. aureus* by day 7, unlike the unselected control C3 (**grey**). (**B**) PO treatment (**dotted**) increased the survival rate of *T. molitor* infected with *nsa-rpo* mutant (**purple**) while decreased those infected with the unselected control strain, C3 (**grey**).

Fig. 5	(**A**) Bacterial load of the ancestor *Staphylococcus aureus* strain (SH1000). The T1T2T4 knockdown (**dotted**) did not affect *in vivo* colonization (**black**) at any time point. (**B**) Survival of *T. molitor* infected with ancestor strain (**black**) decreased by T1T2T4 knockdown, while the sham infected (**grey**) did not vary significantly by knockdown treatment.
Fig. 6	(**A**) Bacterial load of *Staphylococcus aureus* harbouring a mutation in *rpo* (**yellow**) operon. The T1T2T4 knockdown (**dotted**) did not affect colonization of AMP-resistant *S. aureus* by day 7, unlike the unselected control C1 (**grey**) which increased significantly. (**B**) T1T2T4 knockdown treatment (**dotted**) increased the survival rate of *T. molitor* infected with *rpo* mutant (**yellow)** in contrary to those infected with the unselected control strain, C1 (**grey**).
Fig. 7	(**A**) Bacterial load of *Staphylococcus aureus* harbouring a mutation in the *pmt* (**green**)*, pmt&rpo* (**red**) or *nsa* (**blue**) operon. The T1T2T4 knockdown (**dotted**) increased colonization of AMP-resistant *S. aureus* by day 7 for the *pmt* mutant and the unselected control C2 (**grey**). (**B**) T1T2T4 knockdown treatment (**dotted**) decreased the survival rate of *T. molitor* infected with the unselected control strain, C2 (**grey**). The mortality rate of *T. molitor* infected with *pmt* (**green**)*, pmt-rpo* (**red**) and *nsa* (**blue**) mutants was not by T1T2T4 knockdown.
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III. Chapter 3

El Shazely, B., Yu, G., Johnston, P. R., & Rolff, J. (2020). Resistance Evolution Against Antimicrobial Peptides in *Staphylococcus aureus* Alters Pharmacodynamics Beyond the MIC. *Front Microbiol, 11*(103), 103. doi:10.3389/fmicb.2020.00103

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11. List of tables

I. Chapter 1 (El Shazely et al., 2019)

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12. List of abbreviations

µl	M**icrolitre(s)**
µM	**m**icromolar
Abbreviation	Stands for
ACB	**A**nti-**c**oagulant **b**uffer
AMP	A**ntim**i**crobial** p**eptide**
ATP-binding cassette (ABC) transporters	**A**denosine **t**ri**p**hosphate **b**inding **c**assette transporters
Bra	**B**acitracin **r**esistance-**a**ssociated two-component system (Nsa)
BSA	**B**ovine **s**erum **a**lbumin
Cat #	**Cat**alogue **number**
cDNA	**C**omplementary **d**eoxyribo**n**ucleic **a**cid
CFU	**C**olony-**f**orming **u**nit
Ct	**C**ycle quantification value at a **t**hreshold
df	**D**egrees of **f**reedom
Dscam	**D**own **s**yndrome **c**ell **a**dhesion **m**olecule
dsRNA	**D**ouble-**s**tranded **r**ibo**n**ucleic **a**cid
g	**G**ram(s)
g	**G**ravitational force (also known as RCF: revolutions per minute)
g/l	**G**ram(s) per **l**itre
glmm	**G**eneral **l**inear **m**ixed **m**odel
GNBPs	**G**ram-**n**egative **b**inding **p**roteins
h	**H**our(s)
Hz	**H**ertz
IgG	**I**mmuno**g**lobulin **G**
IMD pathway	**Im**mune **d**eficiency pathway
JAK-STAT	**Ja**nus **k**inase/**S**ignal **T**ransducer and **A**ctivator of **t**ranscription proteins
JNK	c-**J**un **N**-terminal **k**inase
KD	**K**nock**d**own
kDa	**K**ilo**Da**lton(s)
lmm	**L**inear **m**ixed **m**odel
LPS	**L**ipo**p**oly**s**accharides
Lys	**L**ysozyme
MAMPs	**M**icrobe-**a**ssociated **m**olecular **p**atterns
MCMC	**M**arkov **c**hain **M**onte **C**arlo
MH agar or broth	**M**űller **H**inton agar or broth
MIC	**M**inimum **i**nhibition **c**oncentration
min	**M**inute(s)
mRNA	**M**essenger **R**ibo**n**ucleic **a**cid
NF-κB	Nuclear **f**actor **k**appa-light-chain-enhancer of activated **B** cells
ng	**N**ano**g**ram(s)
ng/µl	**N**ano**g**ram(s) per **m**icro**l**itre(s)
ng/ml	**N**ano**g**ram(s) per **m**illi**l**itre(s)
nm	**N**ano**m**eter(s)
Nsa	**N**isin **s**usceptibility-**a**ssociated two-component system
OD	**O**ptical **d**ensity
PBS	**P**hosphate **b**uffer **s**aline
PBST	**P**hosphate **b**uffer **s**aline containing 0.1% **T**ween
PCR	**P**olymerase **c**hain **r**eaction
PGN	**P**eptido**g**lyca**n**s
PGRPs	**P**eptido**g**lycan **r**ecognition **p**roteins

pH	**P**ower of **h**ydrogen or **p**otential for **h**ydrogen
Pmt	**P**henol-soluble modulin transporter system
PO	**P**henol**o**xidase
PPO	**P**ro**p**henol**o**xidase
PRRs	**P**attern **r**ecognition **r**eceptors
PSM	**P**henol **s**oluble **m**odulins
qRTPCR	**Q**uantitative **r**eal-**t**ime **p**olymerase **c**hain **r**eaction
RF	**R**inger **fl**uid
rpm	**R**ound **p**er **m**inute
s and **sec**	**S**econd(s)
SR-C	**S**cavenger **R**eceptor Class **C**
T1	**T**enecin **1**
T2	**T**enecin **2**
T3	**T**enecin **3**
T4	**T**enecin **4**
$\mathbf{V_{max}}$	**Max**imum growth **v**elocity
Vra	**V**ancomycin-**r**esistance-**a**ssociated system
zMIC	Pharmacodynamic **m**inimum **i**nhibition **c**oncentration
ΔCt	**Delta Ct**
ΔΔ Ct	**Delta delta Ct**
κ	**Kappa**, the Hill coefficient, refer to the steepness (slope) of dose-response curve
$\mathbf{\Psi_{max}}$	**Psi max**, the maximal bacterial growth rate in the absence of antimicrobial
$\mathbf{\Psi_{min}}$	**Psi min**, the minimal bacterial growth rate at high concentrations of antimicrobial

13. Thesis outline

The herein study is a cumulative thesis consisting of three chapters. Chapter 1 and chapter 3 have been published in insect biochemistry and molecular biology and frontiers in microbiology, respectively. Chapter 2 will be submitted for publication. At the beginning of the thesis, a general introduction includes a literature review that starts the ball rolling and uttermost, a general discussion to the findings of the three chapters collectively.

Authors contribution

Manuscript 1:

El Shazely, B., Urbanski, A., Johnston, P. R., & Rolff, J. (2019). ***In vivo*** **exposure of insect AMP resistant *Staphylococcus aureus* to an insect immune system.** *Insect Biochem Mol Biol, 110*, 60-68. doi:10.1016/j.ibmb.2019.04.017

BE and JR conceived the study. BE carried out the experiments, led the statistical analysis and interpretation of the result. PJ help to troubleshoot methods related problems when found. UA led the in vitro phagocytosis assay and microscopical analysis. BE and JR wrote the manuscript.

Manuscript 2:

El Shazely, B. & Rolff, J. **Collateral sensitivity of experimentally evolved AMP resistant *Staphylococcus aureus* to host immune effectors.** In preparation

BE and JR conceived the study. BE carried out the experiments, led the statistical analysis, interpreted the results and wrote the current draft. JR revised the manuscript.

Manuscript 3:

El Shazely, B., Yu, G., Johnston, P. R., & Rolff, J. (2020). **Resistance Evolution Against Antimicrobial Peptides in *Staphylococcus aureus* Alters Pharmacodynamics Beyond the MIC.** *Front Microbiol, 11*(103), 103. doi:10.3389/fmicb.2020.00103

BE, GY, and JR conceived the study. BE carried out the experiments and led the statistical analysis. PJ led the genomic sequencing. PJ and BE analyzed the genomic data. GY and BE carried out modelling of killing curves (MCMC predictions). BE and JR wrote the manuscript.

List of publications

- **B. El Shazely**, J. Rolff (in prep) "Collateral sensitivity of experimentally evolved AMP resistant *Staphylococcus aureus* to host immune effectors"

- A. Rodriguez-Rojas, A. Nath, **B. El Shazely**, J.J. Kim, G. Santi, B. Kuropka, C. Weise, J. Rolff (2020) "Antimicrobial peptide induced-stress renders *Staphylococcus aureus* susceptible to toxic nucleoside analogues" Front. Immunol. - Microbial Immunology. (Accepted 24.06.2020)

- **B. El Shazely**, G. Yu, P. Johnston, J. Rolff (2020) "Resistance evolution against antimicrobial peptides in *Staphylococcus aureus* alters pharmacodynamics beyond the MIC" Front Microbiol. 11(103). DOI: 10.3389/fmicb.2020.00103

- **B. El Shazely**, A. Urbański, P. Johnston, J. Rolff (2019) "*In vivo* exposure of insect AMP resistant *Staphylococcus aureus* to an insect immune system" Insect Biochem Mol Biol,110: 60-68. DOI: 10.1016/j.ibmb.2019.04.017

- O. Makarova, P. Johnston, A. Rodriguez Rojas, **B. El Shazely**, J.M. Morales and J. Rolff (2018) "Genomics of experimental adaptation of *Staphylococcus aureus* to a natural combination of insect antimicrobial peptides" Sci Rep, 8:15359. DOI:10.1038/s41598-018-33593-7

- **B. El Shazely**, V. Veverka, V. Fucˇı´K, Z. Voburka, J. Zˇ Dˇ A´ Rek and V.Cˇerˇovsky´ (2013) "Lucifensin II, a Defensin of Medicinal Maggots of the Blowfly *Lucilia cuprina* (Diptera: Calliphoridae) " J. Med. Entomol. 50(3): 571-578. DOI: 10.1603/ME12208.

- V. Čeřovský, **B. El Shazely**, V. Fučík, Z. Voburka, J. Ņďárek, J. Slaninová, T. Chrudimská, L. Grubhoffer (2012)" The study of defensins of biomedical importance isolated from arthropods" Proceedings of the 32nd European Peptide Symposium 190-191

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Declaration

Hereby I confirm that I have prepared my doctoral degree thesis independently and without any impermissible help. All direct or indirect sources used are given as references. Contributions of co-authors are stated and acknowledged.

Berlin July 1st 2020 **Signature**

Baydaa Mohamed Ahmed El Shazely