







# *In Vitro* Evaluation of Antimicrobial Peptides from the Black Soldier Fly (*Hermetia Illucens*) against a Selection of Human Pathogens

 Laurence Van Moll,<sup>a,b,c</sup>  Jeroen De Smet,<sup>b,c</sup> Anne Paas,<sup>d,f</sup> Dorothee Tegtmeier,<sup>d</sup> Andreas Vilcinskas,<sup>d,e,f</sup>  Paul Cos,<sup>a</sup>  
 Leen Van Campenhout<sup>b,c</sup>

<sup>a</sup>Laboratory for Microbiology, Parasitology and Hygiene (LMPH), Faculty of Pharmaceutical, Biomedical and Veterinary Sciences, University of Antwerp, Antwerp, Belgium

<sup>b</sup>Department of Microbial and Molecular Systems (M<sup>2</sup>S), Research Group for Insect Production and Processing, KU Leuven, Geel, Belgium

<sup>c</sup>Leuven Food Science and Nutrition Research Centre (LForCe), KU Leuven, Leuven, Belgium

<sup>d</sup>Fraunhofer Institute for Molecular Biology and Applied Ecology, Branch for Bioresources, Gießen, Germany

<sup>e</sup>Institute for Insect Biotechnology, Justus Liebig University of Gießen, Gießen, Germany

<sup>f</sup>LOEWE Centre for Translational Biodiversity Genomics (LOEWE-TBG), Frankfurt am Main, Germany

**ABSTRACT** Antimicrobial peptides (AMPs) are being explored as alternatives to traditional antibiotics to combat the rising antimicrobial resistance. Insects have proven to be a valuable source of new, potent AMPs with large structural diversity. For example, the black soldier fly has one of the largest AMP repertoires ever recorded in insects. Currently, however, this AMP collection has not yet undergone antimicrobial evaluation or in-depth *in vitro* characterization. This study evaluated the activity of a library of 36 black soldier fly AMPs against a panel of human pathogens (*Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*, and *Aspergillus fumigatus*) and a human cell line (MRC5-SV2). The activity profile of two cecropins (Hill-Cec1 and Hill-Cec10) with potent Gram-negative activity, was further explored by characterizing their hemolysis, time-to-kill kinetics, membrane-permeabilization properties, and anti-biofilm activity. Hill-Cec1 and Hill-Cec10 also showed high activity against other bacterial species, including *Klebsiella pneumoniae* and multi-drug resistant *P. aeruginosa*. Both AMPs are bactericidal and have a rapid onset of action with membrane-permeabilizing effects. Hill-Cec1 and Hill-Cec10 were also able to prevent *P. aeruginosa* biofilm formation, but no relevant effect was seen on biofilm eradication. Overall, Hill-Cec1 and Hill-Cec10 are promising leads for new antimicrobial development to treat critical infections caused by Gram-negative pathogens such as *P. aeruginosa*.

**IMPORTANCE** With the ever growing antimicrobial resistance, finding new candidates for antimicrobial drug development is indispensable. Antimicrobial peptides have steadily gained attention as alternatives for conventional antibiotics, due to some highly desirable characteristics, such as their low propensity for resistance development. With this article, we aim to upgrade the knowledge on the activity of black soldier fly antimicrobial peptides and their potential as future therapeutics. To achieve this, we have evaluated for the first time a library of 36 synthetically produced peptides from the black soldier fly against a range of human pathogens and a human cell line. Two selected peptides have undergone additional testing to characterize their antimicrobial profile against *P. aeruginosa*, a clinically important Gram-negative pathogen with a high established resistance. Overall, this research has contributed to the search for new peptide drug leads to combat the rising antimicrobial resistance.

**KEYWORDS** *Hermetia illucens*, *Pseudomonas aeruginosa*, antimicrobial agents, antimicrobial peptides, Gram-negative bacteria

**Editor** Neha Garg, Georgia Institute of Technology

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Address correspondence to Leen Van Campenhout, leen.vancampenhout@kuleuven.be.

The authors declare no conflict of interest.

**Received** 27 September 2021

**Accepted** 6 December 2021

**Published** 5 January 2022

In the search for new antimicrobials to counteract the rising drug resistance, antimicrobial peptides (AMPs) have gathered a substantial amount of interest. Ever since the discovery of cecropin from the pupae of the silk moth *Hyalophora cecropia* in 1980 (1), insects have steadily gained attention as AMP producers (2–4). Currently, the Antimicrobial Peptide Database reports on 324 AMPs from insect origin, which is the largest repertoire among all animal classes (4, 5). AMPs are small, evolutionary conserved peptides with antimicrobial activity (6). In insects, they are the main effector molecules of the innate immune system and increase their resistance to bacterial infections (2). It is suggested that exposure to pathogens in the insect's environmental niche is a driving factor behind the evolutionary adaptation of the size and diversity of their AMP repertoire (4, 7).

An insect with an AMP repertoire of remarkable size is the black soldier fly (BSF). The BSF (*Hermetia illucens*, Diptera: Stratiomyidae) expresses over 50 genes encoding putative AMPs, a number that so far has only been recorded for the harlequin *Harmonia axyridis* (8, 9). The larvae of the BSF are saprophagous, feeding preferably on decaying organic matter, including food and agricultural waste, manure, and animal and plant remains (10, 11). Their expansive AMP gene collection has been linked to their survival in these substrates with a high microbial load (8). Apart from their role as defenders against infections, AMPs are also involved in maintaining and shaping the bacterial gut community of the BSF (8, 12). For example, AMP expression in the BSF is diet-dependent and adapts both the feed or substrate microbiota as well as the gut microbiota to allow flexible digestion of the wide range of substrates they encounter in their environment (8, 13). Overall, the use of BSF AMPs could be exploited beyond antimicrobial drug development to applications in the industrial insect farming sector. For instance, AMP addition could help eliminate food pathogens in the insect rearing cycle or stimulate bioconversion of organic waste by *H. illucens* larvae (8). This study, however, focuses on the potential use of AMPs in the development of novel antimicrobial drugs.

To date, the antimicrobial activity of 14 different BSF AMPs has been confirmed *in vitro*, most of these being defensin AMPs (Table 1) (14–22). However, a detailed characterization of their antimicrobial activity is often missing. So far, no *in vitro* antimicrobial evaluation of a full library of BSF AMPs has been performed, leaving much potential of BSF AMPs to be uncovered. This study aims to upgrade the knowledge on the antimicrobial properties of BSF AMPs. This is done first by evaluating a large library of 36 synthetically produced AMPs against a range of pathogenic organisms, and in second by carrying out a deeper *in vitro* characterization of the antimicrobial profile of a selection of two peptides against *Pseudomonas aeruginosa*.

## RESULTS

**Antimicrobial activity of the BSF peptide library.** To identify antimicrobial activity of the AMPs, a screening against one Gram-positive, two Gram-negative, and two fungal species was performed (Table 2, Table S2). One peptide (Hill-Stom2) was not included in the screening due to poor dimethyl sulfoxide (DMSO) solubility. Among the tested peptides, the most promising activity was found within the cecropin family of AMPs. Aside from one peptide (Hill-Cec6), all cecropins showed activity against *Escherichia coli* and *P. aeruginosa* at low micromolar concentrations, with minimum inhibitory concentrations (MIC) values ranging from 0.50  $\mu\text{M}$  to 2  $\mu\text{M}$ . Cecropins are  $\alpha$ -helical AMPs without cysteine residues and  $\beta$ -sheet motifs (2, 3). Potent activity of insect cecropins with a strict Gram-negative spectrum has been reported for other insect species earlier, such as for *Lucilia sericata* (23, 24). One of the earlier described BSF cecropins (CLP1) has a 95% structure homology with the closest related AMP of our library (Hill-Cec2), and exhibits antibacterial activity in the same concentration range (17). Next, one dipterin (Hill-Dip6) also showed activity against *E. coli* (MIC of 2  $\mu\text{M}$ ), but activity against *P. aeruginosa* was not recorded. Antibacterial activity against *Staphylococcus aureus* was reported for three defensins (Hill-Def2a, Hill-Def2b, and Hill-

**TABLE 1** Overview of antimicrobial peptides of the black soldier fly with *in vitro* verified antimicrobial activity

AMP	AMP family	Study	Activity against	Strength of activity (MIC) <sup>a</sup>
DLP4	Defensin	Park et al. (2015)	MRSA <sup>b</sup>	0.59 to 1.17 $\mu$ M
			<i>S. aureus</i> KCCM 40881	0.59 to 1.17 $\mu$ M
			<i>S. aureus</i> KCCM 12256	1.17 to 2.34 $\mu$ M
			<i>S. epidermidis</i> KCCM 35494	0.59 to 1.17 $\mu$ M
			<i>B. subtilis</i> KCCM 11316	0.02 to 0.04 $\mu$ M
		Li et al. (2017)	<i>S. aureus</i> ATCC 25923	0.01 $\mu$ M
			<i>S. aureus</i> ATCC 43300	0.23 $\mu$ M
			<i>S. aureus</i> ATCC 6538	0.47 $\mu$ M
			<i>S. aureus</i> CICC 546	0.47 $\mu$ M
			<i>S. suis</i> CVCC 606	1.88 $\mu$ M
		Li et al. (2020)	<i>L. ivanovii</i> ATCC 19119	0.12 $\mu$ M
			<i>S. aureus</i> CVCC 546	3.75 $\mu$ M
			<i>S. epidermis</i> ATCC 12228	14.99 $\mu$ M
DLP2	Defensin	Li et al. (2017)	<i>S. aureus</i> ATCC 25923	0.01 $\mu$ M
			<i>S. aureus</i> ATCC 43300	0.12 $\mu$ M
			<i>S. aureus</i> ATCC 6538	0.12 $\mu$ M
			<i>S. aureus</i> CICC 546	0.23 $\mu$ M
			<i>S. suis</i> CVCC 606	0.93 $\mu$ M
			<i>L. ivanovii</i> ATCC 19119	0.12 $\mu$ M
			Park et al. (2017)	MRSA <sup>b</sup>
		<i>S. aureus</i> KCCM 40881		5 $\mu$ g/mL
		<i>S. aureus</i> KCCM 12256		10 $\mu$ g/mL
		<i>S. epidermis</i> KCCM 25494		10 $\mu$ g/mL
		<i>E. coli</i> KCCM 11234		10 $\mu$ g/mL
		<i>P. aeruginosa</i> KCCM 11328		40 $\mu$ g/mL
		ID13	Defensin	Li et al. (2020)
<i>S. epidermis</i> ATCC 12228	1.91 $\mu$ M			
<i>S. pneumoniae</i> CVCC 2350	0.95 $\mu$ M			
<i>S. suis</i> CVCC 3928	0.95 $\mu$ M			
CLP1	Cecropin	Park et al. (2017)	<i>E. coli</i> KCCM 11234	0.52 to 1.03 $\mu$ M
			<i>E. aerogenes</i> KCCM 12177	1.03 to 2.07 $\mu$ M
			<i>P. aeruginosa</i> KCCM 11328	1.03 to 2.07 $\mu$ M
Trx-stomoxynZH1a	Cecropin	Elhag et al. 2016)	<i>E. coli</i>	15 to 30 $\mu$ g/mL
			<i>S. aureus</i>	27 to 54 $\mu$ g/mL
HI-attacin	Attacin	Shin et al. (2019)	<i>E. coli</i> KCCM 11234	No MIC given
			MRSA <sup>b</sup>	No MIC given
HiCG13551	IATP	Xu et al. (2020)	<i>E. coli</i>	No MIC given
			<i>S. aureus</i>	No MIC given
			<i>S. pneumoniae</i>	No MIC given
Hidefensin-1	Defensin	Xu et al. (2020)	<i>E. coli</i>	No MIC given
Hidiptericin-1	Diptericin	Xu et al. (2020)	<i>E. coli</i> <i>S. pneumoniae</i>	No MIC given No MIC given
Hill_BB_C6571	Defensin	Moretta et al. (2020)	<i>E. coli</i>	No MIC given
Hill_BB_C16634	Defensin	Moretta et al. (2020)	<i>E. coli</i>	No MIC given
Hill_BB_C46948	Defensin	Moretta et al. (2020)	<i>E. coli</i>	No MIC given
Hill_BB_C7985	Defensin	Moretta et al. (2020)	<i>E. coli</i>	No MIC given

<sup>a</sup>MIC, minimum inhibitory concentration.<sup>b</sup>MRSA, methicillin-resistant *Staphylococcus aureus*.

Def4) but was absent for all other AMPs. Interestingly, the free cysteine residues of Hill-Def2a seem crucial for its potent antibacterial activity, as its counterpart with disulfide bridges (Hill-Def2b) needed much higher concentrations (32  $\mu$ M and 64  $\mu$ M) for 90% growth inhibition of *S. aureus*. Finally, no peptide showed signs of activity against *Aspergillus fumigatus* and *Candida albicans* within the tested concentration ranges.

**TABLE 2** IC<sub>50</sub> values (concentrations causing 50% growth inhibition of the pathogen), MIC values, and MBC values obtained in two individual screenings: the first screening starting at 64 μM, the second screening starting at 32 μM<sup>a</sup>

AMP	Repeat	IC <sub>50</sub> (μM)										MIC (μM)										MBC (μM)									
		MRC5-SV2	<i>S. aureus</i> ATCC 6538	<i>E. coli</i> ATCC 8739	<i>P. aeruginosa</i> ATCC 9027	<i>C. albicans</i> B59630	<i>A. fumigatus</i> B42928	<i>S. aureus</i> ATCC 6538	<i>E. coli</i> ATCC 8739	<i>P. aeruginosa</i> ATCC 9027	<i>S. aureus</i> ATCC 6538	<i>E. coli</i> ATCC 8739	<i>P. aeruginosa</i> ATCC 9027	<i>S. aureus</i> ATCC 6538	<i>E. coli</i> ATCC 8739	<i>P. aeruginosa</i> ATCC 9027	<i>S. aureus</i> ATCC 6538	<i>E. coli</i> ATCC 8739	<i>P. aeruginosa</i> ATCC 9027												
Hill-Knot2	1	9.73	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00												
	2	3.76	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00												
Hill-Knot3	1	>64.00	>64.00	>64.00	22.63	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00												
	2	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00												
Hill-Def2a	1	>64.00	<0.25	35.33	32.86	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	0.25	>64.00	>64.00	>64.00	>64.00	>64.00	static												
	2	>32.00	0.19	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	2.00	>32.00	>32.00	>32.00	>32.00	>32.00	static												
Hill-Def4	1	>64.00	0.50	>64.00	32.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	1.00	>64.00	>64.00	>64.00	>64.00	>64.00	static												
	2	>32.00	0.70	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	8.00	>32.00	>32.00	>32.00	>32.00	>32.00	static												
Hill-Def2b	1	>64.00	8.50	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	64.00	>64.00	>64.00	>64.00	>64.00	>64.00	static												
	2	>32.00	13.45	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	32.00	>32.00	>32.00	>32.00	>32.00	>32.00	static												
Hill-Cec1	1	>64.00	>64.00	<0.25	<0.25	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	1.00												
	2	>32.00	>32.00	0.21	0.20	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	0.25	>32.00	>32.00	>32.00	>32.00	0.50												
Hill-Cec2	1	>64.00	>64.00	0.50	0.93	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	1.00	>64.00	>64.00	>64.00	>64.00	1.00												
	2	>32.00	4.36	>32.00	0.76	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	2.00	>32.00	>32.00	>32.00	>32.00	2.00												
Hill-Cec3	1	>64.00	>64.00	0.49	0.40	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	1.00	>64.00	>64.00	>64.00	>64.00	1.00												
	2	>32.00	>32.00	0.22	0.79	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	0.50	>32.00	>32.00	>32.00	>32.00	32.00												
Hill-Cec4	1	37.37	>64.00	0.50	<0.25	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	1.00	>64.00	>64.00	>64.00	>64.00	1.00												
	2	>32.00	>32.00	0.32	0.22	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	2.00	>32.00	>32.00	>32.00	>32.00	2.00												
Hill-Cec5	1	>64.00	>64.00	0.50	2.61	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	1.00	>64.00	>64.00	>64.00	>64.00	static												
	2	>32.00	>32.00	0.23	3.60	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	0.50	>32.00	>32.00	>32.00	>32.00	32.00												
Hill-Cec7	1	>64.00	>64.00	0.38	0.40	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	1.00	>64.00	>64.00	>64.00	>64.00	1.00												
	2	>32.00	>32.00	0.32	0.78	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	2.00	>32.00	>32.00	>32.00	>32.00	2.00												
Hill-Cec8	1	>64.00	>64.00	0.45	1.29	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	1.00	>64.00	>64.00	>64.00	>64.00	1.00												
	2	>32.00	>32.00	0.68	1.73	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	2.00	>32.00	>32.00	>32.00	>32.00	8.00												
Hill-Cec9	1	>64.00	>64.00	0.43	0.38	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	1.00	>64.00	>64.00	>64.00	>64.00	static												
	2	>32.00	>32.00	0.21	0.88	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	0.50	>32.00	>32.00	>32.00	>32.00	2.00												
Hill-Cec10	1	>64.00	32.74	<0.25	<0.25	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	0.25	>64.00	>64.00	>64.00	>64.00	16.00												
	2	>32.00	>32.00	0.22	0.22	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	0.50	>32.00	>32.00	>32.00	>32.00	32.00												
Hill-Cec11	1	>64.00	>64.00	<0.25	<0.25	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	1.00	>64.00	>64.00	>64.00	>64.00	4.00												
	2	>32.00	>32.00	0.22	0.24	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	0.50	>32.00	>32.00	>32.00	>32.00	8.00												
Hill-Cec12	1	>64.00	>64.00	<0.25	<0.25	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	0.25	>64.00	>64.00	>64.00	>64.00	static												
	2	19.45	>32.00	0.32	0.23	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	2.00	>32.00	>32.00	>32.00	>32.00	8.00												
Hill-Cec13	1	>64.00	>64.00	<0.25	<0.25	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	1.00	>64.00	>64.00	>64.00	>64.00	64.00												
	2	>32.00	>32.00	0.23	0.25	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	0.50	>32.00	>32.00	>32.00	>32.00	2.00												

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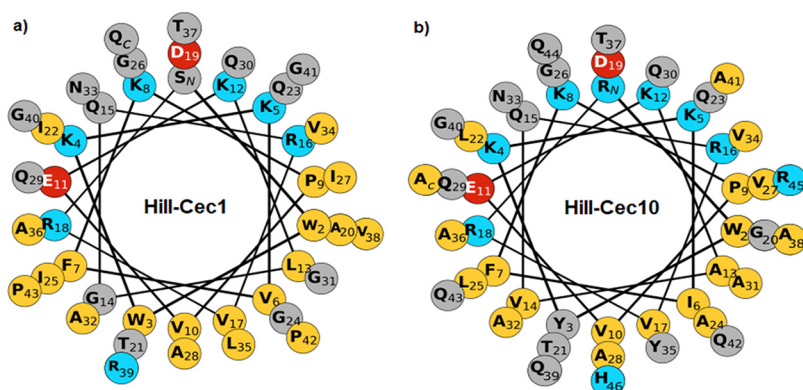
**TABLE 2** (Continued)

AMP	Repeat	IC <sub>50</sub> (μM)						MIC (μM)						MBC (μM)		
		MRC5-SV2	<i>S. aureus</i> ATCC 6538	<i>E. coli</i> ATCC 8739	<i>P. aeruginosa</i> ATCC 9027	<i>C. albicans</i> B59630	<i>A. fumigatus</i> B42928	<i>S. aureus</i> ATCC 6538	<i>E. coli</i> ATCC 8739	<i>P. aeruginosa</i> ATCC 9027	<i>S. aureus</i> ATCC 6538	<i>E. coli</i> ATCC 8739	<i>P. aeruginosa</i> ATCC 9027	<i>S. aureus</i> ATCC 6538	<i>E. coli</i> ATCC 8739	<i>P. aeruginosa</i> ATCC 9027
Hill-Stom1	1	<b>11.65</b>	<b>58.58</b>	>64.00	<b>16.00</b>	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00
	2	<b>24.42</b>	>32.00	>32.00	<b>17.67</b>	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00
Hill-Dip6	1	<b>4.54</b>	>32.00	<b>0.85</b>	>32.00	>64.00	>64.00	>64.00	>64.00	<b>2.00</b>	>32.00	>32.00	>64.00	<b>2.00</b>	>32.00	static
	2	<b>7.73</b>	>32.00	<b>1.00</b>	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>64.00	>32.00	<b>2.00</b>	8.00	8.00

<sup>a</sup>Peptides with activity < 32 μM against either the cell line (MRC5-SV2) or the microbial reference strains are shown. Static, bacteriostatic activity. Values in bold text indicate that antimicrobial activity could be measured within the tested concentration ranges for the AMP.







**FIG 2** (a) Helical wheel projection of Hill-Cec1. (b) Helical wheel projection of Hill-Cec10. Projections were constructed using the online Galaxy CPT software available at <https://cpt.tamu.edu/galaxy-pub>. Polar residues with a positive charge are indicated in blue, negatively charged polar residues are red. Uncharged polar amino acids are indicated in gray, and hydrophobic residues have a yellow color.

*uginosa* ATCC 15442, an environmental strain (29), were also susceptible to the cecropins. The activity of Hill-Cec1 was comparable with its activity against the non-virulent test strain *P. aeruginosa* ATCC 9027 (30). The MIC of Hill-Cec10, however, increased by a factor 2 to 4. Hill-Cec10 also required notably higher concentrations for bactericidal activity. Additionally, Hill-Cec1 and Hill-Cec10 were both highly active against *K. pneumoniae*, another species known to cause critical lung infections (31), with MIC values between 0.25 and 0.5  $\mu\text{M}$  for Hill-Cec1 and between 0.5 and 1  $\mu\text{M}$  for Hill-Cec10. No activity against *Burkholderia cenocepacia* or *Mycobacterium tuberculosis* was found within the tested concentration range.

**Hemolysis analysis.** In addition to the primary toxicity screening against human fibroblasts, a hemolytic assay was performed using human red blood cells. The outer membrane leaflets of red blood cells are rich in sialic acid residues, which gives them a lower negative charge than other mammalian cell types (32, 33). This negative charge makes them prone to interactions with the positively charged AMPs. Hence, a hemolysis analysis is often routinely included in AMP research. Apart from cationicity, hydrophobicity is also positively correlated with the hemolytic capacity of AMPs (34). Both cecropins showed hemolysis of less than 10% at the highest concentration tested of

**TABLE 3** IC<sub>50</sub> values (concentrations leading to 50% growth inhibition of the pathogen), MIC values (needed for visual absence of bacterial growth), and MBC values obtained in two individual screenings of the selected cecropins Hill-Cec1 and Hill-Cec10 performed at concentrations starting at 32  $\mu\text{M}$

Strain	Repeat	IC <sub>50</sub> ( $\mu\text{M}$ )		MIC ( $\mu\text{M}$ )		MBC ( $\mu\text{M}$ )	
		Hill-Cec1	Hill-Cec10	Hill-Cec1	Hill-Cec10	Hill-Cec1	Hill-Cec10
<i>E. coli</i>	1	0.15	0.73	0.5	1	0.5	1
ATCC 8739	2	0.15	0.63	0.25	1	0.25	32
<i>P. aeruginosa</i>	1	0.64	0.75	1	1	1	8
ATCC 9027	2	0.37	0.75	0.5	2	16	16
<i>P. aeruginosa</i>	1	0.77	2.48	1	8	4	16
ATCC 15692 (PAO1)	2	0.39	0.76	0.5	2	0.5	16
<i>P. aeruginosa</i>	1	1.34	2.96	2	4	4	32
LMG 27650 (MDR) <sup>a</sup>	2	0.66	3.35	1	8	16	16
<i>P. aeruginosa</i> ATCC 15442	1	0.73	2.82	1	4	4	16
	2	0.38	2.71	1	4	8	32
<i>K. pneumoniae</i>	1	0.36	0.75	0.5	1	2	4
ATCC 13883	2	0.18	0.31	0.25	0.5	4	8
<i>B. cenocepacia</i>	1	>32	>32	>32	>32		
LMG 16656	2	>32	>32	>32	>32		
<i>M. tuberculosis</i>	1	>32	>32	>32	>32		
ATCC 25177 (H37Ra)	2	>32	>32	>32	>32		

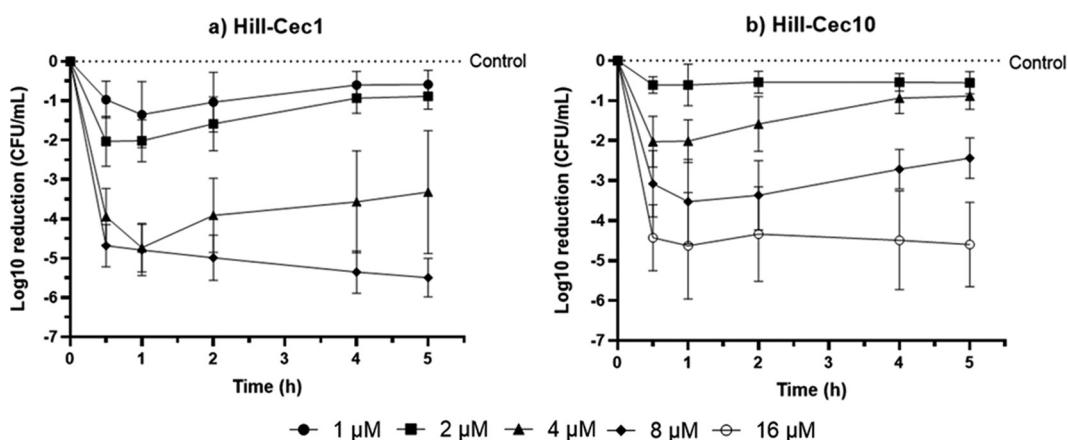
<sup>a</sup>MDR, multi-drug-resistant.

64  $\mu\text{M}$ . These results predict low hemolysis *in vivo*. However, results will need to be confirmed later on *in vivo*, as the experimental conditions differ from the protein-rich environment of whole blood (35). Results of the hemolysis analysis were in line with the bio-informatical predictions of HemoPred which identified the two cecropins as “nonhemolytic” (36).

**Time-to-kill analysis.** To investigate the onset of action and the bactericidal activity of the peptides, a time-to-kill analysis was performed. Both cecropins have a rapid onset of action and cause growth inhibition of *P. aeruginosa* within 30 min (Fig. 3a and b). To obtain bactericidal activity ( $\geq 3\text{-log}_{10}$  reduction compared with the growth control), both AMPs require at least a concentration of four times their MIC values. At 4  $\mu\text{M}$  ( $4 \times \text{MIC}$ ), Hill-Cec1 showed a rapid bactericidal activity, leading to a  $\log_{10}$  reduction of  $4.74 \pm 0.55$  after 1 h. However, afterwards there was a clear increase in *P. aeruginosa* growth, and at 5 h, Hill-Cec1 had variable activity throughout the different experiments, causing on average a  $3.32 \pm 1.40 \log_{10}$  reduction. In contrast, a higher Hill-Cec1 concentration of 8  $\mu\text{M}$  ( $8 \times \text{MIC}$ ) consistently achieved bactericidal activity at all time points tested, leading to a  $\log_{10}$  reduction in bacteria of  $5.50 \pm 0.44$  after 5 h. For Hill-Cec10, a similar pattern was observed. The highest concentration of 16  $\mu\text{M}$  ( $8 \times \text{MIC}$ ) always caused at least a 3- $\log_{10}$  reduction of bacteria, although the exact amount of bacterial killed varied throughout the independent experiments, leading to a  $\log_{10}$  reduction of  $4.60 \pm 0.94$  after 5 h. At 8  $\mu\text{M}$  ( $4 \times \text{MIC}$ ), the AMP was bactericidal up until 1 h, but was not able to sustain this killing effect for the next time points tested ( $\log_{10}$  reduction of  $2.44 \pm 0.46$  after 5 h).

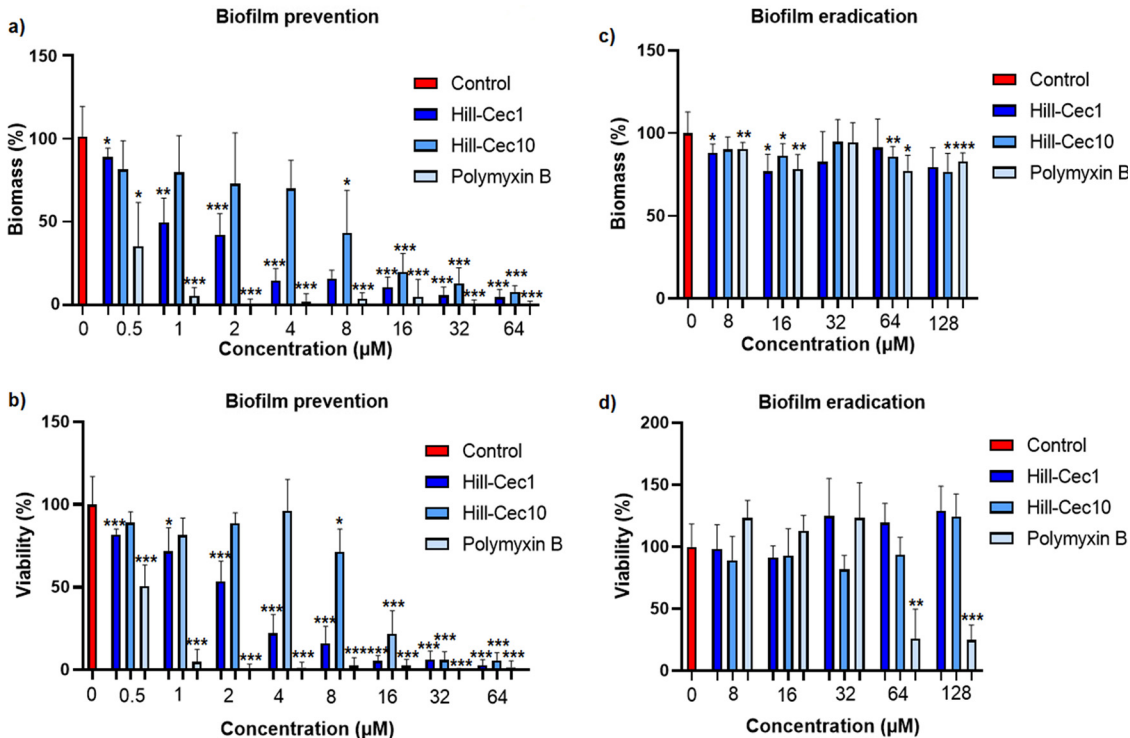
Important to mention is the variability in bactericidal activity noticed in the time-to-kill experiments. This variability was also seen in our earlier MBC experiments, and is suspected to be caused by natural variation in the peptides' MIC and MBC values in-between experiments.

**Inhibition of biofilm formation.** As biofilm formation of *P. aeruginosa* strains contributes to their tolerance and resistance to antibiotics (37), effects of the cecropin AMPs on biofilm formation was investigated. *P. aeruginosa* ATCC 15442 was used as it showed less variability in biofilm formation in our assay than *P. aeruginosa* PAO1, as observed in preliminary experiments (data not shown). Both peptides exhibited a concentration-dependent inhibition of biofilm formation. For Hill-Cec1, a statistically significant decrease, compared with the untreated control, of biofilm mass ( $P < 0.05$ ) and viability ( $P < 0.001$ ) at sub-MICs (0.5  $\mu\text{M}$ ) was noted. Hill-Cec1 achieved 50% reduction in biofilm mass at a concentration close to its MIC ( $\text{IC}_{50}$  of  $1.3 \pm 0.57 \mu\text{M}$ ), and 50% reduction of biofilm viability at supra-MICs ( $\text{IC}_{50}$  of  $2.1 \pm 0.52 \mu\text{M}$ ) (Fig. 4a). For Hill-Cec10, however, higher concentrations were needed to obtain 50% reduction or more of biofilm mass ( $\text{IC}_{50}$  of  $7.5 \pm 3.5 \mu\text{M}$ ) or biofilm viability ( $\text{IC}_{50}$  of  $11 \pm 1.7 \mu\text{M}$ ) (Fig. 4b).



**FIG 3** Time-to-kill curves showing the  $\log_{10}$  reductions in *P. aeruginosa* ATCC 9027 caused by the selected cecropins. (a) Killing kinetics of Hill-Cec1 at 1  $\mu\text{M}$ , 2  $\mu\text{M}$ , 4  $\mu\text{M}$ , and 8  $\mu\text{M}$ . (b) Killing kinetics of Hill-Cec10 at 2  $\mu\text{M}$ , 4  $\mu\text{M}$ , 8  $\mu\text{M}$ , and 16  $\mu\text{M}$ . Graphs represent the mean of five independent experiments.



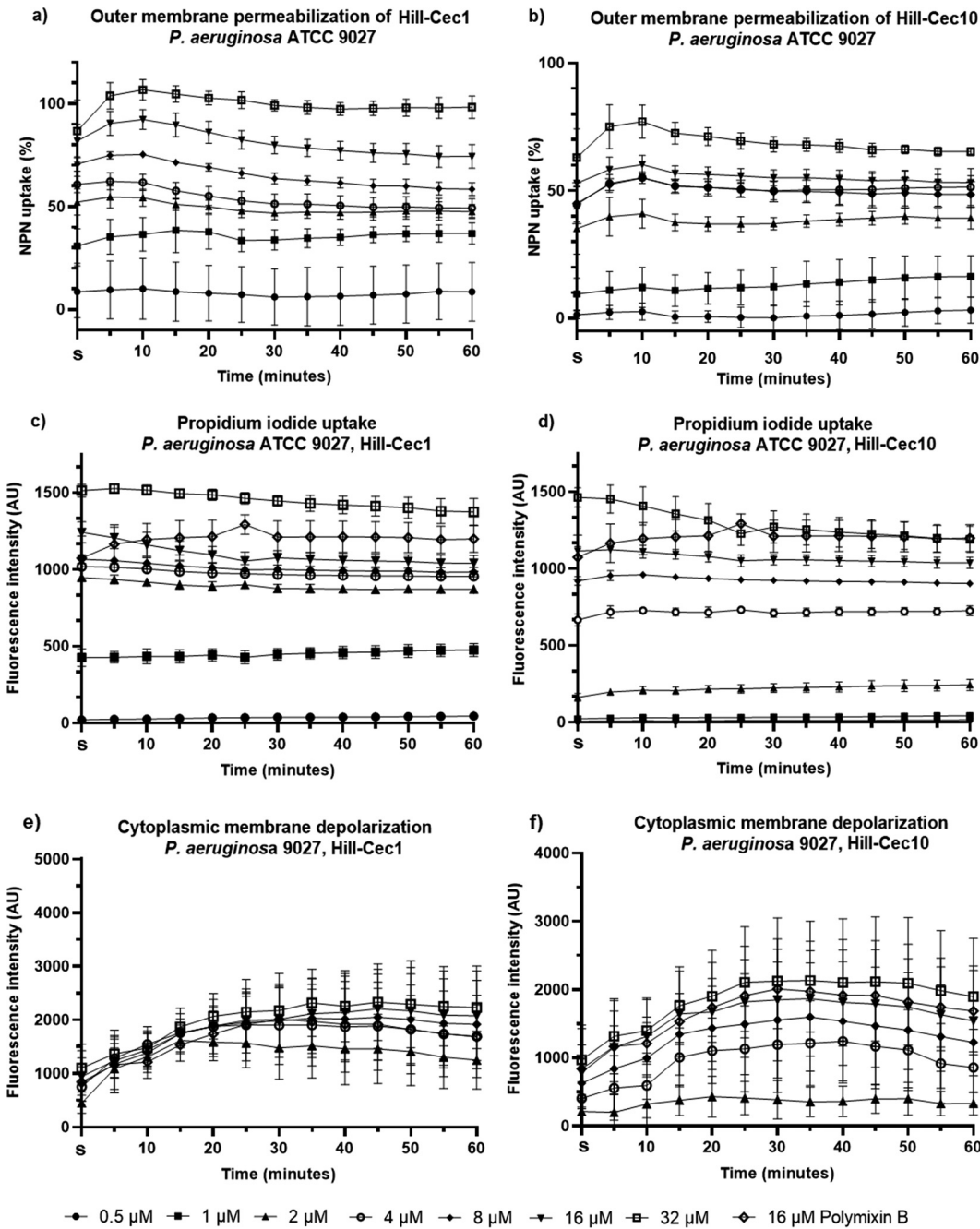


**FIG 4** Anti-biofilm activity of cecropins Hill-Cec1 and Hill-Cec10 against *P. aeruginosa* ATCC 15442. (a) Effect of Hill-Cec1 and Hill-Cec10 on *P. aeruginosa* biofilm formation as measured by biofilm mass. (b) Effect of Hill-Cec1 and Hill-Cec10 on *P. aeruginosa* biofilm formation as measured by biofilm viability. (c) Effect of Hill-Cec1 and Hill-Cec10 on preformed *P. aeruginosa* biofilms as measured by biofilm mass. (d) Effect of Hill-Cec1 and Hill-Cec10 on preformed *P. aeruginosa* biofilms as measured by biofilm viability. Bars represent the mean  $\pm$  SD of three independent experiments. \*,  $P \leq 0.05$ ; \*\*,  $P \leq 0.01$ ; \*\*\*,  $P \leq 0.001$ .

The difference in bactericidal concentrations between both cecropins could explain their difference in inhibition of biofilm formation.

**Biofilm eradication.** Hill-Cec1 and Hill-Cec10 were not able to eradicate a preformed *P. aeruginosa* biofilm. Only marginal reductions of biofilm mass were seen for Hill-Cec1 (between 9% and 23%) and Hill-Cec10 (between 10% and 23%), and the decrease was not concentration-dependent (Fig. 4c). No significant effect on biofilm viability compared with the untreated control was detected (Fig. 4d). In comparison, the reference polymyxin B, a peptide antibiotic, shows over 70% reduction in viability at concentrations of 64  $\mu\text{M}$  and 128  $\mu\text{M}$ .

**Membrane permeabilization and disruption.** Both cecropins are able to permeabilize the cell membranes of *P. aeruginosa*, as confirmed by the N-phenyl-naphthylamine (NPN) and propidium iodide (PI) uptake assays (Fig. 5). NPN, a hydrophobic probe, is normally excluded from bacterial membranes. It will, however, accumulate in the outer membrane (OM) of Gram-negative bacteria when the barrier is compromised, leading to an increase in fluorescence (38, 39). NPN uptake in the OM occurs within the first 5 min, after which the fluorescent signal stabilizes (Fig. 5a and b). The NPN uptake is concentration-dependent and reaches a maximum of 100% (compared with 16  $\mu\text{M}$  polymyxin B) at 32  $\mu\text{M}$  for Hill-Cec1, and a maximum of 75% at 32  $\mu\text{M}$  for Hill-Cec10. Supra-MICs are needed to reach 50% or more NPN uptake. Upon treatment with PI, a sharp increase in fluorescence is also seen immediately after exposure, after which the signal quickly stabilizes (Fig. 5c and d). PI is a DNA intercalating dye, which is not able to traverse intact bacterial membranes (40). When the membranes are permeabilized, PI is able to reach the cytoplasm where it binds to nucleic acids, which increases its fluorescent signal (38). PI fluorescence is therefore an indicator of inner membrane (IM) permeabilization. The PI uptake is concentration-dependent for both cecropins. At high concentrations (32  $\mu\text{M}$ ) the membrane permeabilization exceeds that of a high



**FIG 5** Membrane activity of Hill-Cec1 and Hill-Cec10. (a) Outer membrane (OM) permeabilization of *P. aeruginosa* caused by Hill-Cec1 measured with N-phenyl-naphthylamine (NPN). (b) OM permeabilization of *P. aeruginosa* caused by Hill-Cec10 measured with NPN. NPN uptake is expressed as a percentage of the maximal uptake recorded with a high dose (16 μM) of polymyxin B. (c) Fluorescence caused by propidium iodide (PI) uptake in *P. aeruginosa* after addition of Hill-Cec1. (d) Fluorescence caused by PI uptake in *P. aeruginosa* after addition of Hill-Cec10. Values were normalized with the negative control. (e) Fluorescent signal of 3,3'-dipropylthiadicarbocyanine iodide (diSC<sub>3</sub>(5)) as an indicator of cytoplasmic membrane depolarization of *P. aeruginosa* caused by Hill-Cec1. (f) Fluorescent signal of diSC<sub>3</sub>(5) as an indicator of cytoplasmic membrane depolarization of *P. aeruginosa* caused by Hill-Cec10. Fluorescent signals were normalized with the negative control. AU, arbitrary units; S, start of measurement immediately after dye exposure.

dose of polymyxin B (16 μM). The NPN and PI uptake assays show that Hill-Cec1 and Hill-Cec10 can permeabilize both the OM and IM of *P. aeruginosa*. The majority of AMPs with a characterized mechanism of action work by decreasing the integrity of the bacterial membranes through, for example, pore formation or complete membrane lysis (41). As the increase in fluorescence for both the NPN and PI uptake occurs rapidly

(within 5 min) and is present at non-lethal concentrations, the membrane permeabilization is likely directly linked to the mechanism of action of the BSF AMPs, and not a secondary effect of the bacteria dying through other, non-related intracellular mechanisms (42).

**Cytoplasmic membrane depolarization.** DiSC3(5), a voltage-sensitive cationic dye, was used to study cytoplasmic membrane depolarization. Under normal conditions, the dye is concentrated in the (hyper)polarized cytoplasmic membrane, leading to self-quenching of its fluorescence. Upon depolarization, however, diSC3(5) is released into the cytoplasm causing an increase in fluorescence (43). Both Hill-Cec1 and Hill-Cec10 cause cytoplasmic membrane depolarization of *P. aeruginosa*, as indicated by the increase in diSC3(5) fluorescence (Fig. 5e and f). The depolarization is largely concentration-dependent, although more outspoken for Hill-Cec10. At high concentrations, cytoplasmic depolarization exceeds that of polymyxin B (16  $\mu$ M), especially for Hill-Cec1. As the PI uptake assay showed that the cecropins permeabilize the IM, it is possible that the membrane depolarization is largely caused by membrane damage.

## DISCUSSION

**Biological function of cecropins in the black soldier fly.** As a decomposer of bio-waste, the BSF larva lives in close contact with potentially hazardous microorganisms (16). The evolutionary expansion and diversification of the BSF AMP repertoire could explain their successful colonization of these microbe-rich substrates (8). After a broad antimicrobial screening of a library of BSF peptides, we found the majority of the activity situated in the cecropin family. Apart from Hill-Cec6, they all showed strong activity against the Gram-negative test strains at low micromolar concentrations. Cecropin AMPs are widely distributed among the insect orders, and have so far been identified in the Coleoptera, Diptera, and Lepidoptera. It is suggested, however, that they are produced by all holometabolous insects with the exception of Hymenopteran species (4). The amount and diversity of cecropins found in these insects hints at more preserved functions of these AMPs. In the BSF, AMPs are involved in maintaining and shaping the bacterial gut communities and maintaining eubiosis in the gut (8, 12), but cecropins could have additional biological roles as well. For example, the *Aedes aegypti* cecropin B has been shown to be involved in the formation of the cuticle of adult mosquitoes (44). Knockdown of the cecropin B gene in the pupae led to high mortality, deformed adults, and impaired cuticle lamellae with disorganized chitin fibrils (44). It is suggested that cecropin B works through upregulating the expression of prophenoloxidasases, which are involved in cuticle formation. Prophenoloxidasases also play a crucial role in insect immunity. The enzymes are, for example, involved in the induction of the melanization process, which leads to encapsulation of invading pathogens (45, 46).

**Potential of black soldier fly cecropins in drug development.** The activity against Gram-negative pathogens of cecropins has made them compounds of interest for new antimicrobial drug development (41). In this study, we selected two cecropin AMPs, Hill-Cec1 and Hill-Cec10, for *in vitro* characterization. In line with other cecropins, they have an alpha-helical, amphiphatic domain and a strong positive net charge. As AMPs are known to be toxic to human cells due to unspecific membrane interactions, we studied their effect on lung fibroblasts and erythrocytes (35). Both peptides showed no signs of *in vitro* hemolysis or cytotoxicity. The antimicrobial activity of the cecropins was further explored, and apart from the *E. coli* and *P. aeruginosa* test strains, the cecropins were also active against other *P. aeruginosa* species, including a multidrug-resistant strain, and *K. pneumoniae*.

Next, we characterized the antimicrobial profile of Hill-Cec1 and Hill-Cec10 against *P. aeruginosa* more in-depth. *P. aeruginosa* is an opportunistic pathogen that is a major cause of nosocomial infections (47). Vulnerable patients, such as cystic fibrosis and burn wound patients, are especially at risk of *Pseudomonas* infections (47, 48). Eradication of *P. aeruginosa* has become increasingly difficult due to the rise of multidrug-resistant strains (49). AMPs with activity against these drug-resistant strains could be valuable in the treatment of these critical infections (41). Both cecropins are able to

kill *P. aeruginosa* bacteria within the first 30 min of exposure. However, to maintain bactericidal activity, supra-MICs are needed (4 to 8  $\mu\text{M}$  for Hill-Cec1, 16  $\mu\text{M}$  or higher for Hill-Cec10). Bactericidal activity has been linked to membrane permeabilization for other cecropins (50, 51). Indeed, the NPN uptake assay confirmed that Hill-Cec1 and Hill-Cec10 are able to permeabilize the OM of *P. aeruginosa*. In line with the killing kinetics, the peptides are able to disrupt the OM fast (within 5 min), but need supra-MICs (2  $\mu\text{M}$  for Hill-Cec1, 4  $\mu\text{M}$  for Hill-Cec10) to achieve at least 50% NPN uptake. The PI and diSC<sub>3</sub>(5) assays show that both cecropins are also able to target the inner, cytoplasmic membrane. Membrane disruption by AMPs is one of the main mechanisms that prevents pathogens to develop resistance (52, 53). The combination of their activity against multi-drug resistant *Pseudomonas*, their low cytotoxicity, their fast killing time and the membrane disruptive mechanism of action, make Hill-Cec1 and Hill-Cec10 candidates for new antipseudomonal drug leads to treat acute infections, such as skin or lung infections (54, 55).

Finally, anti-biofilm activity of the cecropins was studied. Biofilm formation of *P. aeruginosa* can lead to persistent infections that respond poorly to antibiotic treatment. The biofilm matrix can slow antibiotic penetration, while sessile bacteria enter into a dormant state characterized by a high antimicrobial tolerance (37). Two strategies to combat biofilms are (i) the prevention of biofilm formation by either inhibiting bacterial adhesion or by reducing initial bacterial growth, and (ii) the eradication of mature biofilms (56). Both strategies were investigated within this study. Hill-Cec1 and Hill-Cec10 showed a significant decrease in biofilm mass and viability when they were added immediately to planktonic *P. aeruginosa* bacteria. Bactericidal concentrations of the cecropins were able to obtain 75% or more reduction of biofilm formation, indicating that the majority of the inhibitory action of the peptides is directly linked to their activity on the planktonic bacteria. Cecropins with inhibitory activity can be useful to prevent biofilms after surgery or as a coating on medical devices (57–59). When the peptides were added to 16-h mature biofilms, no significant effect on biofilm viability was observed at concentrations up to 128  $\mu\text{M}$ . Possibly, their high positive charge leads to electrostatic interactions with anionic polymers in the biofilm matrix, causing them to be trapped (60). For some other cecropins, however, activity against pre-formed biofilms of Gram-negative pathogens has been reported (61, 62).

**Importance in industrial black soldier fly larvae production.** Apart from antimicrobial drug development, there is substantial interest in exploiting active AMPs toward other applications and industries. AMPs could, for example, be used as additives in the agriculture, food, and feed industries (63–65). BSFL are being studied in waste management and recycling in livestock farming, for example in the hygienization of manure (66–70). The reduction of pathogens in these highly contaminated substrates by the BSFL is partially attributed to the larvae's production of AMPs (70). Furthermore, AMPs could also be of use in the insect industry itself. Insect farming is a flourishing sector (71), and the application of AMPs in BSF farming could help to lower the bioburden, including the number of human pathogens, present in the substrate during rearing, the insect biomass and the leftover frass (72, 73). However, in order to develop these AMPs into usable additives, more research needs to be done on the stability of these peptides, for example in the substrate, and possible AMP formulations.

**Concluding remarks.** Overall, our selected cecropin peptides have shown promising activity against Gram-negative pathogens, such as *P. aeruginosa*. This leaves potential for various industrial applications including antimicrobial drug development. As insect AMPs are studied as alternative treatments for infections, such as skin, eye, and lung infections, these BSF cecropins expand the library of peptide templates usable for antimicrobial drug development (54). However, many more aspects, such as synergy with conventional antibiotics, of these cecropin AMPs remain to be characterized (74, 75). Moreover, further development will have to address obstacles commonly associated with peptide-based drugs, including poor metabolic stability (4, 76).

## MATERIALS AND METHODS

**Antimicrobial peptides.** In a previous study, genes were identified in the *H. illucens* transcriptome encoding for putative AMPs (8). All AMPs of this study that could be produced synthetically were produced by solid-phase peptide synthesis and purified by either COVALAB (Bron, France), Proteogenix (Schiltigheim, France), or Genscript (Leiden, the Netherlands). Liquid chromatography-mass spectrometry was used to determine the peptide purity. Peptide type, amino acid sequence, purity, C-terminal modifications, isoelectric point, and molecular weight are summarized in Table S1. For the experiments, peptides were dissolved in DMSO (Acros Organics) at a concentration of 10 mM and further diluted in sterile demineralized water.

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

**Antimicrobial activity assay.** To detect antimicrobial activity of the BSF AMPs, the peptide library underwent screening against a panel of microorganisms, consisting of *S. aureus* ATCC 6538, *E. coli* ATCC 8739, *P. aeruginosa* ATCC 9027, *C. albicans* B59630, and *A. fumigatus* B42928. Serial dilutions of the peptides were prepared from the DMSO stock solutions in sterile demineralized water in 96-well plates using an automated liquid-handling workstation (Beckman Coulter Biomek 3000) in end volumes of 10  $\mu$ L. The final in-plate concentration of DMSO was <1%. Peptide concentrations ranged from 64  $\mu$ M to 0.25  $\mu$ M for the first evaluation, and from 32  $\mu$ M to 0.016  $\mu$ M for the independent repeat. As references, doxycycline (Sigma-Aldrich; *S. aureus*, *E. coli*, *P. aeruginosa*), flucytosine (Sigma-Aldrich; *C. albicans*), and econazole (Sigma-Aldrich; *A. fumigatus*) were used. Suspensions of a starting inoculum of  $5 \times 10^3$  CFU/mL (*E. coli*, *S. aureus*, *C. albicans*, *A. fumigatus*) and  $5 \times 10^4$  CFU/mL (*P. aeruginosa*) were prepared in MHB (bacterial species) or RPMI medium (fungal species) and 190  $\mu$ L was added to the 96-well plates. Plates were incubated for 16 h (*E. coli*, *S. aureus*, *P. aeruginosa*), 24 h (*C. albicans*), or 48 h (*A. fumigatus*) at 37°C. After incubation, read-out of the antimicrobial activity was performed using a resazurin assay. Then, 20  $\mu$ L of a 0.01% (wt/vol) resazurin (Sigma-Aldrich) solution was added to each well and plates were incubated for 15 min (*S. aureus*), 30 min (*E. coli*), 45 min (*P. aeruginosa*), 4 h (*C. albicans*), or 17 h (*A. fumigatus*) to allow resazurin reduction to take place. Fluorescence was read using a microplate reader (Telix) at  $\lambda_{\text{excitation}} = 550$  nm and  $\lambda_{\text{emission}} = 590$ . The results were used to calculate the IC<sub>50</sub> value, defined as the concentration of peptide causing 50% microbial growth inhibition. MIC values were determined visually. Medium in the wells with no visual growth was plated on MHA to detect the MBC, defined as  $\geq 3$  log reduction compared with the growth control.

**Cytotoxicity screening of the peptide library.** To determine early signs of peptide toxicity, the library was tested against the MRC5-SV2 cell line of human, embryonic lung fibroblasts (Sigma-Aldrich). Peptides were serially diluted in 96-well plates as described earlier at concentrations ranging from 64  $\mu$ M to 0.25  $\mu$ M for the first screening, and from 32  $\mu$ M to 0.016  $\mu$ M for the independent repeat. Tamoxifen (Sigma-Aldrich) was included as a reference compound. After preparation of the test plates, cell suspension (190  $\mu$ L) of  $1.5 \times 10^5$  cells/mL in complete minimum essential medium (Gibco) was added to the peptides. Plates were incubated for 72 h at 37°C in a 5% CO<sub>2</sub> incubator (Binder). Afterwards, 50  $\mu$ L of a 0.01% (wt/vol) resazurin (Sigma-Aldrich) solution was added to the wells to detect cell viability. After 4 h of incubation, fluorescence was read using a microplate reader (Telix) at  $\lambda_{\text{excitation}} = 550$  nm and  $\lambda_{\text{emission}} = 590$  and the IC<sub>50</sub> values were calculated.

**Evaluation of selected cecropins against an extended bacterial panel.** To further investigate the antimicrobial spectrum of the selected AMPs, *in vitro* antimicrobial screening against additional microorganisms was performed. The extended panel of microbes included *P. aeruginosa* PAO1, *P. aeruginosa* LMG 27650 (a multidrug-resistant strain), *P. aeruginosa* ATCC 15442, *K. pneumoniae* ATCC 13883, *B. cenocepacia* LMG 16656, and *M. tuberculosis* H37Ra. Peptides were serially diluted in sterile demineralized water in 96-well plates using an automated liquid-handling workstation as described above. Bacterial suspensions were prepared at a concentration of 10<sup>4</sup> CFU/mL (*K. pneumoniae*), 5  $\times$  10<sup>4</sup> CFU/mL (*P. aeruginosa*), 10<sup>5</sup> CFU/mL (*B. cenocepacia*), or 5  $\times$  10<sup>5</sup> CFU/mL (*M. tuberculosis*). In addition, 190  $\mu$ L of this suspension was mixed with the diluted peptides. For the *M. tuberculosis* screen, the outer wells of the test plates were filled with 200  $\mu$ L of demineralized water. As references, doxycycline (Sigma-Aldrich; *P. aeruginosa* PAO1, *P. aeruginosa* ATCC 15442, *K. pneumoniae*), polymyxin B sulfate (Sigma-Aldrich; *P. aeruginosa* LMG 27650), moxifloxacin (Sigma-Aldrich; *B. cenocepacia*), and isoniazide (Sigma-Aldrich; *M. tuberculosis*) were used. Test plates were incubated at 37°C for 16 h (*P. aeruginosa*, *K. pneumoniae*), 48 h (*B. cenocepacia*), or 7 days (*M. tuberculosis*) and read-out of the antimicrobial activity was performed with a resazurin assay as described earlier. Plates with resazurin were incubated 15 min (*K. pneumoniae*), 30 min (*P. aeruginosa*), 4 h (*B. cenocepacia*), or 1 day (*M. tuberculosis*). The fluorescent signal was read using a microplate reader (Promega) at  $\lambda_{\text{excitation}} = 550$  nm and  $\lambda_{\text{emission}} = 590$  and the IC<sub>50</sub> values were calculated.

**Hemolysis analysis.** For the analysis of hemolysis, fresh human whole blood was collected in tubes containing 30 units of heparin (77). Afterwards, the blood was centrifuged (1,000  $\times$  g, 5 min) and washed until the supernatant was clear. The erythrocyte pellet was diluted in phosphate-buffered saline (PBS; Gibco) to obtain a 2% red blood cell suspension. Next, 150  $\mu$ L of serially diluted AMPs in PBS were added to microcentrifuge tubes and mixed with 150  $\mu$ L of the red blood cell suspension. Furthermore,



0.1% Triton-X (Sigma-Aldrich) and PBS were used as positive and negative controls, respectively. The samples were incubated at 37°C for 1 h. Afterwards, the tubes were centrifuged at  $1,000 \times g$  for 5 min and 200  $\mu\text{L}$  of the supernatant was transferred to a 96-well plate. Absorbance was measured with a microplate reader (Promega) at  $\lambda = 570 \text{ nm}$  to detect hemoglobin release. The hemolysis analysis was carried out twice. In addition, hemolysis was predicted using the online software HemoPred (36).

**Time-to-kill analysis.** To investigate the bactericidal kinetics of the selected AMPs, a time-to-kill analysis was performed as described previously by Mascio et al. (78). Briefly, peptide serial dilutions were prepared in 96-well plates (10  $\mu\text{L}$ ) and mixed with 190  $\mu\text{L}$  of a *P. aeruginosa* ATCC 9027 culture of an optical density measured at a wavelength of 600 nm ( $\text{OD}_{600}$ ) of 0.1. Plates were incubated at 37°C and at selected time points (0 h, 0.5 h, 1 h, 2 h, 4 h, and 5 h), aliquots were removed and serially diluted in PBS. The dilutions were plated on TSA and incubated overnight at 37°C. Afterwards, bacterial viability was assessed by performing a standard plate count. Bactericidal activity was defined as a  $\geq 3$ -log reduction compared with the untreated bacterial control. To avoid carry-over effect of the peptides, the undiluted sample was not plated, leading to a quantification limit of  $10^3 \text{ CFU/mL}$  (78). Bacterial counts were calculated for each AMP concentration at each time point using the average plate count. Afterwards,  $\log_{10}$  reductions of the bacterial counts were calculated using the following formula:  $\log_{10} \text{ reduction} = \log_{10} (\text{viable microorganisms without AMP treatment/viable microorganisms with AMP treatment})$ . Next, the average  $\log_{10}$  reductions and the standard deviations of five independent experiments were calculated and visualized using GraphPad Prism 8.

**Inhibition of biofilm formation.** To investigate the effect of the AMPs on biofilm formation, serially diluted peptides were added *in duplo* to 96-well plates, with final concentrations ranging from 64 to 0.5  $\mu\text{M}$ . Next, a *P. aeruginosa* ATCC 15442 culture of approximately  $10^6 \text{ CFU/mL}$  was added. After 16 h of incubation at 37°C and shaking at 110 rpm (New Brunswick Innova 4300), a resazurin assay was used as described by Gilbert-Girard et al. to determine the viability of the bacterial cells in the biofilm (79). After incubation, the growth medium was removed from the well plates and the bacterial biofilms were washed once with PBS to remove remaining planktonic cells. Afterwards, 200  $\mu\text{L}$  of a 20  $\mu\text{M}$  resazurin solution was added to each well and the plates were incubated 4 h at 37°C with agitation at 110 rpm (New Brunswick Innova 4300). The fluorescence of the wells was measured at  $\lambda_{\text{excitation}} = 550 \text{ nm}$  and  $\lambda_{\text{emission}} = 590$  using a microplate reader (Promega). Afterwards, a crystal violet staining was applied to determine the biomass of the biofilms (79). The resazurin solution was removed from the well plates and 200  $\mu\text{L}$  of 100% ethanol (VWR Chemicals) was added to fix the biofilms. After 15 min of incubation, the ethanol was discarded from the wells and the plates were left to air dry for 30 min. Afterwards, 200  $\mu\text{L}$  of a 0.023% (wt/vol) crystal violet (Merck) solution was added to the biofilms. The staining solution was removed after 5 min and the biofilms were washed with PBS. After air-drying for 10 min, the crystal violet stain was solubilized in 200  $\mu\text{L}$  of 100% ethanol. Absorbance was measured at  $\lambda = 595 \text{ nm}$  with a microplate reader (Promega) and, where possible,  $\text{IC}_{50}$  values were calculated. The experiment was carried out three times. Polymyxin B sulfate (Sigma-Aldrich) was included as a reference.

**Biofilm eradication (postexposure).** In addition to biofilm formation, the effect of the peptides on preformed biofilms was investigated to measure the amount of biofilm eradication. *P. aeruginosa* ATCC 15442 biofilms were grown in 96-well plates as described above without the addition of AMPs. After overnight incubation, the medium was removed and 10  $\mu\text{L}$  of serially diluted peptides were added *in duplo* to the plates at concentrations ranging from 128  $\mu\text{M}$  to 0.5  $\mu\text{M}$  (final concentration). New MHB was added and the well plates were incubated again for 16 h at 37°C and shaking at 110 rpm (New Brunswick Innova 4300). The next day, bacterial viability and biomass were determined with a resazurin assay and crystal violet assay as described earlier. The experiment was carried out three times. Polymyxin B sulfate was included as a reference.

**Inner membrane permeabilization by the propidium iodide uptake assay.** To investigate the IM damage induced by the selected AMPs, a PI (Sigma-Aldrich) uptake assay was performed, adapted from Dassanayake et al. (80). *P. aeruginosa* ATCC 9027 was grown until the mid-log phase in MHB. The bacteria were centrifuged ( $3,000 \times g$ , 15 min) and resuspended in 5 mM HEPES (Sigma-Aldrich) buffer (pH 7.4) to an  $\text{OD}_{600}$  of 0.5. Next, 50  $\mu\text{L}$  of serially diluted peptides were added *in duplo* to a 96-well plate and mixed with a 150  $\mu\text{L}$  bacterial suspension containing 4  $\mu\text{M}$  PI (final in-plate concentration of 3  $\mu\text{M}$ ). Peptide test concentrations ranged from 32  $\mu\text{M}$  to 0.25  $\mu\text{M}$ . A high concentration of polymyxin B sulfate (16  $\mu\text{M}$ ) was implemented as a positive control. Fluorescence was measured every 5 min during 1 h using a microplate reader (Promega) at  $\lambda_{\text{excitation}} = 530 \text{ nm}$  and  $\lambda_{\text{emission}} = 620$ . Data were normalized based on the fluorescent signal in the presence of 50  $\mu\text{L}$  HEPES buffer and 150  $\mu\text{L}$  PI bacterial suspension. The assay was carried out in triplicate.

**Outer membrane permeabilization with N-phenyl-naphthylamine.** N-phenyl-naphthylamine (NPN; Tokyo chemical industry [TCI]) was used to study the permeabilization of the OM as described by Helander et al. (81). Briefly, *P. aeruginosa* ATCC 9027 was grown until the mid-log phase in MHB. Afterwards, the cells were centrifuged ( $3,000 \times g$ , 15 min) and resuspended in 5 mM HEPES buffer (pH 7.4) to an  $\text{OD}_{600}$  of 0.5. Next, 50  $\mu\text{L}$  of peptides were added *in duplo* to a 96-well plate (final concentrations ranging from 32  $\mu\text{M}$  to 0.25  $\mu\text{M}$ ). As a positive control, 16  $\mu\text{M}$  polymyxin B was included. The peptides were mixed with 50  $\mu\text{L}$  of a 40  $\mu\text{M}$  NPN solution and 100  $\mu\text{L}$  of *P. aeruginosa* suspension. Fluorescence was measured every 5 min during 1 h using a microplate reader (Promega) with  $\lambda_{\text{excitation}} = 350 \text{ nm}$  and  $\lambda_{\text{emission}} = 420$ . Data were normalized based on the fluorescent signal in the presence of 50  $\mu\text{L}$  HEPES buffer, 50  $\mu\text{L}$  NPN, and 100  $\mu\text{L}$  bacteria. Experiments were carried out in triplicate.

**Cytoplasmic membrane depolarization assay.** To study the effects of the peptides on the membrane potential, 3,3'-dipropylthiadicarbocyanine iodide (diSC3(5); TCI) was used as described by Kwon et al. (82). *P. aeruginosa* ATCC 9027 was grown until the mid-log phase in MHB. Afterwards, the cells



were centrifuged ( $3,000 \times g$ , 15 min), washed and resuspended in 5 mM HEPES buffer (pH 7.4) supplemented with 20 mM glucose and 100 mM KCl to an  $OD_{600}$  of 0.2. DiSC3(5) was added to the *P. aeruginosa* suspension at a concentration of  $1.33 \mu\text{M}$  (final in-plate concentration of  $1 \mu\text{M}$ ), and the mixture was left to stand for 1.5 h to stabilize the fluorescent signal. Serially diluted peptides ( $50 \mu\text{L}$ ) were added in duplo to 96-well plates (final concentrations ranging from  $32 \mu\text{M}$  to  $0.25 \mu\text{M}$ ) and mixed with  $150 \mu\text{L}$  of bacteria with diSC3(5). The fluorescent signal was measured at  $\lambda_{\text{excitation}} = 620 \text{ nm}$  and  $\lambda_{\text{emission}} = 670$  with a microplate reader (Promega) every 5 min during 1 h. Furthermore, 0.1% Triton-X was used as a positive control. Data were normalized based on the fluorescent signal in the presence of  $50 \mu\text{L}$  supplemented HEPES buffer and  $150 \mu\text{L}$  diSC3(5)-bacterial suspension. The assay was carried out in triplicate.

**Statistical analyses.** For the biofilm experiments, the Dunnett's T3 multiple comparison's test was used to compare the means of the treated groups to the mean of the untreated control (GraphPad Prism 8).

## SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, PDF file, 0.3 MB.

## ACKNOWLEDGMENTS

L.V.M. is financed by the Research Foundation Flanders (FWO Vlaanderen) under Grant S008519N. J.D.S. holds a postdoctoral fellowship grant (Grant Number 12V5219N) of the FWO. A.P., D.T., and A.V. acknowledge generous funding by the Hessian Ministry of Higher Education, Research, Science and the Arts (HMWK) via funding the LOEWE Center for Insect Biotechnology and Bioresources.

The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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