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Identification and characterization of amphipathic antimicrobial peptides with broad spectrum activity against multi-drug resistant bacteria.

Mireia López-Siles [a,*,1], Ana Tajuelo [b,c,1], Pau Caravaca-Fuentes [d], Marta Planas [d,*], Lidia Feliu [d,2], Michael J. McConnell [e,f,2]

[a] Microbiology of Intestinal Diseases, Biology Department, Universitat de Girona, Girona, Spain
[b] Intrahospital Infections Laboratory, Instituto de Salud Carlos III (ISCIII), National Centre for Microbiology, Madrid, Spain
[c] Programa de Doctorado en Ciencias Biomédicas y Salud Pública. IMIENS. Universidad Nacional de Educación a Distancia (UNED), Madrid, Spain
[d] LIPPSO, Department of Chemistry, Universitat de Girona, Girona, Spain
[e] Department of Biological Sciences, University of Notre Dame, Notre Dame, USA
[f] Eck Institute for Global Health, Notre Dame, USA

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ABSTRACT

Antimicrobial peptides (AMPs) are potential alternatives to antibiotics given the reduced likelihood of resistance and their high selectivity towards bacteria. AMPs with activity against antibiotic-resistant bacteria have been reported. The aim of this study is to characterize the activity of novel **BP100** analogues against multidrug-resistant bacteria. Eleven bacterial strains representing five pathogenic species were used to evaluate the antimicrobial activity of 26 peptides. An initial screen was performed at 50 μg/ml, and those peptides that inhibited ≈90 % of growth of all strains were selected. Minimum inhibitory concentrations (MIC), minimum bactericidal concentrations (MBC), inhibition in biofilm formation, time kill assays, stability in human serum and *in vivo* toxicity were assessed. **BP607, BP76** and **BP145,** had broad activity against multidrug-resistant bacteria. MICs ranged between 3.13 and 50 μg/ml, whereas MBCs ranged between 6.25 and 100 μg/ml. *Acinetobacter baumannii, Klebsiella pneumoniae* and *Escherichia coli* were the most susceptible species. At 2x the MIC, all compounds were bactericidal after 6h. **BP76** inhibited ≥ 76.77 % of *K. pneumoniae* and *E. coli* biofilm formation at subinhibitory concentrations. **BP145** had improved serum stability and lower toxicity compared to **BP607**. In conclusion, **BP145** and **BP76** demonstrate broad antimicrobial activity, are active at non-toxic concentrations, feature bactericidal activity at 6h and inhibit biofilm formation.

1. Introduction

Antibiotic resistance is a major global problem because infections caused by resistant strains can increase hospitalization stays, morbidity and mortality, which in turn negatively impacts the economic costs associated with healthcare (M Campos et al., 2020, de Kraker et al., 2011, Zhen et al., 2019). The World Health Organization (WHO) has established a list of the most worrisome multidrug-resistant microorganisms. *Pseudomonas aeruginosa, Acinetobacter baumannii, Klebsiella pneumoniae, E. coli* and *Staphylococcus aureus* are highlighted among the most critical pathogens (Asokan et al., 2019). These bacterial species are responsible for the majority of nosocomial infections and have the ability to acquire or develop new resistance mechanisms against currently-available antibiotics (Christaki et al., 2020). These five microorganisms are among the six leading pathogens responsible for the 929,000 (660,000–1270,000) deaths associated and 3.57 million (2.62–4.78) deaths attributable to antimicrobial resistance in 2019 (Murray et al., 2022). Therefore, the development of new therapies to treat infections caused by these pathogens is urgently needed.

Antimicrobial peptides (AMPs) have been proposed as an alternative to antibiotics. They are naturally produced by a large variety of organisms, ranging from prokaryotes to humans, and exhibit activity against a wide range of bacteria, viruses, fungi or even cancer cells (Talapko, 2022, Espeche, 2023). Commonly, AMPs consist of less than 100 amino

* Corresponding authors.
E-mail addresses: mireia.lopezs@udg.edu (M. López-Siles), marta.planas@udg.edu (M. Planas).
[1] These authors contributed equally to this work and share the first authorship.
[2] These authors contributed equally to this work and share the last authorship.

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Table 1
Peptides analysed in this study.

Peptide	Sequence	Hemolysis[d] (%)	Reference
CECMEL11 peptides			
BP15	KKLFKKILKVL-NH_2	16±2.9	(Badosa et al., 2007)
BP76	KKLFKKILKFL-NH_2	34±2.1	(Badosa et al., 2007)
BP125	Ts-KKLFKKILKKL-NH_2	8±1.6	(Badosa et al., 2007)
BP270	KKLHKKILKVL-NH_2	0±1.1	(Badosa et al., 2007)
BP273	Ac-FKLHKKILKVL-NH_2	0±0.5	(Badosa et al., 2007)
BP275	Ts-FKLHKKILKVL-NH_2	4±1.3	(Badosa et al., 2007)
Peptides with D-amino acids[a]			
BP142	KKLFkKILKYL-NH_2	0±0.5	(Güell et al., 2011)
BP143	KKLfKKILKYL-NH_2	2±2.0	(Güell et al., 2011)
BP144	KKlFKKILKYL-NH_2	2±2.0	(Güell et al., 2011)
BP145	KkLFKKILKYL-NH_2	27±0.7	(Güell et al., 2011)
BP146	KKLFKkILKYL-NH_2	23±2.0	(Güell et al., 2011)
BP157	KKLFKkilkyl-NH_2	0±0.7	(Güell et al., 2011)
BP164	kklfkkILKYL-NH_2	0±0.5	(Güell et al., 2011)
BP165	kklfkkiLKYL-NH_2	1±0.5	(Güell et al., 2011)
BP166	kklfkkilKYL-NH_2	0±0	(Güell et al., 2011)
BP168	kklfkkilkyL-NH_2	1±1.2	(Güell et al., 2011)
BP607[b]	kklfkkilryl-NH_2	42	(Oddo et al., 2016)
Peptidotriazoles[c]			
BP241	KX_aLFKKILKYL-NH_2	6±1.2	(Güell et al., 2012)
BP248	KKLX_bKKILKYL-NH_2	1±0.2	(Güell et al., 2012)
Lipopeptides			
BP387	Ac-KKLFKKIK(COC_3H_7)KYL-NH_2	11±5.0	(Oliveras et al., 2018)
BP474	Ac-KKLfKKIK(COC_3H_7)KYL-NH_2	0±0	(Oliveras et al., 2021)
BP475	Ac-KKLfKKILKK(COC_3H_7)L-NH_2	0±0	(Oliveras et al., 2021)
Peptide conjugates			
BP171	KKLFKKILKYL-AGPA-TTGLPALISW-OH	16±1.2	(Badosa et al., 2013)
BP179	KKLFKKILKYL-GIGKFLHSAK-KDEL-OH	12±0.8	(Badosa et al., 2013)
BP181	KKLFKKILKYL-AGPA-KFLHSAK-OH	0±0.5	(Badosa et al., 2013)
BP190	AVAVVGQATQIAK-KKLFKKILKYL-KDEL-OH	17±0.4	(Badosa et al., 2013)

[a] The underlined lower case letters refer to D-amino acids;
[b] Corresponds to **BP214** in (Oddo et al., 2016);
[c] X_a stands for a Lys bearing a triazole ring substituted with a 2-aminohexanoic acid and X_b stands for an Ala bearing a triazole ring;
[d] Percent hemolysis at 150 μM plus confidence interval ($\alpha = 0.05$)

acids, typically with a positive net charge and a significant percentage of hydrophobic residues. These features allow them to adopt an amphipathic structure which facilitates their insertion into the lipid bilayer of bacterial cell membranes (Talapko, 2022, Espeche, 2023). Although activity is closely related to their structure (Ciulla and Gelain, 2023), the main mechanism of action of many AMPs is due to their electrostatic interaction with the negatively charged membrane, which leads to membrane disruption, eventually causing cell death (Talapko, 2022, Espeche, 2023). This mechanism of action gives AMPs an advantage compared to conventional antibiotics, since resistance is less likely to occur (Xuan et al., 2023). AMPs can also inhibit cell wall synthesis or target intracellular components such as DNA or RNA (Gottschalk and Thomsen, 2017). Another advantage of AMPs is their selectivity towards bacteria because the negative charge density of mammalian cell membranes is generally lower than that of bacteria (Lobo and Boto, 2022).

A wide variety of AMPs have been shown to display antibacterial activity against different clinically relevant pathogens (Kundu, 2020, Wang et al., 2019, Narayana et al., 2019, Falah et al., 2019, Pavlova et al., 2020). For instance, AMPs produced by the gut microbiota have been shown to exhibit activity against *E. coli* or *P. aeruginosa* (Falah et al., 2019, Pavlova et al., 2020). However, one of the main challenges in this field is finding AMPs with activity against antibiotic-resistant bacteria (Xuan et al., 2023). In this context, derivatives of the human cathelicidin LL-37 have been described to be active against multidrug-resistant pathogens, such as *S. aureus* MRSA USA300, *Enterobacter cloacae* B2366–12 or *A. baumannii* B28–16 (Wang et al., 2019, Narayana et al., 2019). Another example is colistin, which is commonly employed to treat multidrug resistant (MDR) Gram-negative bacteria, such as *A. baumannii, K. pneumoniae, E. coli* and *P. aeruginosa* (El-Sayed Ahmed et al., 2020).

Previous studies have demonstrated that AMPs from the CECMEL11 library and analogues including lipopeptides, peptidotriazoles, peptide conjugates, and derivatives with a D-amino acid, display high activity against plant pathogenic Gram-negative bacteria, low hemolysis and phytotoxicity (Badosa et al., 2007, Badosa et al., 2013, Güell et al., 2011, Güell et al., 2012, Oliveras et al., 2018, Oliveras et al., 2021, Oliveras et al., 2022). Among this collection of peptides, **BP100** has been described to be active against other clinically relevant human bacteria, such as *A. baumannii, Bacillus subtilis, S. aureus, E. coli, P. aeruginosa, Staphylococcus epidermidis, K. pneumoniae* or *Enterococcus faecium* (Oddo et al., 2016, Eales et al., 2018, Carretero et al., 2018, Carretero et al., 2021, Torcato et al., 2013, Ajish, 2022). The activity of diverse analogs of **BP100** incorporating Arg, Trp, D-amino acids or a lipidic chain have also been investigated against these pathogens (Oddo et al., 2016, Carretero et al., 2018, Carretero et al., 2021, Torcato et al., 2013, Ajish, 2022). For instance, the D-amino acid derivative **BP214** (renamed in this manuscript as **BP607**, see Materials and Methods), exhibits good activity against colistin-resistant *A. baumannii* strains (Oddo et al., 2016).

The above findings prompted us to analyze a collection of **BP100** analogues against multidrug-resistant bacteria that are of public health concern, with the aim of identifying AMPs with improved properties such as target microorganisms, minimum inhibitory and bactericidal concentrations, bactericidal capacity over time, inhibition of biofilm formation, stability in human serum, and *in vivo* toxicity.

2. Experimental procedures

2.1. Peptide synthesis and selection

Peptides were synthesized on a solid phase following a standard 9-fluorenylmethoxycarbonyl (Fmoc)/*tert*-butyl (*t*Bu) strategy as previously described (Table 1) (Badosa et al., 2007, Badosa et al., 2013, Güell

et al., 2011, Güell et al., 2012, Oliveras et al., 2018, Oliveras et al., 2021). The peptides were obtained in HPLC purities ranging from 64 to >99 % and were characterized by mass spectrometry (data not shown).

The 25 peptides derived from **BP100** included in this study were selected from previous studies (Badosa et al., 2007, Badosa et al., 2013, Güell et al., 2011, Güell et al., 2012, Oliveras et al., 2018, Oliveras et al., 2021) according to their high activity against Gram-negative plant pathogenic bacteria and their low hemolytic activity. These sequences include: (i) six peptides from the CECMEL11 library (Badosa et al., 2007); (ii) ten D-amino acid-containing peptides (Güell et al., 2011, Oddo et al., 2016); (iii) two peptidotriazoles (Güell et al., 2012); (iv) three lipopeptides (Oliveras et al., 2018, Oliveras et al., 2021); and (v) four peptide conjugates (Badosa et al., 2013). Moreover, the D-amino acid containing peptide **BP607** was selected because it is active against colistin-resistant strains of *A. baumannii* and features low hemolytic activity (Oddo et al., 2016). It must be noted that **BP607** corresponds to the peptide named by Oddo et al. as **BP214** (Oddo et al., 2016). In our study, **BP214** has been renamed **BP607** because before the work by Oddo et al. (Oddo et al., 2016) we had already published a different peptide coded as **BP214** (Badosa et al., 2013). Colistin (A2922, Panreac) a peptide antibiotic was used as control during this study.

2.2. Bacterial strains and culture conditions

Eleven bacterial strains were used to evaluate the antimicrobial activity of the peptides. All strains have been reported previously, including information on antimicrobial susceptibility for each strain. These include the antibiotic susceptible reference strains *S. aureus* ATCC 29,213, *A. baumannii* ATCC 19,606, *K. pneumoniae* 43,816, *E. coli* ATCC 25,922 and *P. aeruginosa* ATCC 27,853 from the ATCC. In addition, a previously reported methicillin-resistant *S. aureus* strain, MRSA USA 300 (Román et al., 2021), a multidrug resistant *P. aeruginosa* clinical isolate, *P. aeruginosa* ST175–1 (Del Barrio-Tofiño et al., 2019), a colistin-resistant derivative of the *A. baumannii* ATCC 19,606 strain with a mutation in the *lpxD* gene, *A. baumannii* IB010 (García-Quintanilla et al., 2014), a multidrug resistant clinical *A. baumannii* clinical isolate, Ab-1 (McConnell et al., 2011), a *K. pneumoniae* clinical isolate, CG258 (Peirano, 2017), and an ESBL-producing *E. coli* clinical isolate, *E. coli* ESBL 1057.1 (López-Siles et al., 2023), were used in the study (Table 2).

Strains were grown on Luria Bertani (LB) agar plates. For the mutant strain *A. baumannii* IB010, LB plates were supplemented with 10 μg/ml of colistin (A2922, Panreac). For long-term storage, strains were kept in LB broth containing 20 % glycerol (v/v) at -80 °C. Bacterial strains were freshly plated from stocks before each experiment.

2.3. Antimicrobial activity screening

An initial screen to evaluate the antimicrobial activity of the 26 peptides and colistin was performed by analyzing inhibition of bacterial growth. Peptides were resuspended in milli-Q water to a concentration of 5 mg/ml and added to the medium to achieve the final concentration. Bacteria were adjusted to 10^6 CFU/ml and then added to the wells of a 96-well flat bottom polystyrene microplate (351,172, Falcon) containing Mueller Hinton broth II (MHB II; 90,922, Merck) and the peptide at 50 μg/ml. Plates were incubated at 37 °C and, after 24 h, the $OD_{620\ nm}$ was measured (M200 Infinite Pro, Tecan). The percentage of growth inhibition was calculated with respect to growth in medium without peptide. Peptides that inhibited at least 90 % of growth for all 11 strains analysed were selected for further testing. Percentages of growth were determined in triplicate.

2.4. Hemolytic activity

The hemolytic activity of the peptides was evaluated by determining hemo-globin release from erythrocyte suspensions of horse blood (5 % vol/vol) (Oxoid) as previously described (Badosa et al., 2007, Badosa et al., 2013, Güell et al., 2011, Güell et al., 2012, Oliveras et al., 2018, Oliveras et al., 2021). The hemolysis of peptide **BP607** is reported by Oddo *et al.* (Oddo et al., 2016).

2.5. Minimum inhibitory concentration (MIC) of BP76, BP145, and BP607

Minimum inhibitory concentrations (MIC) were determined using the broth microdilution method. Bacterial strains were adjusted to 10^6 CFU/ml in MHB II (90,922, Merck), according to Clinical and Laboratory Standards Institute (CLSI) for antimicrobials (CLSI 2017), and added to a 96-well flat bottom polystyrene microplate (351,172, Falcon) containing two-fold serial dilutions of each peptide, from 100 to 0.10 μg/ml. After incubation at 37 °C for 24 h, MICs were determined as the lowest concentration at which there was no bacterial growth. Experiments were performed in triplicate.

2.6. Minimum bactericidal concentration (MBC) of BP76, BP145, and BP607

For minimum bactericidal concentrations (MCB), 20 μl of bacterial culture were incubated with **BP76, BP145, BP607** at the MIC and at 2x MIC and then plated on LB. In addition, the content of the well used as a positive control (assessment of growth without peptide) was serially diluted and plated onto LB to be used as a reference. After incubating the suspensions at 37 °C for 24 h, the number of CFUs was counted and the percentage of growth at both concentrations was calculated with respect to the positive control. The lowest concentration that reduced at least 99.9 % of growth was considered the MBC, in accordance with CLSI protocols (CLSI 2017). The analyses were performed in triplicate.

2.7. Time-kill assay

Time-kill curves of the most susceptible (MIC< 25 μg/ml) strains (i.e. *A. baumannii* ATCC 19,606, *K. pneumoniae* CG258 and *E. coli* ESBL 1057.1) in the presence of **BP76, BP145** and **BP607** were carried out

Table 2
Bacterial strains used to test antimicrobial activity.

Strain	Characteristics	Reference
S. aureus ATCC 29,213	Control strain for antimicrobial activity assays	ATCC, USA
S. aureus MRSA USA 300	Clinical isolate, MRSA	(Román et al., 2021)
P. aeruginosa ATCC 27,853	Control strain for antimicrobial activity assays	ATCC, USA
P. aeruginosa (ST175–1)	Clinical isolate, panresistant	(Del Barrio-Tofiño et al., 2019)
A. baumannii ATCC 19,606	Reference bacterial strain	ATCC, USA
A. baumannii IB010	ATCC 19,606 derivative deficient in lipooligosaccharide due to a large deletion in the *lpxD* gene, colistin resistant	(García-Quintanilla et al., 2014)
A. baumannii Ab-1	Clinical isolate, panresistant	(McConnell et al., 2011)
K. pneumoniae ATCC 43,816	Reference bacterial strain	ATCC, USA
K. pneumoniae CG258	Clinical isolate	(Peirano, 2017)
E. coli ATCC 25,922	Control strain for antimicrobial activity assays	ATCC, USA
E. coli ESBL 1057.1	Clinical isolate, resistant to 18 antibiotics	(López-Siles et al., 2023)

following a previous described protocol (Vinuesa et al., 2021), with some modifications. Overnight cultures of bacterial strains were adjusted to a concentration of 10^5 CFU/ml. Then strains were grown in MHB II medium supplemented with the peptide at the MIC and 2× the MIC. As a control for growth, MHB II without peptide was included. All tests were performed in a final volume of 5 ml at 37 °C under continuous shaking. For bacterial quantification, samples were taken at 0, 3, 6 and 24 h, diluted as appropriate and further plated on MH II agar plates. Each curve was assayed at least in duplicate.

2.8. Inhibition of biofilm formation of BP76, BP145, and BP607

Reductions in biofilm formation were analyzed at ¼ the MIC following a previously-reported protocol (Domenech and García, 2020), with some modifications. After 24 h of incubation of bacterial strains (inoculum adjusted to 10^6 CFU/ml) at 37 °C in LB supplemented with the peptide, the media was discarded from the microplate. Wells were washed with 200 µl of sterile 1x PBS (H3BE17–515Q, Cultek) and the adherent cells were stained with 1 % crystal violet (61,135–25G, Sigma-Aldrich) at room temperature for 15 min. Excess crystal violet solution was discarded and the plates were washed twice with 200 µl of PBS. To elute the stain, 200 µl of 70 % ethanol (v/v) were added to each well followed by measuring the absorbance at 595 nm (M200 Infinite Pro, Tecan). The assay was conducted in triplicate. The specific biofilm formation (SBF) index was calculated following the formula:

$$\frac{OD_{595nm} - OD_{595nm\ control\ without\ peptide}}{OD_{600nm}}$$

SBF index was classified in three categories: weak (SBF $\leq$ 0.5), moderate (0.5 $>$ SBF $\leq$ 1), and strong (SBF $>$ 1) as previously reported (Martinez-Medina et al., 2009).

2.9. Measurement of stability of BP76, BP145 and BP607 in serum

The stability of **BP76, BP145** and **BP607** was evaluated in human serum. Specifically, 50 µL of the peptide stock solution at 1000 µM were added to 100 µL of RPMI supplemented with 37.5 % (v/v) of human serum temperature-equilibrated at 37 ± 1 °C for 15 min. The final peptide concentration was 333.33 µM and the final human serum concentration was 25 % (v/v). Four reaction mixtures were prepared for each peptide which were kept under stirring at 37 °C for 10, 30, 45 and 60 min, respectively. After the corresponding reaction time, 200 µL of 96 % ethanol were added to each of the reaction mixtures to precipitate the serum proteins. Reaction samples were cooled at 4° C for 15 min and then centrifuged at 14,000 g for 2 min to pellet the precipitated serum proteins. The reaction supernatants were analyzed by RP-HPLC using a Kromasil C_{18} column (4.6 × 40 mm; 3 mm particle size). Linear gradients of 0.1 % aqueous TFA and 0.1 % TFA in CH_3CN were run from 0.98:0.02 to 0:1 over 12 min at a flow rate of 1 mL/min with UV detection at 220 nm. Digestion was estimated as the percentage of degraded peptide calculated from the decrease of the HPLC peak area of the native peptide. The analyses were performed in duplicate.

2.10. Toxicity assay of BP76, BP145, BP607 and colistin in vivo

In vivo toxicity of peptides was assessed in larvae of *Galleria mellonella* in the sixth instar (200–300 mg) as previously reported (Gorr et al., 2019, Desbois and Coote, 2012) with minor modifications. Larvae were obtained from local vendors and maintained in sawdust at 4 °C until use.

Injections were performed with a 100 µl Hamilton syringe in the second rear proleg. Ten larvae per group (biological replicates) were injected with either 10 µl of peptide **BP76, BP145, BP607** or colistin at 1–10 µg/ml and maintained at 30 °C after peptide injection. Control larvae were injected with saline solution consisting of 0.9 % NaCl (w/v). Survival was evaluated for 72h by movement in response to prodding and the lack of melanization.

2.11. Statistical analysis

Data do not have a normal distribution, according to Shapiro-Wilks test, therefore non-parametric statistical tests were used.

Differences in inhibition of biofilm inhibition were analyzed using Kruskal-Wallis test followed by Dunn's Multiple Comparison test. All analyses were performed using SPSS (IBM) and Prism 5 v.5.01 (GraphPad Software).

3. Results

3.1. Antimicrobial activity screening of the peptides

The effect of 26 selected peptides on bacterial growth inhibition was screened against two Gram-positive and nine Gram-negative bacteria of clinical relevance, including multi-drug resistant strains (Table 2). Growth percentages of these bacterial strains exposed to 50 µg/ml of the peptides and colistin are shown in Fig. 1. Screening was also carried out with peptides at 100 µg/ml, but the inhibition profile was similar to that observed at 50 µg/ml (data not shown).

We identified peptides with activity against all species tested, including antibiotic resistant strains. *A. baumannii* strains ATCC 19,606 and Ab-1 and *E. coli* ESBL 1057.1 were the most susceptible strains tested, with most of the peptides producing significant growth inhibition. A total of 13 peptides reduced growth of all *A. baumannii* strains at least 83 %. Eleven peptides reduced growth at least 80 % in *E. coli* strains. Regarding *K. pneumoniae* strains eight compounds were able to reduce over 92 % of growth. *P. aeruginosa* strains were the least susceptible of the Gram-negative species tested, and only five out of the 26 compounds reduced at least 88 % of growth. *S. aureus* was the least susceptible to our collection of AMPs, but we were able to identify five peptides able to inhibit growth ≥83 %.

Notably, the peptides that demonstrated the highest antibacterial activity were **BP76, BP145** and **BP607**, which inhibited growth of all the strains, including both Gram-negative and Gram-positive species, by ≈90 %. Peptide **BP146** reduced at least 89 % growth in all strains except for *P. aeruginosa* ST175–1. **BP125** also inhibited ≥ 83 % of growth in all strains except for both *K. pneumoniae* and *E. coli* ATCC 25,922. Interestingly, **BP143** had ≥83 % growth inhibition for all Gram-negative strains tested. Other peptides were more selective, displaying activity against certain strains, for example **BP475, BP181, BP179, BP241** and **BP171** mainly reduced growth of *A. baumannii, K. pneumoniae* and *E. coli* strains with values ranging between 74 % and 95 %. Peptide **BP190** had activity only against *A. baumannii* strains with up to 75 % of growth inhibition. Peptides **BP275, BP248** and **BP474** were the most specific, exhibiting ≥ 93 % growth inhibition mainly against *A. baumannii* ATCC 19,606. Given the broad spectrum of activity of **BP76, BP145** and **BP607**, they were selected for further experiments.

3.2. MIC and MBC of BP76, BP145, and BP607

MIC and MBC values were determined using the broth microdilution method for the antimicrobial peptides with best antibacterial activity in the screening (Table 3). Peptides **BP76, BP145** and **BP607** showed very similar activity for each strain with MICs ranging between 3.13 and 50 µg/ml. They displayed the highest MICs for *S. aureus* MRSA USA 300 and *P. aeruginosa* (ST175–1), whereas the lowest MIC values were found for *A. baumannii* ATCC 19,606. Of note, **BP76** was slightly more active against *S. aureus* ATCC 29,213 and *K. pneumoniae* CG258 compared to the other two peptides. In contrast, **BP607** showed the lowest MIC values for *P. aeruginosa* ATCC 27,853, *A. baumannii* ATCC 19,606 and *A. baumannii* Ab-1.

The MBC values indicated that **BP76** was bactericidal for all strains at the MIC. **BP145** also showed bactericidal activity at the MIC for all strains except for *A. baumannii* ATCC 19,606, **BP607** had the lowest bactericidal activity at the MIC, as 2 × the MIC concentration was

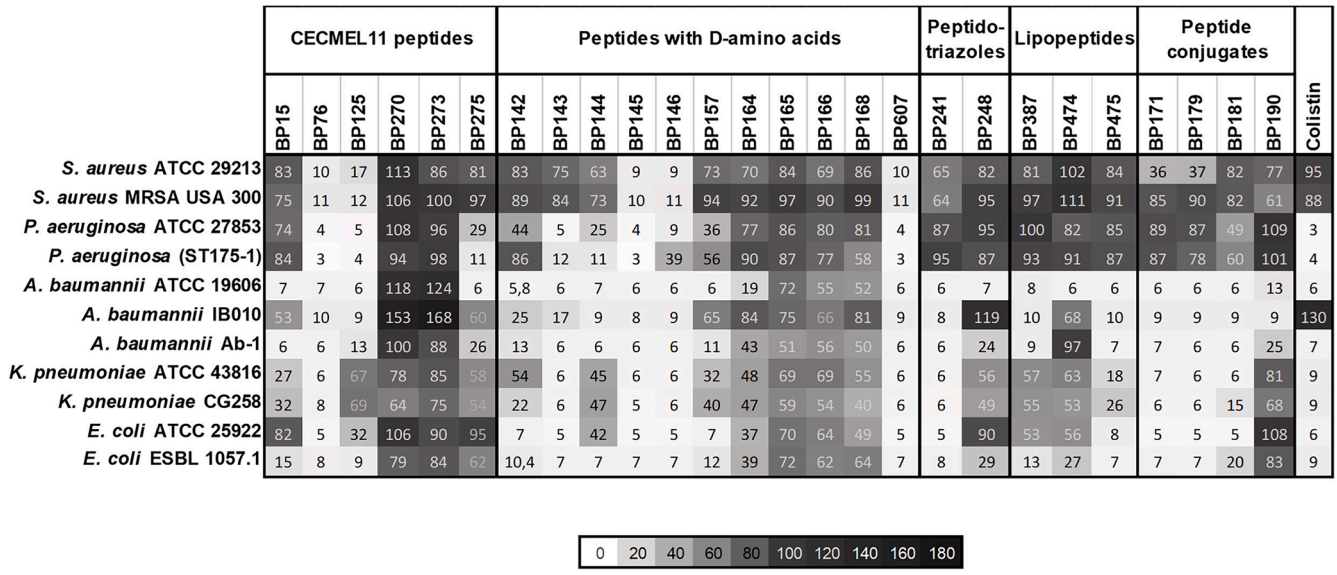

Fig. 1. Heatmap showing the initial screening of bacterial growth in the presence of the different peptides. Color intensity represents the magnitude of the percentage of growth of each bacterial strain when 50 μg/ml of the peptide is present in the medium. Legend shows the percentage of growth after 24 h compared to medium without the peptide. Growth percentages showed represent the mean of three independent assays.

Table 3
Minimum inhibitory concentration (MIC) and Minimum Bactericidal Concentration (MBC) of peptides **BP76, BP145** and **BP607** against the different bacterial strains.

	BP76		**BP145**		**BP607**	
	MIC (μg/ml)	**MBC** (μg/ml)	**MIC** (μg/ml)	**MBC** (μg/ml)	**MIC** (μg/ml)	**MBC** (μg/ml)
S. aureus ATCC 29,213	25	25	50	50	50	50
S. aureus MRSA USA 300	50	50	50	50	50	50
P. aeruginosa ATCC 27,853	50	50	50	50	25	25
P. aeruginosa (ST175–1)	50	50	50	50	50	100
A. baumannii ATCC 19,606	6.25	6.25	6.25	12.5	3.13	6.25
A. baumannii IB010	25	25	25	25	25	50
A. baumannii Ab-1	12.5	12.5	12.5	12.5	6.25	6.25
K. pneumoniae ATCC 43,816	12.5	12.5	25	25	12.5	12.5
K. pneumoniae CG258	6.25	6.25	12.5	12.5	12.5	12.5
E. coli ATCC 25,922	6.25	6.25	6.25	6.25	6.25	6.25
E. coli ESBL 1057.1	12.5	12.5	12.5	12.5	12.5	12.5

necessary to reach this activity in three of the strains (*P. aeruginosa* ST175–1, *A. baumannii* ATCC 19,606 and *A. baumannii* IB010).

3.3. Time-kill activity against mdr gram-negative species

The antimicrobial activity of **BP607, BP145** and **BP76** was further characterized by performing time-kill experiments with the strains *A. baumannii* ATCC 19,606, *K. pneumoniae* CG258 and *E. coli* ESBL 1057.1. For this, growth at 0, 3, 6 and 24 h was analysed by CFU counting when peptides were present in the medium at the MIC and 2× the MIC (Fig. 2).

Experiments carried out at the MIC revealed that none of the peptides were bactericidal for *A. baumannii* ATCC 19,606 at 6 h or at 24 h. Regarding *K. pneumoniae* strain CG258 all three peptides displayed bactericidal activity at 6 h, but only **BP607** maintained this bactericidal effect at 24 h. In contrast, all three peptides were bactericidal for *E. coli* ESBL 1057.1 at 6 and 24 h.

At 2× the MIC, an increased bactericidal effect was observed with the three peptides against the three bacterial strains, with all of them bactericidal for *A. baumannii* ATCC 19,606 and *E. coli* ESBL 1057.1 at 6 and 24 h. Regarding *K. pneumoniae* CG258, **BP145** and **BP607** were also bactericidal at 2× MIC after 24 h whereas **BP76** was able to reduce growth to bactericidal level at 6 h, but not at 24 h.

3.4. Inhibition of biofilm formation

The effect of **BP76, BP145** and **BP607** on biofilm formation inhibition was analyzed at subinhibitory concentration (¼ MIC) for all strains except for *E. coli* ESBL 1057.1 as it is a non-biofilm-forming strain (Fig. 3).

In the presence of any of the three peptides, *S. aureus* MRSA USA 300, all *K. pneumoniae* strains and *E. coli* ATCC 25,922 were weak to moderate biofilm producers. In contrast, *A. baumannii* strains, *P. aeruginosa* ATCC 27,853 and *S. aureus* ATCC 29,213 featured strong SBF index in the presence of any of the three peptides.

Specifically, **BP76** and **BP145** reduced SBF indices compared to **BP607** for *P. aeruginosa* ST175–1, *A. baumannii* ATCC 19,606, *A. baumannii* Ab-1 and *K. pneumoniae* ATCC 43,816. **BP76** also reduced production of biofilm for *E. coli* ATCC 25,922 whereas **BP145** inhibited biofilm formation of *K. pneumoniae* CG258.

3.5. Stability in serum

The stability of **BP76, BP145** and **BP607** in human serum was analyzed. Degradation was monitored by reversed-phase HPLC over time (Fig. 4). Unexpectedly, **BP607** was the least stable peptide, being completely degraded after 10 min. Peptides **BP76** and **BP145** showed higher stability values, with a 15 % and 35 % of peptide remaining after 60 min, respectively.

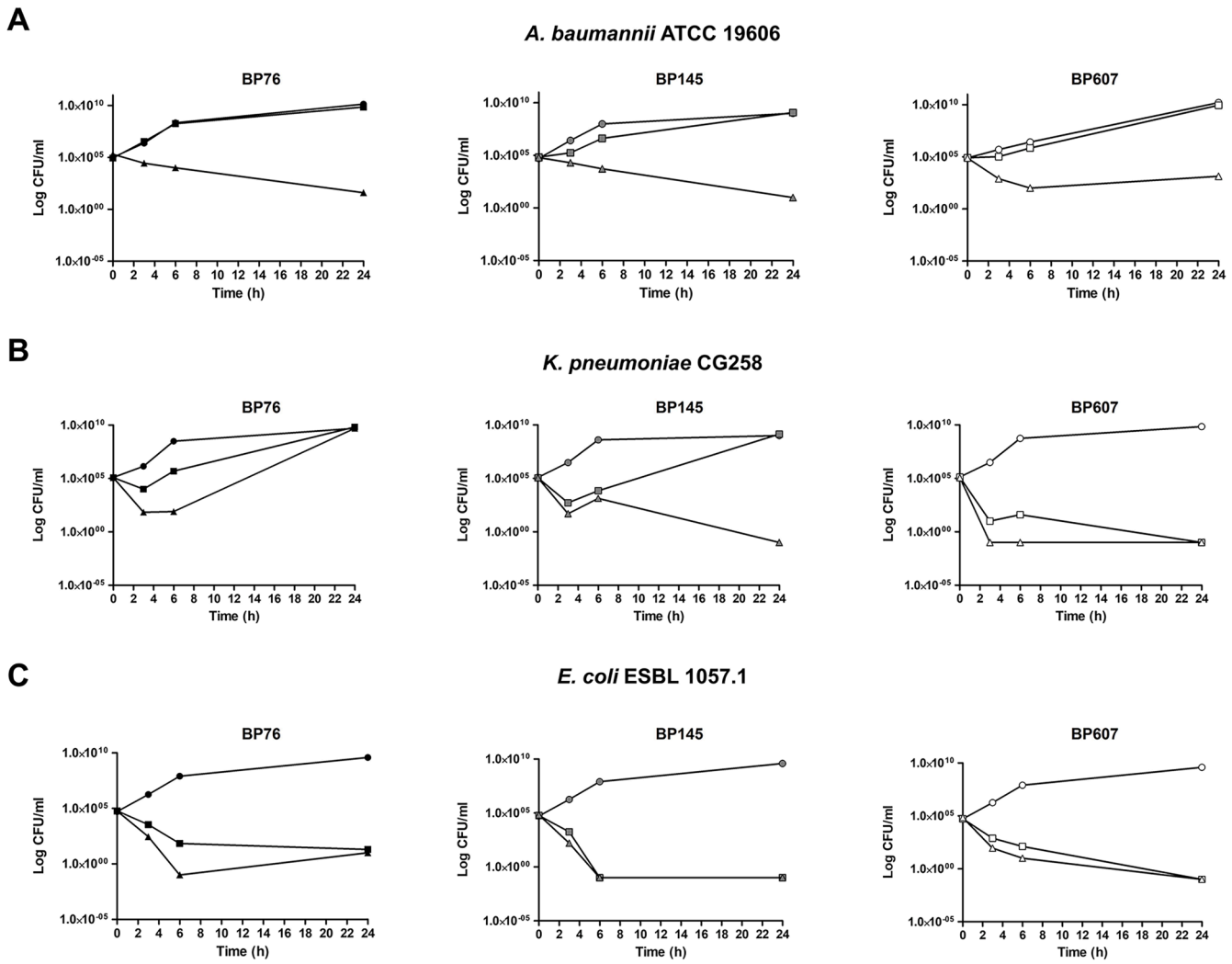

Fig. 2. Time-killing assays for BP76, BP145, and BP607. Time-killing curves represent CFU counts in cultures at 0, 3, 6 and 24 h of the strains *A. baumannii* ATCC 19,606 (A), *K. pneumoniae* CG258 (B) and *E. coli* ESBL 1057.1 (C) in the presence of MIC (square) and 2× MIC (triangle) of peptides **BP607** (white), **BP145** (grey) and **BP76** (black). Time-killing curves in MHBII (circles) were carried out as a control.

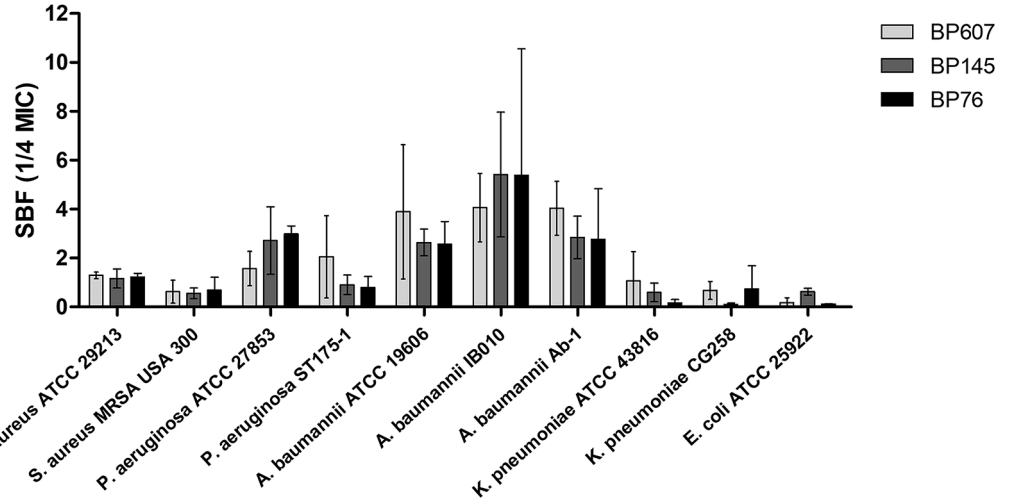

Fig. 3. Specific biofilm formation (SBF) index of the strains in the presence of **BP76, BP145** and **BP607** at subinhibitory concentration (¼ MIC). Bars represent the mean of the results of three independent assays with error bars representing the standard deviation.

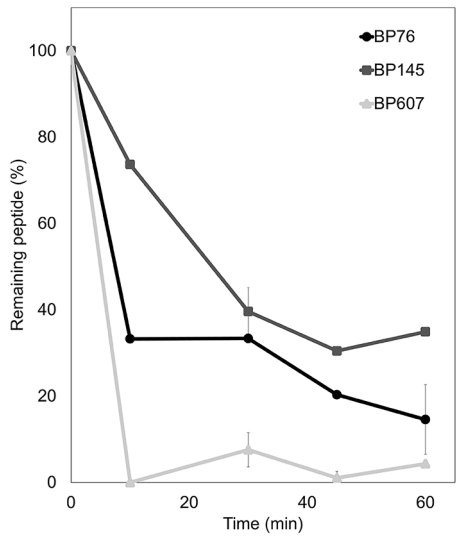

Fig. 4. Peptides stability in human serum. Percentage of remaining peptide at the end of the experiment (1h) is indicated. The average results from two independent replicates are shown.

3.6. Toxicity of BP76, BP145, BP607 and colistin

The percentage of hemolysis of peptides (Table 1) showed that the hemolytic activity of **BP76** and **BP145** is lower as compared to **BP607**.

Peptide toxicity was assessed in *G. mellonella*. First, a dose-response study was performed with colistin. Viability was assessed at 48h and LD_{50} established at 5mg/ml (Fig. 5A). Based on these results, the time course of mortality was determined for **BP76, BP145, BP607** and colistin at 50 μg/larvae (Fig. 5B). Of note, **BP76** and **BP145** presented less toxicity than colistin with 100 % and 70 % of survival after 24h, respectively. In contrast, **BP607** resulted in an 80 % of mortality after 24h, which was greater than values obtained for colistin (50 %).

4. Discussion

The search for novel antimicrobial compounds is of critical importance due to the rise in infections caused by resistant bacteria. Colistin has been successfully used as a last resource treatment for infections caused by multidrug resistant Gram-negative bacteria such as *A. baumannii, K. pneumoniae, E. coli* and *P. aeruginosa.* However, its toxicity and the increasing emergence of resistance has encouraged the search for novel alternatives (Eales et al., 2018). In a previous study, **BP100** analogues containing D-amino acids demonstrated activity against these pathogens, and highlighted **BP214** (renamed as **BP607** in this work) activity against *A. baumannii* (Oddo et al., 2016). Prompted by these results, a collection of 25 AMPs derived from **BP100** with antimicrobial properties against plant pathogens (Badosa et al., 2007, Badosa et al., 2013, Güell et al., 2011, Güell et al., 2012, Oliveras et al., 2018, Oliveras et al., 2021, Oddo et al., 2016) together with **BP607** (Oddo et al., 2016) were screened for their bactericidal and antibiofilm activity against 11 bacterial strains, including species of clinical concern and multidrug resistant bacteria.

Our initial screen revealed that susceptibility was highly species dependent. In general, *S. aureus* strains were the least susceptible to colistin, which was used as reference peptide throughout the study, and to the **BP100** analogues assayed in this study. The higher susceptibility of Gram-negative strains compared to Gram-positive strains could be attributed to the positive charge of the peptide sequences, which favors their interaction with the negatively charged lipid A of lipopolysaccharide, which is only present in Gram-negative bacteria (Moffatt et al., 2019). Among the Gram-negative bacteria, *P. aeruginosa* and *A. baumannii* strains were the least and the most susceptible, respectively, probably due to differences in membrane components of the target microorganism, *e.g.*, charge and lipid composition that would influence rates of binding of cationic peptides to the membranes. In addition, this result is in accordance with a previous study with **BP100** analogues (Oddo et al., 2016).

Interestingly, it has also been observed that the ability to inhibit bacterial growth is peptide-specific. In the present study we found peptides active against: (i) only *A. baumannii* ATCC 19,606; (ii) the three *A. baumannii* strains; (iii) *A. baumannii, K. pneumoniae* and *E. coli* strains; or (iv) all Gram-negative strains. In contrast, **BP76, BP145** and **BP607** displayed broad-spectrum activity, reducing the growth of either all Gram-negative or Gram-positive bacteria, including resistant strains, by ≈90 % at 50 μg/ml. Results obtained for **BP607** were similar to those previously described (Oddo et al., 2016). Peptides **BP76, BP145** and **BP607** were even more active than colistin, which was not active against *S. aureus* strains and *A. baumannii* IB010. These results confirm previous data indicating that subtle changes in an AMP have an important influence on its antimicrobial activity (Badosa et al., 2007, Badosa et al., 2013, Güell et al., 2011, Güell et al., 2012, Oliveras et al., 2018, Oliveras et al., 2021, Oliveras et al., 2022). Further studies including the design of new peptides incorporating systematic changes at each position are needed to decipher those residues playing a key role in inhibiting bacterial growth, and to determine the mechanism of action of these sequences.

In general, the MBC values of **BP76, BP145** and **BP607** coincided with the MIC, except for **BP145** against *A. baumannii* ATCC 19,606 and for **BP607** against *P. aeruginosa* ST175–1, *A. baumannii* ATCC 19,606 and *A. baumannii* IB010 that required two-fold the MIC to achieve the

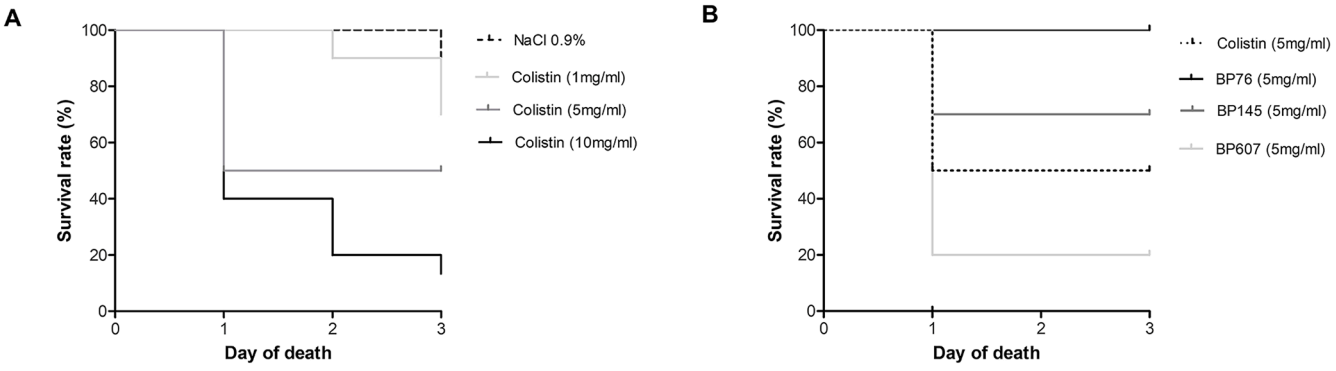

Fig. 5. Peptide toxicity in *G. mellonella*. (A) Survival curves for *G. mellonella* treated with colistin at increasing concentrations: 1mg/ml (light grey), 5mg/ml (dark grey), 10 mg/ml(black). A control group with saline solution (NaCl0.9 % wt/v) was included as negative control (dotted line). (B). Survival curves for *G. mellonella* treated with at 5 mg/ml of colistin (dotted line), **BP 76** (black), **BP145** (dark grey), and **BP607** (light grey). All larvae were injected with 10 μl (N=10 per group) for both experiments. Mortality was assessed as absence of movement upon prodding and heavy melanization.

MBC. Time-kill analysis of representative strains corroborated that **BP607** was bactericidal at the MIC against *K. pneumoniae* CG258 and *E. coli* ESBL 1057.1, but 2 × the MIC was required against *A. baumannii* 19,606, which is in agreement with previous results (Oddo et al., 2016). Regarding **BP145**, a significant reduction in the number of CFU/ml was observed for the three strains after treatment at the MIC for 6 h, but 2 × the MIC was necessary to sustain the bactericidal effect for 24 h. Peptide **BP76** was bactericidal at the MIC against *E. coli* ESBL 1057.1 and at 2 × MIC against *A. baumannii* ATCC 19,606. In contrast, 2 × MIC of **BP76** did not demonstrate bactericidal activity after 24 h against *K. pneumoniae* CG258. For instance, the presence and composition of the capsule in this strain may prevent the penetration of certain peptides to the cell or affect their structure (Fleeman et al., 2020).

It is noteworthy that the selected peptides feature potential to prevent formation of biofilm even below the MIC. Specifically, for resistant strains of *P. aeruginosa, A. baumannii*, and *K. pneumoniae* **BP76** and **BP145** values of SBF indices at subinhibitory concentrations (¼ MIC) were lower than those achieved by **BP607**. Differences observed between peptides may be due to differences in their mechanism of action or to a lesser extent, in the number of active molecules as slight differences in the active concentration have been found between compounds and strains. Further tests on the capacity of these peptides to eradicate biofilm are of interest. If corroborated, this may represent an advantage compared to colistin that performs well against planktonic cells of *A. baumannii* but is less effective against biofilm (Kim et al., 2015).

Of note, one of the novel peptides, **BP145**, had improved stability in serum compared to **BP607**. To a lesser extent, stability of **BP76** was also better than that of **BP607**. Concerning their toxicity, all three peptides displayed a similar hemolytic activity, despite it was slightly lower for **BP145**. Previous studies revealed 50 % cytotoxicity against HELA cells at 49.2 ± 1.4 mmol/L (Torcato et al., 2013) for **BP100**. Although cytotoxic studies to determine this activity for **BP76** and **BP145** are required, the highest MBC observed here was 0.04 mmol/L and 0.035 mmol/L, respectively, suggesting that clinical antimicrobial activity can be achieved. In line with this, *in vivo* toxicity testing of these compounds in the *G. mellonella* model demonstrated that the percentage of survival larvae inoculated with **BP76** and **BP145** is higher than that for colistin and **BP607.** Altogether, the results support the use of **BP76** and **BP145** to control infections by multidrug-resistant biofilm-producing species. Future potency studies with infection models to evaluate the effectiveness of the peptide to tackle the infection would be of interest. Continuation studies to analyze their mechanism of action as membrane permeabilization and the induction of resistance assays are warranted.

5. Conclusion

BP145 and **BP76** have broad-range antimicrobial activity including antibiotic resistant strains. Both are active at concentrations that are not toxic for humans, feature bactericidal activity at 6h and can inhibit biofilm formation. Further studies regarding mechanism of action or synergies with antibiotics would be of interest. In addition, this study lists other AMPs with more selective activity with potential to become group- or species-specific antimicrobial agents.

Author's contribution

MLS: Assisted in antimicrobial activity experiments and data analysis, performed *in vivo* toxicity tests, supervised the study, drafted the manuscript, revised the final version, and granted funding.

AT: Performed antimicrobial activity screening, MIC, MBC, biofilm inhibition experiments and time-kill assays. Analyzed data, drafted the manuscript and revised the final version.

PCF: Synthesized and purified peptides, performed stability assays and revised the manuscript.

MP: Designed, synthesized and purified peptides, supervised the study, drafted the manuscript, and revised the final version.

LF: Designed, synthesized and purified peptides, supervised the study, drafted the manuscript, and revised the final version.

MJM: Supervised the study, revised the manuscript and granted funding.

All authors contributed to the article and approved the submitted version.

Declaration of competing interest

MJM is founder and stockholder of the biotechnology spin-off company Vaxdyn, which develops vaccines for infections caused by MDR bacteria. Vaxdyn had no role in the elaboration of this manuscript. The other authors declare no conflict of interest.

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Data availability

Data will be made available on request.

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