Bat Cathelicidins as Natural Antimicrobial Agents: A Computational and *In Vitro* Investigation

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1. Introduction

Antimicrobial peptides (AMPs) are small proteins that play an important role in the innate immune system of various organisms, including plants, animals, and humans. These natural defense molecules have attracted considerable interest due to their potential as alternative antimicrobial agents to combat infectious diseases. In this study, we investigated the antimicrobial activity of cathelicidin peptides from three bat species with different ecological niches using computational and in vitro methods.

2. Materials and Methods

Bioinformatic workflow

Different bat genome species were studied to identify the sequences of cathelicidin. A comprehensive analysis of their structural and physicochemical properties was then performed. For structural analysis, Alphafold Colab was used to predict secondary structures, and the quality of these predictions was evaluated using the ERRAT program. Finally, the interaction between the peptide and the lipid membrane of bacteria was determined using Chimera X and the PPM web server.
Conversely, the physicochemical properties were evaluated using Expasy. Bioactivity prediction was performed using CRAMPR, Antifp, Meta-IAVP, and HemoPI programs.
After the *in-silico* analysis, three peptides were selected: DR_GL31, Aj2_GD29 and Es_GL33 from *Desmodus rotundus*, *Artibeus jamaicensis* and *Eonycteris spelaea* respectively. (NCBI Reference Sequence: NC_071394.1, JAIVGF010000184.1 and PUFA01000145.1)

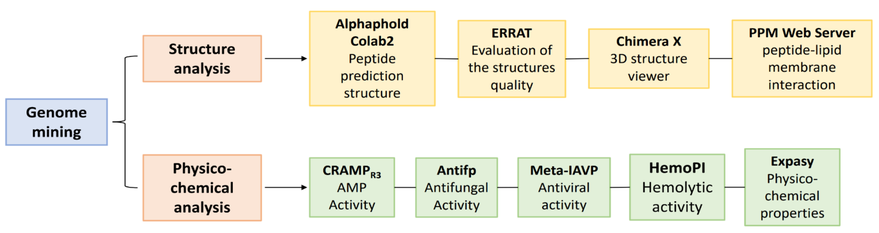

Figure 1. Bioinformatic workflow.

Determination of MIC and MBC values

The minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC) were calculated to determine the concentrations at which the selected peptides were effective against four human pathogens: *Escherichia coli, Staphylococcus aureus, Enterococcus faecalis,* and *Salmonella enterica.*

The initial bacterial count was approximately 10^5 bacteria/ml. The concentrations of the tested peptides ranged from 50 µM to 0.39 µM, and the incubation time was 18 hours at a temperature of 37°C. MBC and MIC values were determined by quantifying the number of bacterial colonies that survived after the incubation period.

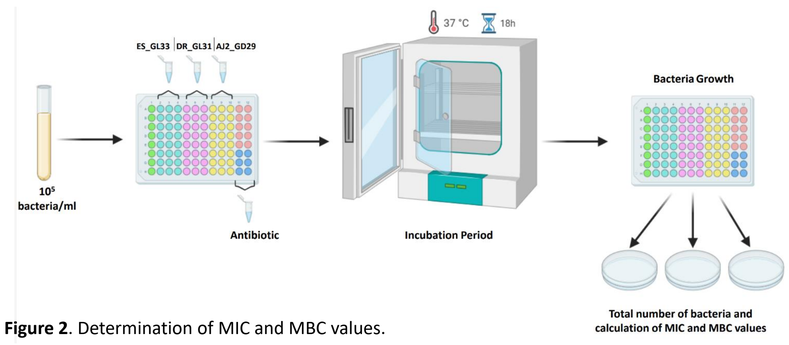

Figure 2. Determination of MIC and MBC values.

Time-kill analysis

To gain a more comprehensive insight into the antimicrobial efficacy of the peptide, kill curves were generated for both the latent and exponential growth phases. Bacterial growth was monitored continuously over a 9-hour period, with measurements taken hourly and again after 24 hours.

The peptide selected for these experiments was DR _GL31, which showed the highest activity against *Salmonella enterica* and *Staphylococcus aureus*.

For the latent phase assay, the antimicrobial peptide was introduced at the beginning of the experiment, when the bacterial population was approximately 10^5 cells/ml, with its minimum inhibitory concentration (MIC) of 1.56 µM. In contrast, in the exponential phase assay, the peptide was added as soon as the bacteria showed signs of an exponential growth phase. Consequently, the initial inoculum was significantly higher and the peptide concentrations used were 1.56 µM and 3.125 µM.

Hemolytic activity

The potential toxicity of peptides may have negative effects on blood cells, making them unsuitable as potential antimicrobial drugs. To address this issue, different concentrations of the peptides were combined with erythrocytes from rats. Tests were performed to determine whether the peptides could induce cell lysis, using Triton X-100 at a concentration of 0.1% as a control.

3. Results

Bioinformatic analysis

	Structure analysis					Physico-chemical analysis			
Selected Peptides	**Hydrophobicity***	**Hydrophobic moment***	**Net charge**	**Hydrophobic face**	**Membrane interaction**	**Antimicrobial activity**	**Antifungal activity**	**Hemolytic activity****	**Antiviral activity****
Dr_GL31	0.109	0.771	+7	L L I I L I		AMP	Not Active	0.51	0.994
Aj2_GD29	-0.003	0.365	+3	-		AMP	Active	0.49	0.878
Es_GL33	0.421	0.654	+5	L L L I L L I I		AMP	Not Active	0.48	0.770

Table 1. Bioinformatic analysis results. Based on the in silico analysis of 41 bat species, we selected three peptides with different properties and structures to perform the in vitro assays. Based on these results, we predicted that the Dr_GL31 peptide would have the highest affinity for biological membranes and consequently exert the most remarkable antimicrobial activity. In contrast, the peptide Aj2_GD29 was predicted to have the lowest activity.
* The values for hydrophobicity and hydrophobic moment were taken from Heliquest (https://heliquest.ipmc.cnrs.fr/)
** Values range from 0 to 1, with 1 representing a very active peptide and 0 representing an inactive peptide.

Determination of MIC and MBC value

Microorganism	Dr_GL31		Aj2_GD29		Es_GL33	
	MIC (µM)	MBC (µM)	MIC (µM)	MBC (µM)	MIC (µM)	MBC (µM)
***E. coli* (CECT 434)**	6.25 – 3.125	12.5 – 6.25	>50	>50	12.5 – 6.25	25 – 12.5
***S. aureus* (CECT 794)**	3.125 – 1.56	3.125 – 1.56	>50	>50	50 - 25	100 - 50
***E. faecalis* (CECT 795)**	50 – 25	>50	>50	>50	>50	>50
***S. enterica* (CECT 456)**	3.125 – 1.56	3.125 – 1.56	>50	>50	50 - 25	50

Table 2. MIC and MBC values. The peptide designated Dr_GL31 exhibited the strongest antibacterial activity, as evidenced by its significantly reduced minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values. This increased activity can be attributed to its specific physicochemical properties. In contrast, the antibacterial activity of the Aj2_GD29 peptide was significantly lower, as shown by the MIC and MBC values above 50 µM.

Time-kill curve analysis

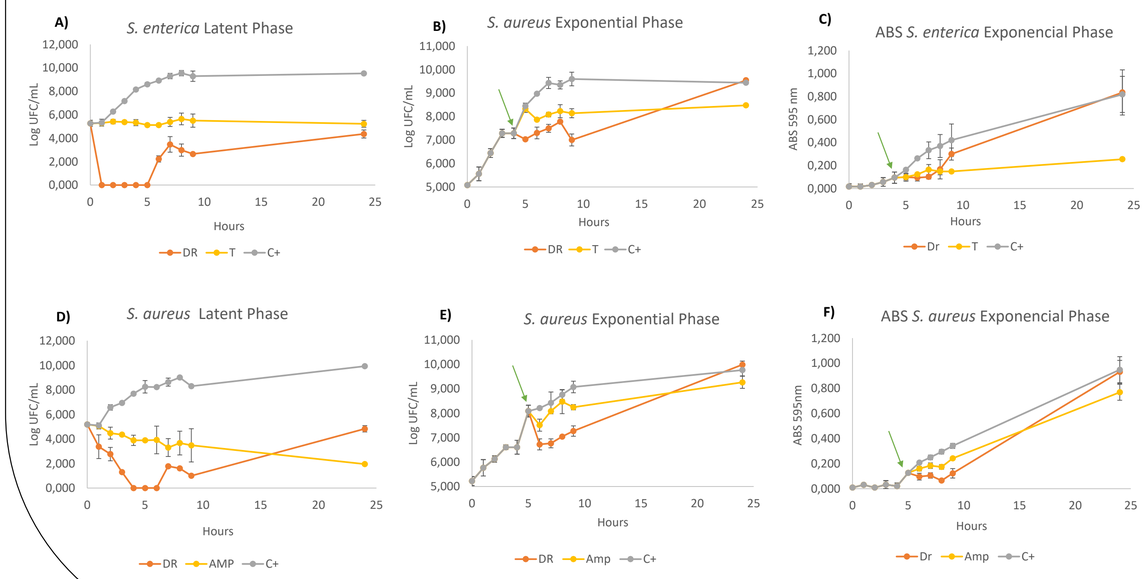

Figure A. *S. enterica* growth culture after 105 bacteria/ml treatment with DR_GL31 at 1.56 µM for 24 hours. Under the above conditions, the selected peptide showed bactericidal activity in every hour.
Figure B. The peptide DR_GL 31 at a concentration of 3.125 µM was added to the culture after 4 hours of incubation (marked with an arrow). In this case, it showed a bacteriostatic effect and inhibited bacterial growth up to the 9-hour mark, corresponding to five hours after the addition of the peptide.
Figure C. During the kill curve in exponential growth phase, absorbance measurements were monitored. The observed decrease in absorbance aligned with the decline in bacterial culture density, indicating that DR_GL31 exerts a lytic effect against *S. enterica.*
The antibiotic used as positive control in *S. enterica* was tetracycline.
Figure D. *S. aureus* growth culture after 10^5 bacteria/ml treatment with DR_GL31 at 1.56µM for 24 hours. After the first 2 hours of incubation, the peptide showed bactericidal behavior during the following 24 hours.
Figure E. The peptide DR_GL31 at a concentration of 3.125 µM was added to the culture after 5 hours of incubation (marked with an arrow). Between hours 5 and 9, the peptide showed a bacteriostatic effect and temporarily stopped bacterial growth. However, the growth of the culture recovered after 24 hours.
Figure F. Absorbance measurements were taken every hour during the exponential growth phase of the kill curve experiment. The significant decrease in absorbance, which parallels the decrease in bacterial culture, indicates that DR _GL31 exerts a lytic effect against *S. aureus*.
Ampicillin was used as a positive control for *S. aureus*.

Hemolytic activity

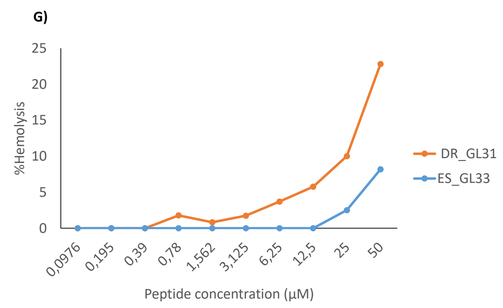

Figure G. Percentage of hemolytic activity at different peptide concentrations. Because the peptide Aj2_GD29 did not show significant antimicrobial activity, we focused on evaluating the hemolytic activity of the peptides DR_GL31 and ES_GL33. Both showed minimal ability to lyse rat erythrocytes, with possible adverse effects at concentrations above 25 µM. Interestingly, neither of these peptides caused damage at the MIC and MBC levels.

4. Conclusions

After a comprehensive in silico analysis of 41 different bat species, three peptides from *Desmodus rotundus*, *Artibeus jamaicensis*, and *Eonycteris spelaea* were selected based on their physicochemical properties. These peptides are designated as DR _GL31, Aj2_GD29, and Es_GL33.
After determining the MIC and MBC values for each peptide, it was found that DR _GL31 had the highest activity against Salmonella and S. aureus. For this reason, we investigated the duration of its activity and its ability to lyse bacterial cells. For this purpose, we plotted the killing curves during both the exponential and latent phases of bacterial growth. Remarkably, DR _GL31 exhibited bactericidal activity during the stationary phase of growth, whereas it exerted bacteriostatic activity during the exponential phase in both human pathogens. In addition, the peptide showed the ability to lyse bacterial cell membranes.
To fully evaluate the safety of the peptide, its potential to induce hemolysis in rat erythrocytes was investigated, and no significant hemolytic activity was observed.

5. Acknowledgments

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