In silico and *in vitro* approach of antimicrobial peptides from fish

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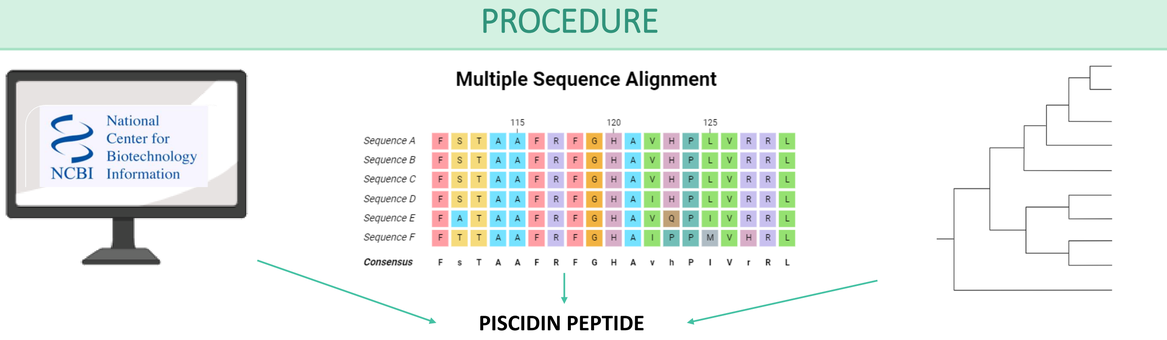
INTRODUCTION

Fish engage in continual interactions with microorganisms within their aquatic environments. As a defense mechanism, they possess innate immune system-derived antimicrobial peptides that combat bacteria, viruses, and fungi [1,2,3]. The piscidin family's characterization has been limited to Teleostei fish. This study aimed to select a subset of peptides annotated as piscidins in NCBI and evaluate their bioactivity and structure through both *in silico* and *in vitro* methodologies.

PROCEDURE



***In silico* predictions:**
- Physicochemical features (DBAASP)
- Structure (Alphafold)
- Membrane interaction (PPM)
- Antibacterial, antifungal, antiviral activity (DBAASP)

***In vitro assays*:**
- Antimicrobial activity (MIC, MBC, time-kill curve assay)
- Toxicity test against rat erytrocites, Vero and Cos7 cell lines.

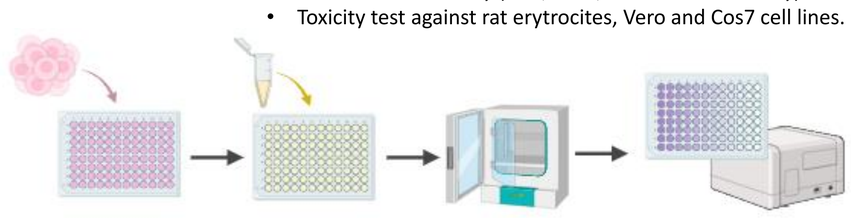


Figure 1: Schematic procedure of the piscidin identification and bioinformatic and in vitro analysis.

IN SILICO RESULTS

Based on 51 annotated piscidin sequences in NCBI, we followed the procedure outlined in several articles [4] to derive the predicted peptides. Alignment with well-characterized piscidin peptides enabled us to obtain peptides ranging from 22 to 25 amino acids, which generally exhibit a positive charge and an alpha-helical structure. The results in the following tables present the bioinformatic analysis of the five selected piscidin peptides and Epinecidin-1 (from *Epinephelus coioides*) as reference peptide: their physico-chemical properties (Table 1) and the predicted bioactivity (Table 2).

Table 1: Results of predicted phisico-chemical features of selected piscidin peptides using DBAASP web server.

	Nº aa	Net Charge	pI	Normalized Hydrophobicity	Normalized Hydrophobic moment
Ea_FF25	25	4	12.27	0.03	0.18
Sd_FI25	25	5	12.42	0.06	0.21
Tm_II22	22	3	11.57	-0.06	0.19
Ar_IW23	23	0	8.12	-0.07	0.11
Dl_FI22	22	3	12.12	-0.06	0.23
Epinecidin-1	25	5	12.42	0.01	0.15

The distinct features of molecules and the amino acids composing them lead to diverse interactions with cell membranes. In Figure 2, we can observe the two predicted types of interactions of the selected peptides with the membrane: transversely or superficially. Tm_II22 exhibit an orientation type B and the other 5 type A.

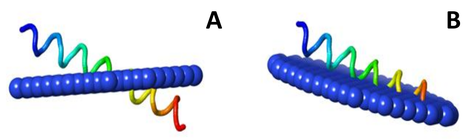
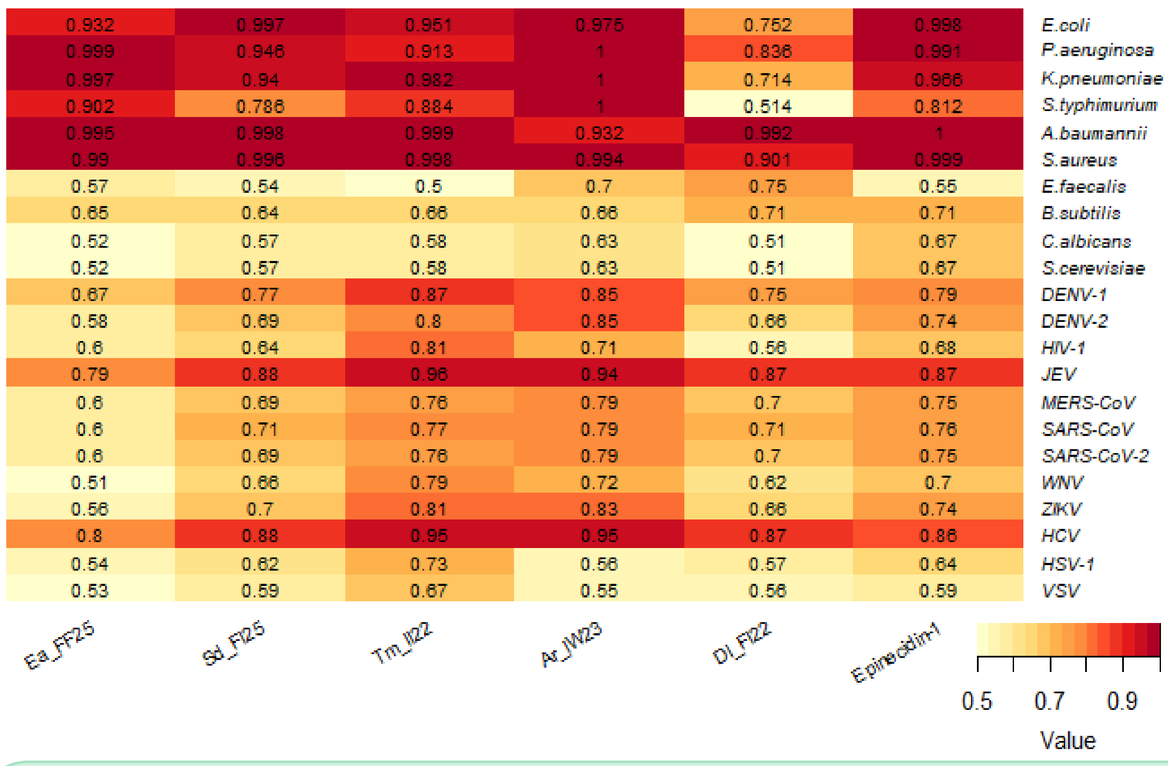


Figure 2: Predicted orientations of Proteins in Membranes using PPM Web Server. **A**: transversal orientation **B**: supeficial orientation.

Table 2: Heatmap of prediction of antibacterial, antifungal, and antiviral activity of piscidin peptides selected using the DBAASP web server. Values indicate antimicrobial score (from 0 to 1).



IN VITRO RESULTS

Microdilution assays were conducted to establish the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) for each peptide using human pathogens (*Escherichia coli, Staphylococcus aureus, Salmonella enterica, Enterococcus faecalis, Pseudomonas aeruginosa, Campylobacter jejuni,* and *Candida albicans*) as test subjects. Notably, the outcomes demonstrated significant antimicrobial activity at low concentrations, including 1.56 µM against *S. aureus,* 3.125-6.25 µM against *P. aeruginosa* or 0.78 against *C. jejuni* (Table 3).

Table 3: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the peptides were determined against 8 human pathogens. The results are expressed in µM.

		Ea_FF25	Sd_FI25	Tm_II22	Ar_IW23	Dl_FI22	Epinecidin-1
E. coli	MIC	25-50	3,125-6,25	25	>50	6,25-12,5	3,125-6,25
	MBC	25-50	3,125-6,25	12,5-25	>50	6,25-12,5	3,125-6,25
S. enterica	MIC	>50	6,25-12,5	>50	>50	12,5-25	12,5-25
	MBC	>50	6,25-12,5	>50	>50	12,5-25	12,5-25
S. aureus	MIC	25-50	1,56-3,125	3,125	12,5	**1,56**	3,125-6,25
	MBC	25-50	1,56-3,125	6,25-12,5	25-50	1,56-3,125	3,125-6,25
C. jejuni	MIC	12,5-25	3,125-6,25	1,56-3,125	6,25-12,5	0,39-0,78	3,125-6,25
	MBC	12,5-25	3,125-6,25	3,125-6,25	12,5-25	0,78-1,56	3,125-6,25
E. faecalis	MIC	>50	12,5-25	3,125-6,25	25-50	3,125-6,25	25-50
	MBC	>50	12,5-25	3,125-6,25	25-50	3,125-6,25	25-50
P. aeruginosa	MIC	25-50	3,125-6,25	25-50	>50	>50	12,5-25
	MBC	25-50	3,125-6,25	25-50	>50	>50	12,5-25
C. albicans	MIC	>50	>50	>50	25-50	>50	>50
	MBC	>50	>50	>50	25-50	>50	>50

Time-kill curve assays were conducted against *S. aureus* in both stationary and exponential growth phases for 9 hours to assess the impact of the most active peptide (Dl_FI22) against this bacteria. Viable cell counts were taken hourly, revealing that the peptide exhibited bactericidal effects, reducing the bacterial count by up to 3 logarithms per ml (Figure 3).

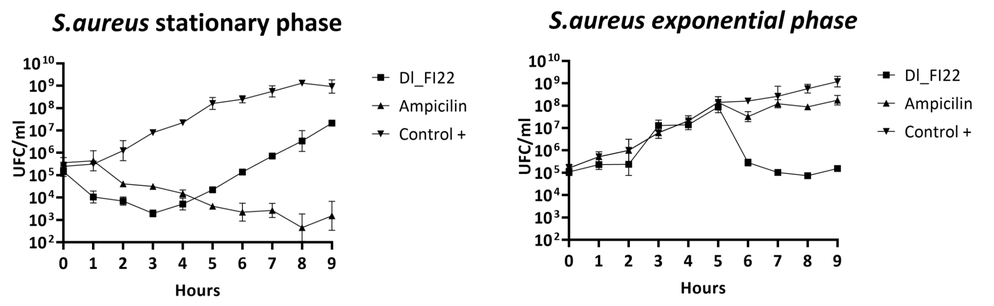


Figure 3: Graphics of time-kill assay for 9 hours introducing the peptide Dl_FI22 1,56 µM (MIC concentration) and Ampicilin 100 µM in the stationary pase (left graph) and Dl_FI22 3,125 µM (Double MIC concentration) and Ampicilin 100 µM in the exponential phase, hour 5 (right graph).

On the other hand, toxicity tests were conducted to observe if the peptides at MIC/MBC concentrations induced hemolysis in rat erythrocytes and toxicity in non-tumoral mammalian cell lines: Vero (kidney epithelial cells from African green monkey) and Cos7 (kidney fibroblast from the African green monkey).

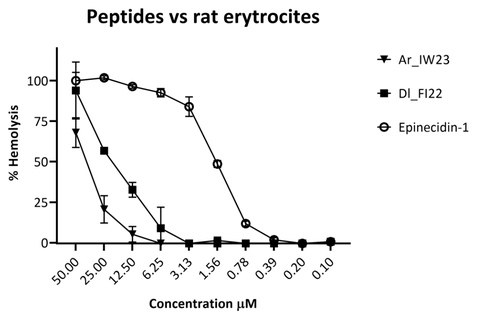
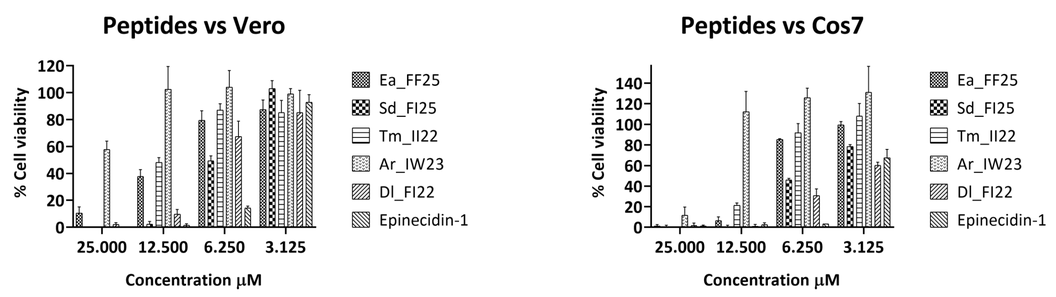


Figure 4: Hemolytic activity graph

Four of the tested peptides exhibited high hemolytic activity against rat erythrocytes, whereas Ar_IW23 and Dl_FI22 demonstrated activity that decreased at lower concentrations (including MIC/MBC values) (Figure 4). At 1.56 µM (MIC against *S. aureus*), Dl_FI22 does not produce hemolysis.

The toxicity test against non-tumoral cell lines was conducted with MTT technique. The results (Figure 5) show low viability of the cells with higher concentrations of peptides (50-25 µM) but less pronounced toxicity at lower concentrations. We can also observe that there are differences between the two types of cells; the Cos7 cells appear to be more sensitive to the peptides.



Vero	Ea_FF25	Sd_FI25	Tm_II22	Ar_IW23	Dl_FI22	Epinecidin-1
IC50	10.21	6.25	11.73	25.42	7.31	4.70

Cos7	Ea_FF25	Sd_FI25	Tm_II22	Ar_IW23	Dl_FI22	Epinecidin-1
IC50	8.24	5.47	9,84	23.05	2.19	3.53

Figure 5: Toxicity analysis of peptides against Vero and Cos7 cell lines: % cell viability relative to the control at each concentration and IC50 values

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CONCLUSION

The six piscidin peptides exhibited *in vitro* antimicrobial activity, particularly against bacteria, which was consistent with the *in silico* predictions (high scores with bacteria). Only one peptide displayed activity against *C. albicans* in vitro below 50 µM. The toxicity tests against erythrocytes and non-tumoral cell lines showed hemolytic activity and reduced cell viability at higher tested concentrations, but not as much at the MIC/MBC values of the peptides. These results strongly suggest the potential of these peptides for combating microorganisms and addressing antibiotic resistance. However, further research is required to explore their mode of action and other immunomodulatory activities.

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