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D-amino acid peptides as antimicrobial agents against vibrio-associated diseases in aquaculture

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ABSTRACT

Vibriosis is one of the most usual infection diseases in bivalve mollusks, particularly affecting seeds and larvae, which are more susceptible than adults to these bacterial infections. The devastating effect of vibriosis in shellfish hatcheries is aggravated by the increasing resistance of many Vibrio species to traditional antibiotics, highlighting the need to find new antimicrobial agents. Antimicrobial peptides (AMPs) are a promising alternative, however, their low stability can be a handicap for their practical application. In this study, the 3D structure and the stability of a synthetic all-D-amino acid peptide (D-Caerin) to proteases and extreme pH conditions have been analyzed and compared with its corresponding natural L-enantiomer peptide, Caerin 1.1. Moreover, the antimicrobial activity of D-Caerin has been tested in vitro against the control bacteria *Micrococcus luteus* CECT 245; and four Vibrio species: *Vibrio aestuarianus* CECT 625 T, *Vibrio anguillarum* CECT 522 T, *Vibrio harveyi* CECT 525 T and *Vibrio tapetis* CECT 4600 T, which are among the most representative causative agents of vibriosis in aquaculture. Our results demonstrate that D-Caerin contains two left-handed alpha helices, and is more stable and effective against the bacterial species tested than its corresponding natural L- counterpart. The bioactivity of D-Caerin has also been tested in vivo, in clam seeds infected with a mixed inoculum of these vibrio species. These preliminary assays show that D-Caerin-treated specimens exhibit higher survival rates after infection, demonstrating the superior stability of D-Caerin and its effectiveness against vibriosis-causative agents.

1. Introduction

Antimicrobial peptides (AMPs) are short, generally amphipathic and cationic defensive gene-encoded peptides, present in most living organisms, from bacteria, insects, and plants to vertebrates, including humans. This wide distribution suggests that AMPs have an essential role in microbial ecology and the innate immunity of most organisms (Lazzaro et al., 2020; Shabir et al., 2018). The mechanism of action of these peptides, which exhibit antimicrobial activity against a large number of bacteria, viruses, fungi, and protozoa, begins with an electrostatic interaction with the negatively charged cell membranes of target cells, resulting in their permeabilization and causing lysis and death of the cells. Eventually, AMPs can pass through the membrane and interact with different intracellular targets. The apparent non-specificity of the interaction with the membrane and the multiple final targets of these peptides seem to be the basis for the difficulties that bacteria find in developing resistance against them (Peschel and Sahl, 2006; Benfield and Henriques, 2020). Many of these AMPs exhibit, in addition to antimicrobial properties, other bioactivities, such as immunomodulatory functions, induction of cytokine production and inflammatory responses, or regulation of chemotaxis and angiogenesis, among others (Hancock and Diamond, 2000; Haney et al., 2019; Mitta et al., 2000; Valero et al., 2020).

Small cationic peptides of different origins are active against a broad

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spectrum of common pathogens in aquaculture. León et al., 2020 showed that the growth of 15 bacteria and 4 of the most devastating viruses in aquaculture was inhibited to a different extent by the peptides tested, indicating their potential as antibacterial and antiviral agents. Similar results were observed for peptides obtained from frog epithelial tissue (Apponyi et al., 2004), mussels (Rey-Campos et al., 2020), sea crab (Zhu et al., 2021), and many other organisms, demonstrating their antimicrobial effect *in vitro* and their low cytotoxic effect on animal cell lines (Boto et al., 2018). In addition, it has been described that the expression of genes encoding certain AMPs, such as NKL in sea bass (Valero et al., 2021) or myticins in mussels (Rey-Campos et al., 2019), is strongly induced in response to infectious processes, wounds and other stress conditions (Rey-Campos et al., 2020; Sendra et al., 2020). Although bivalves lack an adaptive immune system, they have a very powerful innate immune response. Moreover, there is increasing evidence of their memory to recognize pathogens and respond to infections at both cellular and molecular levels, by releasing reactive oxygen species or by the production of AMPs, among others (Allam and Raftos, 2015; Kurtz, 2005). All these data indicate that AMPs are essential in the innate immune response of these invertebrates and suggest their potential to control infections in bivalves. However, the activity of these peptides against usual aquaculture infective species has been poorly explored (Cuesta et al., 2021; Shabir et al., 2018). The number of antimicrobial peptides described has increased exponentially in the last years (Huan et al., 2020) and some of them have been proposed as antibiotics substitutes against drug-resistant microorganisms. However, low stability and supply difficulties have limited their use in clinical or veterinary practice (Erdem Büyükkiraz and Kesmen, 2022).

Vibriosis is among the most usual infection diseases in bivalves, affecting particularly seeds and larvae, which are more susceptible than adults to these bacterial infections. The devastating effect of vibriosis in shellfish hatcheries is aggravated by the increasing resistance of many Vibrio species to traditional antibiotics. This evidences the need to find alternative antimicrobial agents (Sanches-Fernandes et al., 2022).

In this study, we compared the bioactivity of Caerin 1.1, an antimicrobial peptide that has shown high inhibitory capacity against human and animal pathogens (Chen et al., 2021; Lei et al., 2019; León et al., 2020), with that of its all-D-aminoacids counterpart, D-Caerin. D-oligopeptides are expected to be much more resistant to degradation by proteases, for which they are not natural substrates. However, it is necessary to demonstrate its stability under different degrading conditions and its antimicrobial activity, which largely depends on its 3D-structure.

2. Material and methods

2.1. Synthesis of peptides

Peptides: D-Caerin (G{d-L}{d-L}{d-S}{d-V}{dL}G{d-S}{d-V}{d-A}{d-K}{d-H}{d-V}{d-L}{d-P}{d-H}{d-V}{d-V}{d-P}{d-V}{d-I}{d-A}{d-E}{d-H}{d-L}) and Caerin 1.1. (Uniprot accession no. P62568) were synthetized by GenScript Corp. (Piscataway, NJ, USA) with a purity higher than 95%, as checked by HPLC. Lyophilized peptides were resuspended in sterile ultrapure water (10 mg mL^{-1}), aliquoted and stored at −20 °C until their utilization.

2.2. Bacterial strains and culture conditions

The control bacterium *Micrococcus luteus* CECT245 was cultured at 37 °C in Tryptic Soy broth (TSB). The fish pathogens *Vibrio aestuarianus* CECT 625 T, *Vibrio anguillarum* CECT 522 T, *Vibrio harveyi* CECT 525 T and *Vibrio tapetis* CECT 4600 T, from the Spanish collection of type cultures, were cultured in marine agar (MA) or marine broth (MB) at 26 °C. All the strains were incubated with rotatory agitation (150 rpm).

2.3. In vitro antimicrobial activity

Antimicrobial activity was evaluated by measuring the diameters of inhibition growth halos on lawn-grown bacteria, as previously described (León et al., 2020). Agar plates were inoculated with 0.5 mL of the corresponding bacteria ($OD_{530} = 1$), that were spread across the whole plate surface and 5 μL-drops (stock solution 10 mg mL^{-1}) of the tested peptides were spotted on the surface of well-dried plates. After 24 h of incubation at the indicated temperature, the diameters were measured.

The Minimal Inhibitory Concentration (MIC) was determined as described by Wiegand et al., 2008, with minor modifications. The essays were carried out in 96-well microplates (sterile and flat bottom), where 100 μL of suspension of the corresponding log-phase bacterial culture, containing about 10^4 CFU, were mixed with serial dilutions of the peptides to obtain concentrations from 0.125 μg mL^{-1} to 835 μg mL^{-1} in a final total volume of 150 μL. A negative control containing sterile culture medium without inoculum, and a bacterial-growth control with the bacterial inoculum and no peptide, were also included. Microplates were incubated for 48 h under constant shaking at 37 °C for *M. luteus* and at 26 °C for the of *Vibrio* strains. Bacterial cell density was registered at 0 and 48 h in a microplate reader (Fluostar Omega, BMG Labtech) at $\lambda =$ 530 nm and the MIC, defined as the lowest peptide concentration at which no bacterial growth was detected, was calculated. To determine the Minimal Bactericidal Concentration (MBC), aliquots of each well (100 μL) were diluted with the corresponding medium and plated out in triplicate on solidified-agar plates with TSB medium for *M. luteus* and MA medium for the *Vibrio* species. After overnight incubation at optimal temperatures, the CFU were counted to estimate the MBC, defined as the peptide concentration at which no colony growth was observed. All determinations were done in triplicate.

2.4. Enzymatic digestion of peptides with immobilized trypsin

D- and L- peptides were dissolved in the digestion buffer, consisting in 0.1 M NH_4HCO_3 pH 8.0, at a concentration of 1 mg mL^{-1}. The trypsin enzyme preparation (0.2 mL) was washed with 3 × 500 μL of digestion buffer and resuspended in 0.2 mL of digestion buffer. 1 mL of each peptide solution (1 mg mL^{-1}) was mixed with the washed enzyme preparation and incubated at 37 °C during the indicated time.

2.5. Circular dichroism studies

Circular dichroism (CD) spectra of the studied peptides were recorded in a Biologic Mos-450 spectropolarimeter using a standard quartz cell of 10 mm path length. The spectra show the average observed ellipticity of 10 runs, expressed in millidegrees (Θ_{obs}, mdeg) and plotted against wavelength. All measurements were performed at 25 °C in 50% (*v*/v) 2,2,2-Trifluoroethanol (TFE).

2.6. Chromatographic peptide analysis

Quantification of the studied peptides and their derived peptide fractions was performed in a Hitachi Chromaster HPLC equipped with an auto sampler and a UV–Vis DAD detector (Hitachi High-Tech Corporation, Tokyo, Japan), using a RP-18 column (ODS 3 μm 250 × 4,6) and a continuous gradient of 0%–80% acetonitrile in 0.1% trifluoroacetic acid (TFA), for 20 min at 1 mL min^{-1}. Peptides were detected at 214 nm and quantified on the basis of calibration curves. Chromaster System Manager software was used for data processing.

2.7. Optimizing D-Caerin dose for Ruditapes philippinarum seeds

Healthy *R. philippinarum* seeds, with a mean size and weight of 6 ± 1 mm and 38 ± 3 mg, respectively, obtained from the company Culmasur (Huelva, Spain), were acclimated to lab conditions in continuously aerated filtered seawater seawater (FSW) at room temperature (25 °C)

for 5 days. Then, clam seeds were distributed in glass containers with 10 mL of FSW, at a density of 20 clam seeds per container, and incubated in triplicate with two different doses of D-Caerin (0.1 mg mL^{-1} and 1 mg mL^{-1}) or without D-Caerin (Control assay) during 2 h or 24 h. After the treatment, clam seeds were rinsed with filtered seawater (FSW) without the peptide and maintained at continuous aeration in FSW. Clams mortality was checked periodically during the 7 days following the treatment.

2.8. Infection assays

Lab-acclimated healthy clams, pretreated with 0.1 mg mL^{-1} of D-Caerin for 2 h, as described above, were distributed in glass containers with 10 mL of FSW at a density of 1 clam seed per mL of FSW. After 24 h, D-Caerin-pretreated clams were infected with a mixed inoculum consisting of *V. aestuarianus* ($2.2{\cdot}10^{10}$ CFU mL^{-1}), *V. anguillarum* ($9.5{\cdot}10^{10}$ CFU mL^{-1}), *V. harveyi* ($1.2{\cdot}10^{10}$ CFU mL^{-1}) and *V. tapetis* ($7{\cdot}10^{10}$ CFU mL^{-1}). Initial bacterial concentrations were calculated by counting the CFU obtained after spreading serial dilution suspensions of the indicated bacteria on marine agar plates. The final volume was maintained at 1 mL per clam in all the infection essays, adding FSW when necessary to compensate evaporation. A control assay with non-pretreated clam seeds inoculated with the same Vibrios mixture (NP-Infected control) was run in parallel. Control essays with pretreated (DCAE-non infected) and non-pretreated (NP-non infected) clam seeds not exposed to the infection were also run. All assays were done in triplicate. Evolution of the infection essays was daily monitored for 7 days, removing dead clams when they were detected.

3. Results

3.1. Peptide stability and functionality at different extreme conditions

Most peptides have low stability, being easily degraded when exposed to oxidative conditions, extreme pH, high temperatures, or the action of proteases. To check if the substitution of L-amino acids by the corresponding D-enantiomers makes Caerin less susceptible to degradation, the stability of D-Caerin was compared with that of the corresponding natural L-Caerin. Both peptides were incubated at three different conditions: i) with the proteolytic enzyme Trypsin at 37 °C, ii) at high salinity and pH 8 at room temperature, to simulate seawater and iii) at pH 2 and 37 °C, to mimic the acidic conditions of animal stomachs. Both, D- and L-Caerin peptides were incubated at the indicated conditions, and their integrity followed during 2 h, in the case of incubation with the protease, and during 12 days in the other essays. Samples were periodically withdrawn at different times and the percentage of non-degraded peptide was calculated from the chromatographic analysis of the incubation mixtures (Fig. 1).

Immobilized TPCK trypsin (Thermo Scientific) was chosen to check the stability of the peptides against proteases. Trypsin is a serine endonuclease able to cleave peptide bonds on the carboxylic extreme of arginine or lysine residues. This enzyme has been treated with a reagent to specifically inhibit the activity of the contaminant chymotrypsin and is supplied immobilized by crosslinking in agarose resin. This facilitates sampling and allows the identification of the original and hydrolysis-derived peptides by HPLC, avoiding the interference of the own protease and their hydrolysis products. 0.2 mL of trypsin enzymatic preparation were added to 1 mL of the AMPs solution (1 mg mL^{-1}), as indicated in materials and methods, and incubated at 37 °C for 2 h. Samples were taken at 0, 15, 30, 45, 60, and 120 min after the addition of the enzyme, centrifuged at 13,000 rpm for 1 min, and analyzed by HPLC. Digestion of Caerin with trypsin is predicted by PeptideCutter tool (Gasteiger et al., 2005) to generate 2 fragments by cleavage of the peptidic bond between K11 and H12. Caerin, either in L- or D- isoform, can be detected by its absorbance at 214 nm. The lack of aromatic amino acids prevents the peptide from absorbing at the typical protein wavelength of $\lambda = 280$ nm. HPLC chromatogram, followed at $\lambda = 214$ nm, allows identification of Caerin, with a retention time of around 26 min in our chromatographic conditions. It can be observed that after incubation with trypsin, L-Caerin disappears and two new peaks, corresponding to the smaller peptides generated by trypsin treatment, arise. D-Caerin, on the contrary, stays uncut during the trypsin treatment (Fig. 1A-1D). The structure of the obtained peptides was predicted with PEPFOLD-3 software (Shen et al., 2014) and visualized with Chimera (Pettersen et al., 2004). The two fragments generated are predicted to adopt alpha helix structures, as the original L-Caerin, from which they are generated (Fig. 1E). Tryptic digestion of L-Caerin, which contains a Lys residue in position K11, is very fast. 15 min after the addition of trypsin, 100% of the peptide has been hydrolyzed. D-Caerin, on the other hand, is not hydrolyzed by trypsin and practically all the peptide remains uncut after two hours of enzymatic treatment (Fig. 1; Fig. 2A). This demonstrates the superior stability to proteases of D-Caerin, which does not contain L-amino acid residues.

D-Caerin is, not only less susceptible to enzymatic degradation, but also much more stable at acidic conditions and high saline concentrations, as demonstrated when both L- and D- Caerin were incubated in sterile seawater with a salinity of 33 g L^{-1} at pH 8 (Fig. 2B) and in sterile distilled water at pH 2 and 37 °C (Fig. 2C). After 2 h of incubation at pH 8 in seawater, 60% of L-Caerin has been degraded. After the same time of incubation at pH 2, 40% of L-Caerin has been hydrolyzed. While the

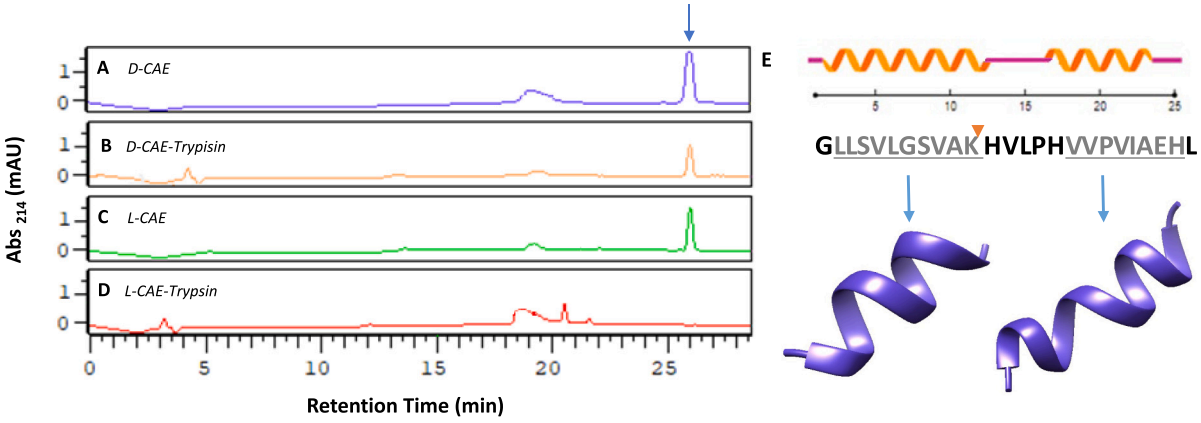


Fig. 1. Chromatographic assessment of the stability of trypsin-treated peptides. L- and D-Caerin were incubated with trypsin at 37 °C with continuous agitation and the chromatograms of the incubation mixtures registered immediately before the addition of the enzyme (A, C) and 15 min after the treatment (B, D). L-Caerin sequence indicating the target of the hydrolytic enzyme (▼) and the predicted 3D structure of the peptide fragments generated by tryptic treatment (E). Pepfold3 and Chimera software were employed for 3D structure prediction and visualization, respectively.

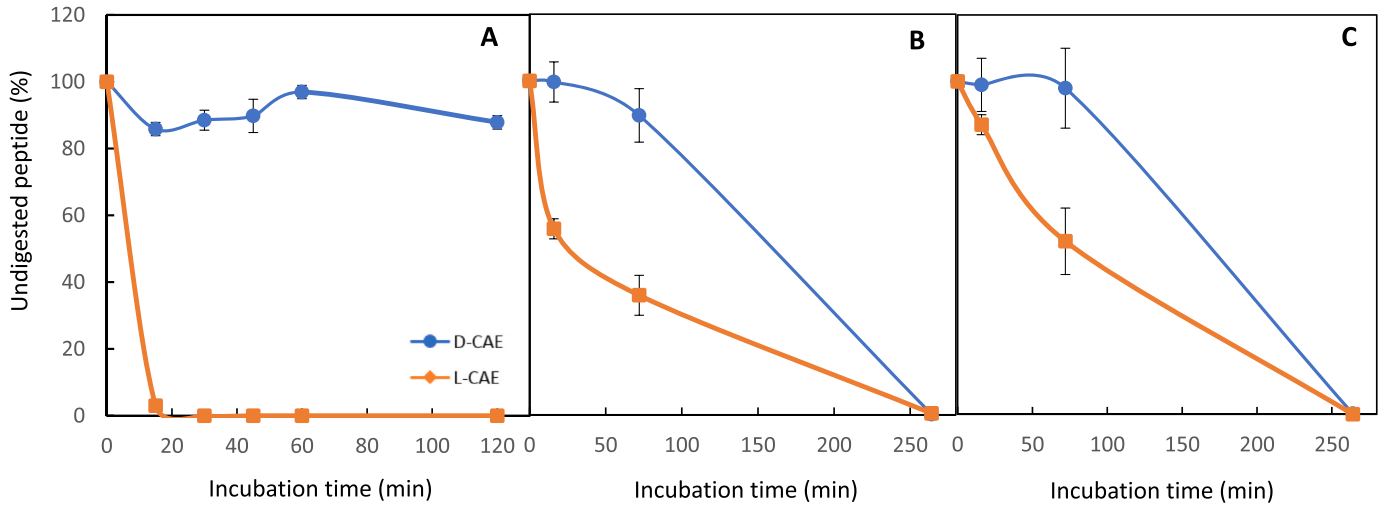


Fig. 2. Stability of L- and D- Caerin. L- (■) and D- (●) Caerin were incubated with trypsin (A); in sterile seawater with a salinity of 33 g L^{-1} at pH 8 and room temperature (B); and in sterile distilled water at pH 2 and 37 °C (C).The percentage of undigested peptide was calculated from HPLC determinations.

D-isomer maintained its stability after 2 h of incubation, either at high salinity or at acidic pH. Degradation of D-Caerin started after 4 h at pH 2 and after 72 h in saline water, indicating the high stability of this artificial isomer of Caerin.

3.2. *Structural assays of the synthetic peptide D-Caerin*

The synthetic peptide D-Caerin is composed of dextrorotatory residues, enantiomers of the corresponding L-aminoacids, chemically linked by peptidic bonds as in the natural L-Caerin. We have demonstrated that D-Caerin is much more stable to degradation than its natural corresponding isomer. However, it is necessary to check if D-Caerin possesses the 3D structure of its natural L-isomeric form and maintains its antimicrobial capacity.

Circular dichroism (CD) is usually used to monitor conformational changes in protein structures. The CD spectrum of a protein is the result of the different secondary structures present in the protein. For example, an alpha-helix spectrum has two typical negative bands with minima centered at 208 and 222 nm, while a beta-turn appears as a spectrum with a positive band with a maximum at 208 nm and a negative band with a minimum at 228 nm. The typical spectrum of a non-ordered structure (random coil) is characterized by a negative band around 200 nm and another weak band that can be positive or negative (Kelly et al., 2005). The CD spectrum observed for L-Caerin in the presence of TFE 50% has two negative minima around 208 and 222 nm and is compatible with the existence of an α-helix. Although the short length of the peptide and the presence of other structures, such as the loop between the two alpha helices, can cause additional interactions and alterations in the spectrum, as previously reported (Montserret et al., 2000). Synthetic D-Caerin shows a spectrum opposed to that of L-Caerin, with two positive maxima around 208 and 222 nm, as expected for a left-handed alpha helix (Fig. 3A). These results confirm that the substitution of all the chiral L-amino acids causes a modification of the alpha helices, which in natural Caerin are right-handed and are converted into left-handed alpha helices in D-Caerin. The CD spectrum indicates that the helix-hinge-helix, although specularly opposed to that of L-Caerin, is very probably maintained in D-Caerin. This implies that the dextrorotatory form of Caerin can maintain its bioactivity. The 3D structure predicted for L-Caerin by Pepfold3 tool (Fig. 3B) is very similar to that previously resolved by Pukala et al., 2004. The 3D structure of D-Caerin has not been described before. However, the structure obtained when the spatial coordinates of each atom of the structure are manually converted into its specular image, is compatible with two left-handed alpha-helices (Fig. 3C), in concordance with the data obtained from circular dichroism.

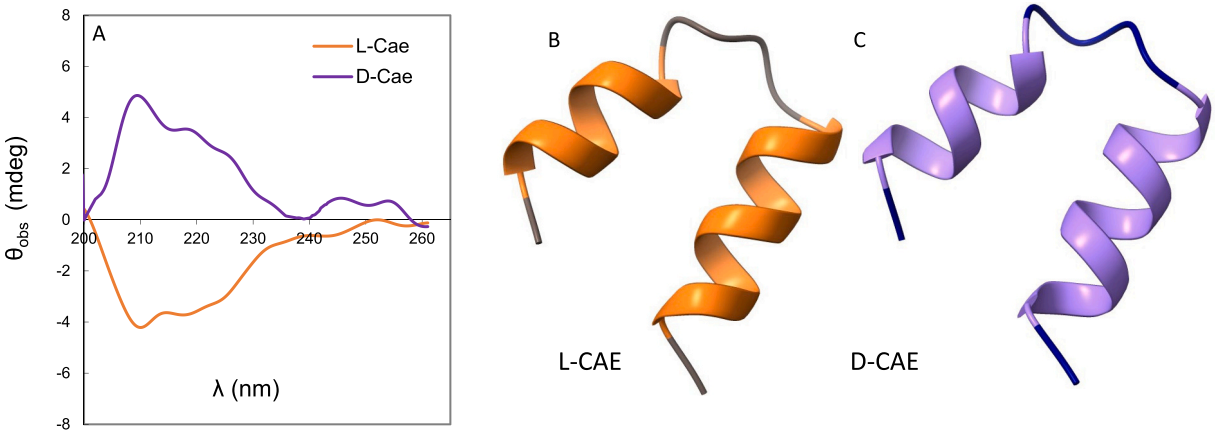


Fig. 3. Circular dichroism spectra and predicted 3D structure of the L- and D-Caerin.
Far UV-CD spectra registered in the 200–260 nm range for L-Caerin (—) and D-Caerin (—) with 50% TFE (A). 3D predicted structure for L-Caerin (B) and D-Caerin (C). Pepfold3 and Chimera software were employed for 3D structure prediction and visualization, respectively. D-Caerin structure was generated by converting the spatial coordinates of each atom into its corresponding specular image by Chem3D software.

3.3. Antimicrobial ability of D-Caerin in vitro

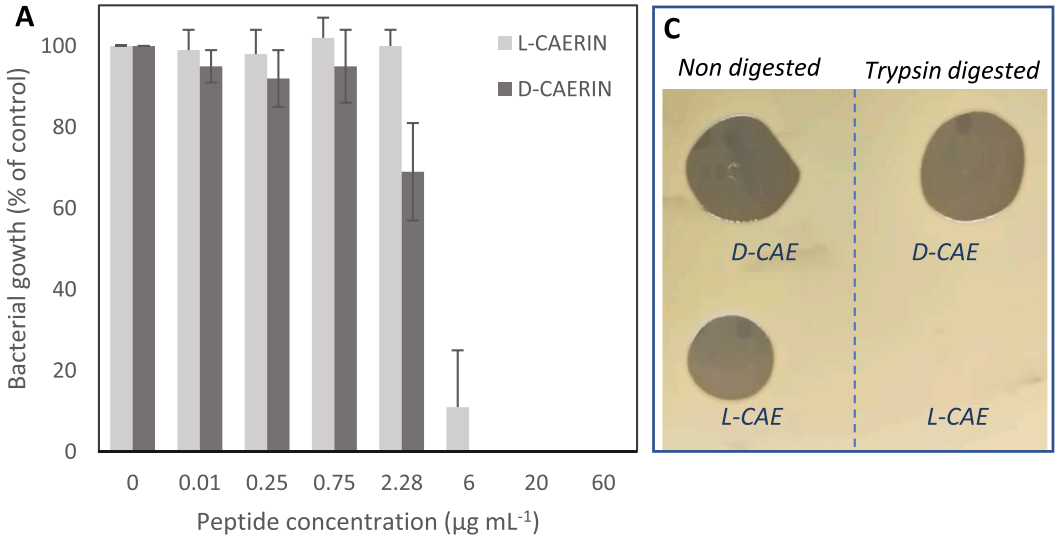
To check if D-Caerin maintains the antimicrobial activity previously demonstrated for the L-isomeric form of the peptide (Cuesta et al., 2021; León et al., 2020), the control species *Micrococcus luteus* was selected and the diameter of the growth inhibition halo, the minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) of L- and D-Caerin were determined as described in material and methods (Fig. 4). The obtained results demonstrate that the dextrorotatory form of Caerin 1.1 has a superior antimicrobial activity compared to L-Caerin, with slightly larger growth inhibition halo (Fig. 4) and lower MIC and MBC values. When *M. luteus* was grown with increasing quantities of both peptides, the minimum peptide doses at which no growth was detected were 20 and 6 μg mL^{-1} for L- and D-Caerin, respectively (Fig. 4A). Similarly, MBC value was lower for D-Caerin (Fig. 4B). The lower MIC and BIC values exhibited by D-Caerin are probably due to its higher stability. The MIC values observed for L-Caerin are of the same order as those previously reported for this peptide (León et al., 2020; Steinborner et al., 2009; Xiao et al., 2022). The superior bioactivity of dextrorotatory isomers has been described for other peptides, as discussed below.

Furthermore, the antimicrobial activity of L- and D-Caerin peptides submitted to the trypsin enzymatic treatment for 2 h was also analyzed, using *M. luteus* as control strain, in agar plates as indicated in material and methods. It was concluded that the proteolytic peptides resulting from the tryptic digestion of L-Caerin have no antimicrobial activity. On the other hand, D-Caerin treated for 2 h with trypsin caused a big lytic halo in *M. luteus* plates, similar to that occasioned by untreated D-Caerin (Fig. 4C). This confirms that after the hydrolytic treatment with trypsin, D-Caerin, not only keeps its primary structure, but also its 2D structure on which the antimicrobial activity of the peptide depends.

Then, the antimicrobial activity of both D- and L- Caerin peptides was assayed on the vibrio species *V. anguillarum, V. aestuarianus, V. harveyi,* and *V. tapetis*, which are among the most usual *causative agents of vibriosis in aquaculture.* The diameter of the growth inhibition halo, and MIC and MBC values were determined for these species as described in the material and methods (Table 1). The obtained results demonstrate that both, L-and D-Caerin, exhibit an antimicrobial activity against all vibrio species studied, being especially strong against *V. aesturarianus* and *V. anguillarum*. For the four vibrio species, the diameter of the inhibition halo was greater for D-Caerin, while the MIC and MBC values were lower for D-Caerin, indicating higher inhibitory activity of the non-natural D-Caerin peptide. This is the same pattern observed for *M. luteus,* and is consistent with the lower stability observed for L-Caerin.

3.4. Vibrio infection assays in the Japanese clam (Ruditapes phillipinarum) pretreated with D-Caerin

Before performing *in vivo* infection assays, the toxicity of D-Caerin on the clam seeds was studied, and optimal non-toxic peptide concentration and incubation time were established. Groups of 20 clam seeds (*Ruditapes phillipinarum*) were incubated with two doses of the peptide at a final concentration of 0.1 and 1 mg mL^{-1}, as described in material and methods (Fig. 5). Clams survival was followed after the treatment during a 7-days period and compared with that of control groups maintained in the same conditions without peptide. A concentration of 1 mg mL^{-1} resulted very toxic for clams, which stopped filtering immediately after exposition to such concentration of peptide (Fig. 5A), and registered a high mortality (35% 24 h after the treatment). No additional mortality was detected in the following days. A reduction of the peptide concentration to 0.1 mg mL^{-1} caused a reduction in the mortality (25% 24 h after the treatment). No more deaths were detected in subsequent days. When the incubation time was reduced to 2 h, clams with 0.1 mg mL^{-1}



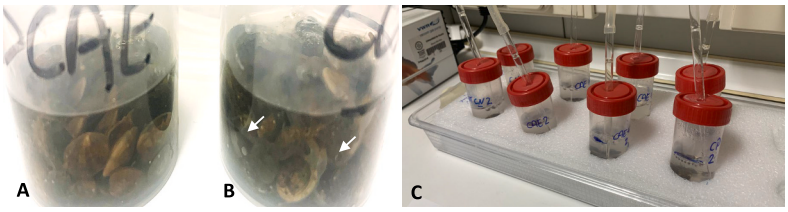
B

	Undigested peptide		Trypsin digested peptide	
	L-CAE	D-CAE	L-CAE	D-CAE
Halo (mm)	11 ±0.5	12 ±0.4	0	12 ±0.5
MIC (μg mL^{-1})	20	6	nd	nd
MBI (μg mL^{-1})	180	60	nd	nd

Fig. 4. Antibacterial activity of L-and D-Caerin on *M. luteus*. Growth of the selected bacteria in the presence of increasing concentrations of the indicated peptide (A). MIC and MBC values and diameter of the inhibition growth halo of L- and D-Caerin on *M.luteus* (B). Agar diffusion susceptibility tests for *M. luteus* treated with L- and D- Caerin, before and after being submitted to 2 h of incubation with trypsin at 37 °C (C).

Table 1
Relative antibacterial activity of L-Caerin and D-Caerin diameter of inhibition halos in 24 h-antibacterial agar diffusion essays, minimal inhibition concentration (MIC) and minimal bactericidal concentration (MBC) calculated after 48 h of incubation for the species *Vibrio aestuarianus, Vibrio anguillarum, Vibrio harveyi; Vibrio tapetis.*

	V. aestuarianus		*V. anguillarum*		*V. harveyi*		*V. tapetis*	
	L-CAE	D-CAE	L-CAE	D-CAE	L-CAE	D-CAE	L-CAE	D-CAE
Halo (mm)	8.2 ± 1	9.7 ± 0.6	8.7 ± 1	9.1 ± 0.5	6.9 ± 0.1	7.2 ± 0.3	5.5 ± 0.7	6.6 ± 0.3
MIC (mg mL^{-1})	0.3	0.1	0.8	0.2	0.8	0.3	0.8	0.3
MBC (mg mL^{-1})	> 0.8	0.8	> 0.8	0.8	> 0.8	> 0.8	0.8	> 0.8



D

Treatment	Siphon activity	24h Mortality (%)
No peptide	normal	0
0.1 mg mL^{-1} D-CAE	normal	25 ±5
1 mg mL^{-1} D-CAE	stopped	35 ±4

Fig. 5. Effect of D-Caerin on Japanese clam (*Ruditapes phillipinarum) seeds.* Clam seeds groups ($n = 10$) were incubated with 1 mg mL^{-1} (A) and 0.1 mg mL^{-1} (B) of D-Caerin in plastic recipients with continuous aeration (C). Immediate clam response and survival rate after 24 h of treatment were registered (D). A white arrow point visible active siphons.

D-Caerin showed their *siphons out and filtered normally*, while those treated with 1 mg mL^{-1} of D-Caerin immediately retreated siphons and closed their valves, indicating toxicity of this dose of Caerin. Control untreated clam seeds filtered normally and did not register any death during the 7 days of the experiment. D-Caerin dose stablished for the infection assays was 0.1 mg mL^{-1} for 2 h.

To evaluate the protective effect against vibrio infections of D-Caerin on the Japanese clam (*Ruditapes phillipinarum*), clam seeds pre-treated with D-Caerin (0.1 mg mL^{-1}) for 2 h were subjected to an infection challenge. After 2 h of incubation with D-Caerin (0.1 mg mL^{-1}), clam seeds were rinsed with FSW to eliminate excess of peptide, maintained in FSW continuously aerated for 24 h, and then infected with a mixed inoculum consisting of *V. aestuarianus*, *V. anguillarum*, *V. harveyi* and *V. tapetis*, as detailed in material and methods. The results show that when pre-treated clams were exposed to vibriosis, no mortalities were detected. In parallel, in the non-pretreated group inoculated with the same vibrios mixture (NP-Infected control), mortality started to be evident 2 days after infection and reached 80% of cumulative mortality on the fourth day (Fig. 6). In the negative control, clams pretreated with D-Caerin and no submitted to the infections (NP-non infected) registered no mortalities during the time of the experiment (Fig. 6).

4. Discussion

Concerns about the excessive use of antibiotics in medicine and veterinary have stimulated research to find alternative antimicrobial agents active against multidrug-resistant and persistent bacteria, and AMPs have been noted as an attractive alternative option (Wang et al., 2016a, 2016b). An increasing number of peptides with antibiotic characteristics has been described, and many of them have been compiled in databases. Such as the APD3 data base, that compiles 3425 antimicrobial peptides from six different life kingdoms (Wang et al., 2016a); CAMP$_{R4}$ (http://www.camp.bicnirrh.res.in), which contains information about 11,298 natural and 6607 synthetic peptide; or DRAMP http://dramp.cpu-bioinfor.org/), a Data Repository of Antimicrobial Peptides with 22,480 entries reviewed by Ramazi et al., 2022.

Some of these databases include useful tools to predict the characteristics, expected structure or potential activity of peptides, which can be a support for their rational design. However, non-standard amino acids are not usually accepted as input for most of these computational

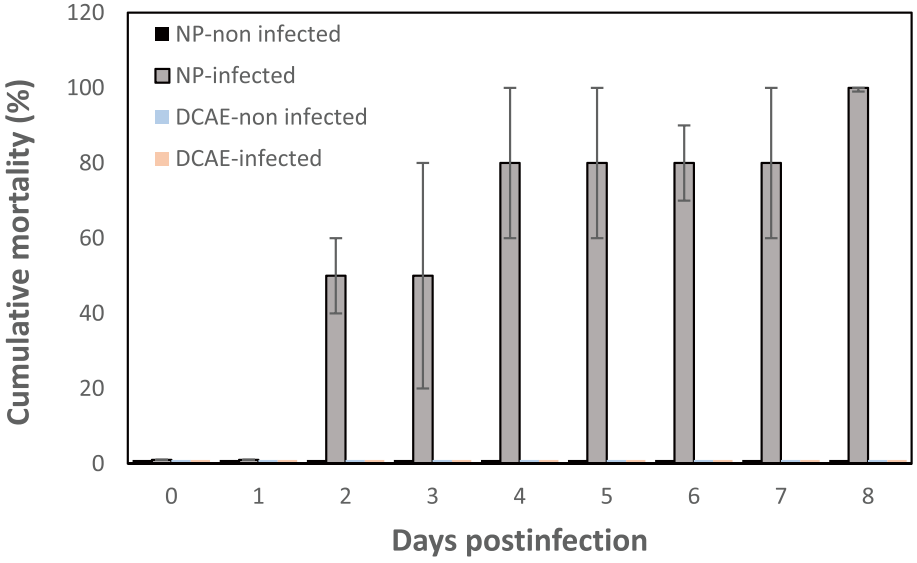


Fig. 6. *In vivo* protective effect of D-Caerin on *R. philippinarum* seeds. Clam seeds incubated pre-treated D-Caerin (0.1 mg mL^{-1} for 2 h) were infected with a mixed inoculum consisting of *V. aestuarianus*, *V. anguillarum*, *V. harveyi* and *V. tapetis* (DCAE-infected). Clam mortality was followed during 9 days after the infection. Control essays with non-pretreated clam seeds inoculated with the same Vibrios mixture (NP-Infected control), pretreated non-infected (DCAE-non infected) and non-pretreated non-infected (NP-non infected) were run in parallel in the same conditions. All treatments were done in triplicate ($n = 10$ for each group). Bars represent standard deviation among replicas.

tools reducing the utility for *in-silico* design of modified peptides with non-natural amino acids. Hopefully, the reduction of the peptides synthesis price makes the empirical modification of promising peptides affordable to improve their characteristics, stability, or functionality. With this goal, we designed an artificial peptide composed of the dextrorotatory isomers of the corresponding L-amino acids of the antimicrobial peptide Caerin 1.1, which was identified and isolated from the skin of the Australian frog *Ranoidea splendida* (Bevins and Zasloff, 1990). Caerin 1.1 has been actively investigated due to its activity against viruses (Rollins-Smith et al., 2020) and bacteria (Chen et al., 2021), including vibrio species (León et al., 2020). Caerin peptides have also been shown to exhibit anticancer (Ni et al., 2020; Xiao et al., 2022) or immune-stimulation activities (Ni et al., 2021).

Despite its proven bioactivity, Caerin 1.1, as many short peptides, has low stability when exposed to harsh conditions, like acidic pH, high temperature, or proteases (Chen et al., 2021). Finding strategies to avoid or minimize the degradation of peptides is one of the main challenges for their application. Peptides are more susceptible to enzymatic degradation than other compounds and present low chemical stability under extreme pH, high temperatures, or other conditions that can promote the hydrolysis of the peptide bonds (Bellotti and Remelli, 2022). Different peptide modifications *have been proposed to enhance their stability and, on many occasions, improve their bioactivity*. Substitution of the amino acid residues most susceptible to proteolytic cleavage, chemical modification of N- or C- terminus by acetylation or amidation, addition of specific functional groups or new residues to the extreme of the peptide, modification of the natural peptide bonds, or peptide cyclization are some of the chemical modifications employed, with different degree of success, to increase stability of AMPs (Evans et al., 2020; Li et al., 2021a). As an example, Hazam et al. (2022) showed that two synthetic peptides, redesigned from a tilapia inactive piscidin-2 peptide to improve their cationicity and amphipathic balance, presented enhanced antimicrobial activity and higher stability when incubated in human serum and at high temperatures. Similarly, the addition to the N-terminal end of Caerin of a poly-histidine (6xHis) tag, usually used for purification of target peptides by Ni-resin chromatography, caused an increase in Caerin activity against certain bacteria (Cuesta et al., 2021).

The incorporation of non-natural amino acids, such as dextrorotatory isomers of the corresponding L-amino acids, is another possible approach to increase peptide stability. Several commercialized antimicrobial peptides contain D-amino acids in their structure, such as Daptomycin, a cyclic acyl-peptide with 13 amino acids which includes D-alanine and D-serine (Robbel and Marahiel, 2010); gramicidin S, an acyclic decapeptide containing D-amino acids that has showed activity against *Bacillus subtilis* and *Staphylococcus aureus* (Pavithrra and Rajasekaran, 2020); or Octreotide, a peptide drug based in the human hormone somatostatin, with improved stability due to the substitution of two amino acids by their corresponding D-aa (Evans et al., 2020). However, many studies have shown that the introduction of D-amino acids can alter side-chain interactions in the molecule and affect the structure and functionality of the peptides (Evans et al., 2020; C. Li et al., 2013; Powers and Hancock, 2003).

Our study indicates that the all-D-amino acid version of Caerin is more resistant to proteases and harsh environmental conditions than its natural counterpart (Fig. 1, Fig. 2). And suggests that D-Caerin could maintain its viability for several days when delivered through seawater or subjected to the usual acidic conditions in the digestive tract of animals. These observations are consistent with those of Mohamed et al. (2017), who described that the all-D-aa enantiomeric form of a short α-synthetic bioactive helical synthetic peptide named RR4 maintained its activity in serum, under high salt concentration and acidic pH. Similarly, the pioneering studies of Pritsker and coworkers demonstrated that a synthetic all-D-amino acids peptide maintained the ability of its corresponding L- isomer to inhibit the HIV-1 envelope glycoprotein-mediated cell fusion, while exhibiting superior resistance to proteolytic digestion (Pritsker et al., 1998).

The antimicrobial activity of many AMPs is highly dependent on their 3D structure (Huan et al., 2020; Li et al., 2021b). In the case of Caerin, its two amphipathic alpha helices, separated by a flexible hinge, are essential to interact with bacterial cell membranes and eventually cause cell death. The structure of natural Caerin has been resolved by NMR and the role of the proline residues, P15 and P19, has been proved to be essential to maintain the this flexible region and bioactivity of the peptide (Pukala et al., 2004). Moreover, the substitution of either of these proline residues results in structure alterations and loss of bactericidal activity, corroborating their key role (Pukala et al., 2004).

Substitution of all the chiral L-amino acids by the corresponding D-amino acids can alter the secondary structures of the peptide, as we have observed for D-Caerin, which shows a CD spectrum totally opposed to that of L-Caerin and compatible with a left-handed alpha-helix (Fig. 3). Right-handed alpha helices of natural L-peptides have been described to turn into left-handed in several all-D-amino acids peptides. Moreover, Garton et al. (2018) proposed the *in silico* generation of a library of D-peptides, obtained as mirror images of helical structures of the Protein Data Bank, for the mining of bioactive D-peptides. In this way, they successfully obtained dextrorotatory peptides with potential to treat diabetes and osteoporosis that exhibited a higher half-life that their corresponding natural counterparts.

Although antibacterial, antiviral and other biological activities of the natural L-Caerin peptide have been clearly stated, the functionality of D-Caerin (the dextrorotatory corresponding peptide) has never been tested until now. We have demonstrated, using *M. luteus* as control species, that D-Caerin, not only is less susceptible to degradation than its L- counterpart peptide, but also has a higher antimicrobial ability (Fig. 4). This is in agreement with the results previously reported for other all-D-peptides (Mohamed et al., 2017). The superior bioactivity of dextrorotatory peptides in comparison with their L-isomers has also been described for antiviral (Pritsker et al., 1998; Valiente et al., 2021) and other therapeutic peptides (Garton et al., 2018).

Different species of *Vibrio* have been identified as pathogenic and responsible for major disease outbreaks in seeds and larvae of bivalve species worldwide (Prado et al., 2005; Travers et al., 2014; Rojas et al., 2015; Dubert et al., 2016). These episodes affect more severely to seeds and larvae than to adult specimens, which can act as reservoirs, being an important risk factor for larvae. The devastating effects of vibriosis, besides its worldwide occurrence and the *increasing resistance of many Vibrio species to traditional antibiotics,* have encouraged the search for more effective therapeutic approaches. Phage therapy (Kalatzis et al., 2018), supplementation of diets with probiotics (Hoseinifar et al., 2018), or vaccination in the case of fish species (Aly et al., 2021) are some of the most popular.

Although antimicrobial peptides, such as Caerin, have been previously shown to inhibit vibrio species *in vitro* (Cuesta et al., 2021), their low stability has made it difficult to assay their activity *in vivo*. In the present study, the antimicrobial activity of the dextrorotatory isomer, D-Caerin has been assayed *in vivo* on *R. philippinarum* seeds. Our results indicate that, at low incubation times, D-Caerin is well tolerated by *R. philippinarum* seeds (Fig. 5). Moreover, we have demonstrated the ability of the peptide D-Caerin to inhibit the growth of four vibrio species *in vitro* (Table 1) and to ameliorate the response to vibriosis in *R. philippinarum* seeds *in vivo*, decreasing mortality (Fig. 6). Although the effectiveness of D-Caerin is probed with these experiments, further studies should be done to determine its mode of action and clarify whether other protective effects are involved, in addition to D-Caerin's antimicrobial activity. For example, the peptide Sparanegtin, which did not exhibit activity against *V. alginolyticus in vitro*, was shown to play an immunoprotective role in *S. paramamosain*, modulating certain genes related with the response against *V. alginolyticus* infection, reducing the bacterial load and increasing the survival rate of crabs (Zhu et al., 2021).

A peptide of the Caerin 1 family, Caerin 1.9, has been tested in rats submitted to a subcutaneous injection of Caerin 1.9 (100 mg kg^{-1}). The authors concluded that Caerin 1.9 is well tolerated in rats and propose

its therapeutic use to treat solid tumors and genital warts (Yang et al., 2022). L-Caerin peptides have shown low cytotoxicity for cell fibroblast or epithelial fish cell lines E-11 and EPC, respectively (León et al., 2020). However, the cytotoxicity of D-Caerin has not been previously tested, either *in vivo* nor *in vitro*. In this study, we have demonstrated the tolerance of *R. philippinarum,* when exposed to Caerin by immersion for 2 h.

5. Conclusion

Bivalves, which do not possess an adaptive immune system and cannot be protected by vaccines are good candidates for peptide therapies. The high stability demonstrated in this study for the D-Caerin, besides the tolerance exhibited by R. *philippinarum* to this peptide, suggests its potential against pathogens in this species. Although more studies are necessary to understand the basis of this protective effect, from our results, it can be confirmed that D-Caerin attenuates the effect of vibriosis on clams. It has to be established whether the increased survival is exclusively due to an antimicrobial activity *in vivo* of D-Caerin, or whether a prebiotic or an immune-stimulator effect of the peptide contributes to improving the survival of clams upon infection by vibrios. Moreover, D-Caerin and other dextrorotatory peptides could be used to prevent infections in other bivalve or aquaculture species. However, efficient ways for their administration have to be addressed, and the effect of the peptides on other environmental bacteria has to be investigated, before adopting this therapy strategy.

Author contribution

R.L and M. L-S designed research; R.L, M. L-S and J. V. wrote the paper and were involved in funding acquisition; M.L-S, R.R. and A M-M. carried out antibacterial assays; R.L. and R.R. did peptides structure predictions; M. L-L; P. L-C and J.A. L. performed CD studies and contributed to structure studies. M.L-S.; I. R. and A.C. preformed *in vivo* infection assays. All authors contributed to review and edit the manuscript. All authors have read and agreed to the published version of the manuscript.

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Writing - Review & Editing All authors.

Declaration of Competing Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data.

Data availability

Data will be made available on request.

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References

Allam, B., Raftos, D., 2015. Immune responses to infectious diseases in bivalves. J. Invertebr. Pathol. 131, 121–136. https://doi.org/10.1016/j.jip.2015.05.005.
Aly, S.M., Eissa, A.E., ElBanna, N.I., Albutti, A., 2021. Efficiency of monovalent and polyvalent *Vibrio alginolyticus* and *Vibrio parahaemolyticus* vaccines on the immune response and protection in gilthead sea bream, *Sparus aurata* (L.) against vibriosis. Fish Shellfish Immunol. 111, 145–151. https://doi.org/10.1016/j.fsi.2020.10.011.
Apponyi, Margit, A., Pukala, Tara, L., Brinkworth, Craig, S., Maselli, Vita, M., Bowie, John, H., Tyler, Michael, J., Booker, Grant, W., Wallace, John, C., Carver, John, A., Separovic, Frances, Doyle, Jason, Llewellyn, Lyndon E., 2004. Host-defence peptides of Australian anurans: structure, mechanism of action and evolutionary significance. Peptides 25 (6), 1035–1054. https://doi.org/10.1016/j.peptides.2004.03.006.
Bellotti, D., Remelli, M., 2022. Lights and shadows on the therapeutic use of antimicrobial peptides. Molecules 27 (14), 4584. https://doi.org/10.3390/molecules27144584.
Benfield, A.H., Henriques, S.T., 2020. Mode-of-action of antimicrobial peptides: membrane disruption vs. intracellular mechanisms. Front. Med. Technol. 2 https://doi.org/10.3389/fmedt.2020.610997.
Bevins, C.L., Zasloff, M., 1990. Peptides from frog skin. Annu. Rev. Biochem. 59 (1), 395–414. https://doi.org/10.1146/annurev.bi.59.070190.002143.
Boto, A., Pérez de la Lastra, J., González, C., 2018. The road from host-defense peptides to a new generation of antimicrobial drugs. Molecules 23 (2), 311. https://doi.org/10.3390/molecules23020311.
Chen, S., Zhang, P., Xiao, L., Liu, Y., Wu, K., Ni, G., Li, H., Wang, T., Wu, X., Chen, G., Liu, X., 2021. Caerin 1.1 and 1.9 peptides from Australian tree frog inhibit antibiotic-resistant bacteria growth in a murine skin infection model. Microbiol. Spectrum 9 (1). https://doi.org/10.1128/Spectrum.00051-21.
Cuesta, A., Vila, M., Molina-Marquez, A., Carbajo, C., Lebron, J.A., López-López, M., López-Cornejo, P., Moyá, M.L., López-Sanmartín, M., López, J.R., Navas, J.I., Vigara, J., León, R., 2021. Influence of adding terminal tags on the structural and antimicrobial properties of the peptide Caerin 1.1. Aquaculture 532, 736035. https://doi.org/10.1016/j.aquaculture.2020.736035.
Dubert, J., Nelson, D.R., Spinard, E.J., Kessner, L., Gomez-Chiarri, M., da Costa, F., Prado, S., Barja, J.L., 2016. Following the infection process of vibriosis in Manila clam (*Ruditapes philippinarum*) larvae through GFP-tagged pathogenic *Vibrio* species. J. Invertebr. Pathol. 133, 27–33. https://doi.org/10.1016/j.jip.2015.11.008.
Erdem Büyükkiraz, M., Kesmen, Z., 2022. Antimicrobial peptides (AMPs): a promising class of antimicrobial compounds. J. Appl. Microbiol. 132 (3), 1573–1596. https://doi.org/10.1111/jam.15314.
Evans, B.J., King, A.T., Katsifis, A., Matesic, L., Jamie, J.F., 2020. Methods to enhance the metabolic stability of peptide-based PET radiopharmaceuticals. Molecules 25 (10), 2314. https://doi.org/10.3390/molecules25102314.
Garton, M., Nim, S., Stone, T.A., Wang, K.E., Deber, C.M., Kim, P.M., 2018. Method to generate highly stable D-amino acid analogs of bioactive helical peptides using a mirror image of the entire PDB. Proc. Natl. Acad. Sci. 115 (7), 1505–1510. https://doi.org/10.1073/pnas.1711837115.
Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S., Wilkins, M.R., Appel, R.D., Bairoch, A., 2005. Protein identification and analysis tools on the ExPASy server. In: The Proteomics Protocols Handbook. Humana Press, pp. 571–607. https://doi.org/10.1385/1-59259-890-0:571.
Hancock, R.E., Diamond, G., 2000. The role of cationic antimicrobial peptides in innate host defences. Trends Microbiol. 8 (9), 402–410. https://doi.org/10.1016/S0966-842X(00)01823-0.
Haney, E.F., Straus, S.K., Hancock, R.E.W., 2019. Reassessing the host defense peptide landscape. Front. Chem. 7 https://doi.org/10.3389/fchem.2019.00043.
Hazam, P.K., Cheng, C.-C., Hsieh, C.-Y., Lin, W.-C., Hsu, P.-H., Chen, T.-L., Lee, Y.-T., Chen, J.-Y., 2022. Development of bactericidal peptides against multidrug-resistant *Acinetobacter baumannii* with enhanced stability and low toxicity. Int. J. Mol. Sci. 23 (4), 2191. https://doi.org/10.3390/ijms23042191.
Hoseinifar, S.H., Sun, Y.-Z., Wang, A., Zhou, Z., 2018. Probiotics as means of diseases control in aquaculture, a review of current knowledge and future perspectives. Front. Microbiol. 9 https://doi.org/10.3389/fmicb.2018.02429.
Huan, Y., Kong, Q., Mou, H., Yi, H., 2020. Antimicrobial peptides: classification, design, application and research progress in multiple fields. Front. Microbiol. 11 https://doi.org/10.3389/fmicb.2020.582779.
Kalatzis, P., Castillo, D., Katharios, P., Middelboe, M., 2018. Bacteriophage interactions with marine pathogenic vibrios: implications for phage therapy. Antibiotics 7 (1), 15. https://doi.org/10.3390/antibiotics7010015.
Kelly, S.M., Jess, T.J., Price, N.C., 2005. How to study proteins by circular dichroism. Biochim. Biophys. Acta (BBA) - Proteins Proteom. 1751 (2), 119–139. https://doi.org/10.1016/j.bbapap.2005.06.005.
Kurtz, J., 2005. Specific memory within innate immune systems. Trends Immunol. 26 (4), 186–192. https://doi.org/10.1016/j.it.2005.02.001.
Lazzaro, B.P., Zasloff, M., Rolff, J., 2020. Antimicrobial peptides: application informed by evolution. Science 368 (6490). https://doi.org/10.1126/science.aau5480.

Lei, J., Sun, L., Huang, S., Zhu, C., Li, P., He, J., Mackey, V., Coy, D.H., He, Q., 2019. The antimicrobial peptides and their potential clinical applications. Am. J. Transl. Res. 11 (7), 3919–3931. http://www.ncbi.nlm.nih.gov/pubmed/31396309.

León, R., Ruiz, M., Valero, Y., Cárdenas, C., Guzman, F., Vila, M., Cuesta, A., 2020. Exploring small cationic peptides of different origin as potential antimicrobial agents in aquaculture. Fish Shellfish Immunol. 98, 720–727. https://doi.org/10.1016/j.fsi.2019.11.019.

Li, C., Zhan, C., Zhao, L., Chen, X., Lu, W.-Y., Lu, W., 2013. Functional consequences of retro-inverso isomerization of a miniature protein inhibitor of the p53–MDM2 interaction. Bioorg. Med. Chem. 21 (14), 4045–4050. https://doi.org/10.1016/j.bmc.2013.04.039.

Li, R., He, S., Yin, K., Zhang, B., Yi, Y., Zhang, M., Pei, N., Huang, L., 2021a. Effects of N-terminal modifications on the stability of antimicrobial peptide SAMP-A4 analogues against protease degradation. J. Pept. Sci. 27 (10) https://doi.org/10.1002/psc.3352.

Li, S., Wang, Y., Xue, Z., Jia, Y., Li, R., He, C., Chen, H., 2021b. The structure-mechanism relationship and mode of actions of antimicrobial peptides: a review. Trends Food Sci. Technol. 109, 103–115. https://doi.org/10.1016/j.tifs.2021.01.005.

Mitta, G., Vandenbulcke, F., Roch, P., 2000. Original involvement of antimicrobial peptides in mussel innate immunity. FEBS Lett. 486 (3), 185–190. https://doi.org/10.1016/S0014-5793(00)02192-X.

Mohamed, M.F., Brezden, A., Mohammad, H., Chmielewski, J., Seleem, M.N., 2017. A short D-enantiomeric antimicrobial peptide with potent immunomodulatory and antibiofilm activity against multidrug-resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. Sci. Rep. 7 (1), 6953. https://doi.org/10.1038/s41598-017-07440-0.

Montserret, R., McLeish, M.J., Böckmann, A., Geourjon, C., Penin, F., 2000. Involvement of electrostatic interactions in the mechanism of peptide folding induced by sodium dodecyl sulfate binding. Biochemistry 39 (29), 8362–8373. https://doi.org/10.1021/bi000208x.

Ni, G., Chen, S., Chen, M., Wu, J., Yang, B., Yuan, J., Walton, S.F., Li, H., Wei, M.Q., Wang, Y., Chen, G., Liu, X., Wang, T., 2020. Host-defense peptides caerin 1.1 and 1.9 stimulate TNF-alpha-dependent apoptotic signals in human cervical cancer HeLa cells. Front. Cell Develop. Biol. 8 https://doi.org/10.3389/fcell.2020.00676.

Ni, G., Yang, X., Li, J., Wu, X., Liu, Y., Li, H., Chen, S., Fogarty, C.E., Frazer, I.H., Chen, G., Liu, X., Wang, T., 2021. Intratumoral injection of Caerin 1.1 and 1.9 peptides increases the efficacy of vaccinated TC-1 tumor-bearing mice with PD-1 blockade by modulating macrophage heterogeneity and the activation of CD8 + T cells in the tumor microenvironment. Clin. Trans. Immunol. 10 (8) https://doi.org/10.1002/cti2.1335.

Pavithrra, G., Rajasekaran, R., 2020. Gramicidin peptide to combat antibiotic resistance: a review. Int. J. Pept. Res. Ther. 26 (1), 191–199. https://doi.org/10.1007/s10989-019-09828-0.

Peschel, A., Sahl, H.G., 2006. The co-evolution of host cationic antimicrobial peptides and microbial resistance. Nat. Rev. Microbiol. 4 (7), 529–536. https://doi.org/10.1038/nrmicro1441.

Pettersen, E.F., Goddard, T.D., Huang, C.C., Couch, G.S., Greenblatt, D.M., Meng, E.C., Ferrin, T.E., 2004. UCSF chimera-a visualization system for exploratory research and analysis. J. Comput. Chem. 25 (13), 1605–1612. https://doi.org/10.1002/jcc.20084.

Powers, J.-P.S., Hancock, R.E., 2003. The relationship between peptide structure and antibacterial activity. Peptides 24 (11), 1681–1691. https://doi.org/10.1016/j.peptides.2003.08.023.

Prado, S., Romalde, J., Montes, J., Barja, J., 2005. Pathogenic bacteria isolated from disease outbreaks in shellfish hatcheries. First description of *Vibrio neptunius* as an oyster pathogen. Dis. Aquat. Org. 67, 209–215. https://doi.org/10.3354/dao067209.

Pritsker, M., Jones, P., Blumenthal, R., Shai, Y., 1998. A synthetic all-D-amino acid peptide corresponding to the N-terminal sequence of HIV-1 gp41 recognizes the wild-type fusion peptide in the membrane and inhibits HIV-1 envelope glycoprotein-mediated cell fusion. Proc. Natl. Acad. Sci. 95 (13), 7287–7292. https://doi.org/10.1073/pnas.95.13.7287.

Pukala, T.L., Brinkworth, C.S., Carver, J.A., Bowie, J.H., 2004. Investigating the importance of the flexible hinge in caerin 1.1: solution structures and activity of two synthetically modified caerin peptides. Biochemistry 43 (4), 937–944. https://doi.org/10.1021/bi035760b.

Ramazi, S., Mohammadi, N., Allahverdi, A., Khalili, E., Abdolmaleki, P., 2022. A review on antimicrobial peptides databases and the computational tools. Database 2022. https://doi.org/10.1093/database/baac011.

Rey-Campos, M., Moreira, R., Gerdol, M., Pallavicini, A., Novoa, B., Figueras, A., 2019. Immune tolerance in *Mytilus galloprovincialis* Hemocytes after repeated contact with *Vibrio splendidus*. Front. Immunol. 10 https://doi.org/10.3389/fimmu.2019.01894.

Rey-Campos, M., Moreira, R., Romero, A., Medina-Gali, R.M., Novoa, B., Gasset, M., Figueras, A., 2020. Transcriptomic analysis reveals the wound healing activity of mussel myticin C. Biomolecules 10 (1), 133. https://doi.org/10.3390/biom10010133.

Robbel, L., Marahiel, M.A., 2010. Daptomycin, a bacterial lipopeptide synthesized by a nonribosomal machinery. J. Biol. Chem. 285 (36), 27501–27508. https://doi.org/10.1074/jbc.R110.128181.

Rojas, R., Miranda, C.D., Opazo, R., Romero, J., 2015. Characterization and pathogenicity of *Vibrio splendidus* strains associated with massive mortalities of commercial hatchery-reared larvae of scallop *Argopecten purpuratus* (Lamarck, 1819). J. Invertebr. Pathol. 124, 61–69. https://doi.org/10.1016/j.jip.2014.10.009.

Rollins-Smith, L.A., Smith, P.B., Ledeczi, A.M., Rowe, J.M., Reinert, L.K., 2020. Caerin 1 antimicrobial peptides that inhibit HIV and neisseria may spare protective lactobacilli. Antibiotics 9 (10), 661. https://doi.org/10.3390/antibiotics9100661.

Sanches-Fernandes, G.M.M., Sá-Correia, I., Costa, R., 2022. Vibriosis outbreaks in aquaculture: addressing environmental and public health concerns and preventive therapies using gilthead seabream farming as a model system. Front. Microbiol. 13 https://doi.org/10.3389/fmicb.2022.904815.

Sendra, M., Saco, A., Yeste, M.P., Romero, A., Novoa, B., Figueras, A., 2020. Nanoplastics: from tissue accumulation to cell translocation into *Mytilus galloprovincialis* hemocytes. Resilience of immune cells exposed to nanoplastics and nanoplastics plus *Vibrio splendidus* combination. J. Hazard. Mater. 388, 121788 https://doi.org/10.1016/j.jhazmat.2019.121788.

Shabir, U., Ali, S., Magray, A.R., Ganai, B.A., Firdous, P., Hassan, T., Nazir, R., 2018. Fish antimicrobial peptides (AMP's) as essential and promising molecular therapeutic agents: a review. Microb. Pathog. 114, 50–56. https://doi.org/10.1016/j.micpath.2017.11.039.

Shen, Y., Maupetit, J., Derreumaux, P., Tufféry, P., 2014. Improved PEP-FOLD approach for peptide and miniprotein structure prediction. J. Chem. Theory Comput. 10 (10), 4745–4758. https://doi.org/10.1021/ct500592m.

Steinborner, S.T., Currie, G.J., Bowie, J.H., Wallace, J.C., Tyler, M.J., 2009. New antibiotic Caerin 1 peptides from the skin secretion of the Australian tree frog *Litoria chloris*. Comparison of the activities of the Caerin 1 peptides from the genus Litoria. J. Pept. Res. 51 (2), 121–126. https://doi.org/10.1111/j.1399-3011.1998.tb00629.x.

Travers, M.A., Mersni Achour, R., Haffner, P., Tourbiez, D., Cassone, A.L., Morga, B., Doghri, I., Garcia, C., Renault, T., Fruitier-Arnaudin, I., Saulnier, D., 2014. First description of French *V. tubiashii* strains pathogenic to mollusk: I. characterization of isolates and detection during mortality events. J. Invertebr. Pathol. 123, 38–48. https://doi.org/10.1016/j.jip.2014.04.009.

Valero, Y., Saraiva-Fraga, M., Costas, B., Guardiola, F.A., 2020. Antimicrobial peptides from fish: beyond the fight against pathogens. Rev. Aquac. 12 (1), 224–253. https://doi.org/10.1111/raq.12314.

Valero, Y., González-Fernández, C., Cárdenas, C., Guzmán, F., León, R., Cuesta, A., 2021. NK-lysin peptides ameliorate viral encephalopathy and retinopathy disease signs and provide partial protection against nodavirus infection in European sea bass. Antivir. Res. 192, 105104 https://doi.org/10.1016/j.antiviral.2021.105104.

Valiente, P.A., Wen, H., Nim, S., Lee, J., Kim, H.J., Kim, J., Perez-Riba, A., Paudel, Y.P., Hwang, I., Kim, K.-D., Kim, S., Kim, P.M., 2021. Computational design of potent D-peptide inhibitors of SARS-CoV-2. J. Med. Chem. 64 (20), 14955–14967. https://doi.org/10.1021/acs.jmedchem.1c00655.

Wang, G., Li, X., Wang, Z., 2016a. APD3: the antimicrobial peptide database as a tool for research and education. Nucleic Acids Res. 44 (D1), D1087–D1093. https://doi.org/10.1093/nar/gkv1278.

Wang, S., Zeng, X., Yang, Q., Qiao, S., 2016b. Antimicrobial peptides as potential alternatives to antibiotics in food animal industry. Int. J. Mol. Sci. 17 (5), 603. https://doi.org/10.3390/ijms17050603.

Wiegand, I., Hilpert, K., Hancock, R.E.W., 2008. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat. Protoc. 3 (2), 163–175. https://doi.org/10.1038/nprot.2007.521.

Xiao, L., Yang, X., Li, J., Zhang, P., Tang, S., Cao, D., Chen, S., Li, H., Zhang, W., Chen, G., Ni, G., Wang, T., Liu, X., 2022. Caerin 1 peptides, the potential Jack-of-all-trades for the multiple antibiotic-resistant bacterial infection treatment and cancer immunotherapy. Biomed. Res. Int. 2022, 1–11. https://doi.org/10.1155/2022/7841219.

Yang, X., Li, J., Chen, S., Xiao, L., Cao, D., Wu, X., Li, H., Ni, G., Wang, T., Chen, G., Liu, X., 2022. Preclinical pharmacokinetics, biodistribution, and acute toxicity evaluation of caerin 1.9 peptide in Sprague Dawley rats. Evid. Based Complement. Alternat. Med. 2022, 1–13. https://doi.org/10.1155/2022/9869293.

Zhu, X., Chen, F., Li, S., Peng, H., Wang, K.-J., 2021. A novel antimicrobial peptide Sparanegtin identified in Scylla paramamosain showing antimicrobial activity and Immunoprotective role in vitro and vivo. Int. J. Mol. Sci. 23 (1), 15. https://doi.org/10.3390/ijms23010015.