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A New Combination with D-Cateslytin to Eradicate Root Canal Pathogens

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Abstract

The success of endodontic treatments depends on the elimination of intracanal pathogens. Since irrigation and instrumentation can only partially eliminate bacteria, the use of intracanal medicaments is suggested to improve the eradication of the root canal pathogens. Antimicrobial peptides derived from Chromogranin A display bacteriolytic activities and are potentially excellent candidates to be combined with conventional intracanal medicaments. In this study, we combined D-Cateslytin (D-Ctl), together with calcium hydroxide ($Ca(OH)_2$) to test for enhanced antimicrobial properties against *Enterococcus faecalis.* Antimicrobial activities were determined by broth dilution assays, stability of D-Ctl was assessed by HPLC and MTT tests were used to evaluate cytotoxicity. A saturated solution of $Ca(OH)_2$ (1.7 mg/mL) was able to inhibit 58% (±5%) of *E. faecalis* growth, while the combination of both 0.85 mg/mL of $Ca(OH)_2$ and ½ MIC of D-Ctl was able to fully inhibit its growth. D-Ctl was stable against the proteases secreted by *E. faecalis* and showed no toxicity on human gingival fibroblasts. Besides *E. faecalis*, this combination was also effective in completely killing other endodontic pathogens: *Parvimonas micra, Prevotella intermedia, Fusobacterium nucleatum* and *Candida albicans*. In conclusion, D-Ctl combined with $Ca(OH)_2$ eradicates several endodontic pathogens and could be used as an innovative intracanal medicament to reduce endodontic failures.

Keywords Antimicrobial peptides · Calcium hydroxide · D-Cateslytin · *Enterococcus faecalis* · Endodontic pathogens

Introduction

Apical periodontitis (AP) is a common pathology, defined as an inflammatory process around the apex of a root, causing pain and resorption of periradicular structures (Persoon and Özok 2017). AP affects all populations with a varying prevalence. A recent review showed that AP concerned 7% of individuals in a Spanish population to 86% in a Croatian population, with a median of 52.5%. (Persoon and Özok 2017). Since a correlation between endodontically treated teeth (Joshipura et al. 2006; Caplan et al. 2009) or chronic AP (Caplan et al. 2006) and adverse cardiovascular effects has been proposed, it is essential to treat teeth with AP.

The fundamental role of bacteria within the pulp space in the development, expansion and maintenance of AP has been widely demonstrated (Nair 2006; Peciuliene et al. 2008; Siqueira and Rocas 2009; Ricucci and Siqueira 2010). Endodontic treatment aims to improve periapical health by removing the root canal pathogens (Trope and Bergenholtz 2002; El Karim et al. 2007). Although the chemomechanical preparation realized during endodontic treatment is effective in reducing the number of microorganisms, it cannot completely eliminate pathogens from the root canal (Byström and Sundqvist 1981; Haapasalo et al. 2005). Therefore, if the canal is left empty between appointments, the remaining bacteria can multiply and reach levels similar to the beginning of treatment (Byström and Sundqvist 1981; Sjogren et al. 1997; Mohammadi and Dummer 2011). To overcome this problem, the use of intracanal medicaments with antimicrobial properties is recommended to fill the root canal

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between treatment sessions (Mohammadi and Dummer 2011; Gupta et al. 2015).

Currently, calcium hydroxide ($Ca(OH)_2$) is the most commonly used intracanal medicament. $Ca(OH)_2$ is commercially available as pastes containing various vehicle. Its concentrations in these pastes vary from 0.85 g/mL to 1.7 g/mL (Fava and Saunders 1999).

The popularity of $Ca(OH)_2$ is due to its antimicrobial effect against Gram-positive, Gram-negative bacteria and fungi from the root canal (Law and Messer 2004; Mohammadi and Dummer 2011; Mohammadi et al. 2012). These antimicrobial properties are related to the release of hydroxyl ions, which increase the pH within the root canal and directly affect the cell membrane and proteins structure of microorganisms (Mohammadi and Dummer 2011; Mohammadi et al. 2012). Indeed, the hight pH of $Ca(OH)_2$ modifies the integrity of the cytoplasmic membrane through denaturation of proteins and destruction of phospholipids or unsaturated fatty acids. These chemical modifications may be due to peroxidation process (Mohammadi et al. 2012).

Among the numerous endodontic pathogens, *Enterococcus faecalis* is one of the microorganisms often recovered in root canal with persistent infections (Rocas et al. 2004; Stuart et al. 2006). It is a Gram-positive bacterium able to invade dentinal tubules (Love 2001) and to resist very harsh environmental conditions (starvation, acidic and alkaline pH) (Evans et al. 2002; McHugh et al. 2004), especially by maintaining pH homeostasis (Figdor et al. 2003; Stuart et al. 2006). Because *E. faecalis* has shown resistance to $Ca(OH)_2$ (Evans et al. 2002), its association with other antimicrobial agents was proposed (Mohammadi and Dummer 2011).

In our laboratory, since several years, we have identified antimicrobial peptides (AMPs) derived from the processing of Chromogranin A (CgA) (Aslam et al. 2012). These AMPs are part of the innate immune response and are released into the circulation shortly after an infection (Radek et al. 2008). They are short, stable in a wide range of pH and temperature and not toxic for host cells (Aslam et al. 2012). Furthermore, they display bacteriolytic activity against a broad spectrum of pathogens, including oral microorganisms, play a crucial role in modulating the immune response (Lugardon et al. 2001; Briolat et al. 2005; Aslam et al. 2012, 2013; Zaet et al. 2017) and induce less resistance than antibiotics (Zaet et al. 2017). We recently observed that by substituting all L-amino acids from Cateslytin, one of the CgA-derived AMPs, with D-amino acids, we could generate D-Ctl, which has improved antimicrobial efficiency against several oral pathogens such as *Parvimonas micra*, *Prevotella intermedia*, *Fusobacterium nucleatum* and *Candida albicans* (Zaet et al. 2017; Dartevelle et al. 2018).

In the present study, we analyzed the activity of several CgA-derived peptides including Chromofungin (CHR), Catestatin (CAT) and the L- and D-form of its active domain, the Cateslytin (D-Ctl and L-Ctl), against *E. faecalis*. We then combined D-Ctl with $Ca(OH)_2$ to improve its antimicrobial efficiency and develop a new stable, non-toxic combination therapy efficient against endodontic pathogens, including *E. faecalis*.

Materials and Methods

Antimicrobial Agents

The following peptides were purchased from Proteogenix: CHR ($bCgA_{47-66}$: RILSILRHQNLLKELQDLAL), CAT ($bCgA_{344–364}$: RSMRLSFRARGYGFRGPGLQL), L-Ctl and D-Ctl ($bCgA_{344–358}$: RSMRLSFRARGYGFR).

Preparation of $Ca(OH)_2$ Solutions

$Ca(OH)_2$ was purchased from Sigma-Aldrich. A saturated solution of $Ca(OH)_2$ (1.7 mg/mL) was diluted to ½ (0.85 mg/mL) and ¼ (0.425 mg/mL). $Ca(OH)_2$ was diluted in milli-Q water, Anaerobe Basal Broth, Sabouraud medium or Dulbecco's Modified Eagle's Medium (DMEM F12, Dutscher) depending on the experiment performed.

Microorganisms and Mammalian Cell Line

Fusobacterium nucleatum (ATCC© 49256™), *Prevotella intermedia* (ATCC© 49046™), and *Parvimonas micra* (ATCC© 33270™) were purchased from ATCC. *Enterococcus faecalis* (CCM 2541) was obtained from the Czechoslovac Collection of Microorganisms. Bacteria were cultured in Anaerobe Basal Broth (Oxoid) at 37 °C in anaerobic conditions. *Candida albicans* (ATCC© 10231™) was cultured in Sabouraud medium (Becton–Dickinson, Germany), supplemented with tetracycline (10 µg/mL; Sigma Aldrich) and cefotaxime (10 µg/mL; Sigma Aldrich).

Human gingival fibroblasts (HGF-1; ATCC® CRL-2014™) were commercially obtained from ATCC and cultured at 37 °C and 5% CO_2 in DMEM F12 (Dutscher) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco), 100 units/mL penicillin and 100 µg/mL streptomycin (Thermo Fisher Scientific).

Broth Dilution Assays

An overnight culture of each pathogen was diluted ($OD_{600nm} = 0.001$) and incubated at 37 °C in 96-plates in the presence of different concentrations of antimicrobial agents. After 24 h incubation, the OD_{600nm} was evaluated with a spectrophotometer (Multikan EX, Thermo Fisher Scientific).

Determination of the Minimal Inhibitory Concentration (MIC)

The MIC, defined as the lowest concentration of peptide able to inhibit 100% of the inoculum, was calculated using a modified Gompertz model as described by Lambert and Pearson (2000).

Cytotoxicity Assays

The cytotoxicity of the antimicrobial agents was examined by MTT [3(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide] assays (Sigma Aldrich) using HGF-1 cells as a model. HGF-1 cultured in DMEM F12 (Dutcher) supplemented with 10% FBS (Gibco), 100 units/mL penicillin and 100 µg/mL streptomycin (Thermo Fisher Scientific) were incubated at 37 °C and 5% CO_2 for 24 h in a 96-wells plate before being treated with various concentrations of $Ca(OH)_2$ alone or supplemented with D-Ctl. Untreated cells were used as a control. After 24 h, 48 h and 72 h incubation, cells were carefully washed with phosphate-buffered saline (PBS) and treated with MTT at a final concentration of 0.25 mg/mL. HGF-1 cells were then incubated for 2 h at 37 °C before being lysed with isopropanol/HCl (48:2, v/v). Cell viability was assessed by reading the OD_{540nm} with a Multiskan EX microplate spectrophotometer (Thermo Fisher Scientific).

Stability Assays of D-Ctl in the Supernatant of *E. faecalis*

The supernatant of *E. faecalis* was prepared as follows: a single colony of *E. faecalis* was suspended in 5 mL of Anaerobe Basal Broth and incubated at 37 °C overnight. The culture was centrifuged at 10,000×*g* for 1 min and filtered (Millex GV filter unit 0.22 µm, Millipore SAS). The supernatant (1 mL) was then incubated at 37 °C for 24 h with D-Ctl (300 µg). As controls, bacterial supernatant (1 mL) and D-Ctl (300 µg) diluted in milli-Q water (1 mL) were incubated separately in similar conditions.

The samples (1 mL) were analyzed by HPLC (Dionex, Ultimate 3000) using a Nucleosil reverse-phase 300-5C18-column (4.6 × 250 mm; particle size: 5 µm; porosity, 300 Å) (Macherey–Nagel). The two solvents used were: 0.1% (v/v) Trifluoroacetic acid (TFA) in water (solvent A) and 0.09% (v/v) TFA in 70% (v/v) acetonitrile–water (solvent B). Absorbance was measured at 214 nm (A_{214nm}). The flow rate for elution was 0.7 mL/min with a gradient of solvent B as indicated on the chromatograms. For each sample, material (1 mL) was directly injected on the HPLC column.

Stability Assays of D-Ctl in $Ca(OH)_2$

D-Ctl (156 µg or 78 µg) corresponding to MIC or ½ MIC was incubated in 1 ml of a $Ca(OH)_2$ solution (1.7 mg/mL or 0.85 mg/mL) at 37 °C for 24 h. The pH of the solution was buffered with HCl to be the same as for the broth dilution assays and MTT tests (pH 9). As a control, D-Ctl (156 µg or 78 µg) was also incubated in milli-Q water (1 mL) at 37 °C for 24 h. The samples (1 mL) were analyzed by HPLC as previously reported.

Statistical Analysis and Graphs

Each assay was done at least in triplicate. For broth dilution and cytotoxicity assays, data are presented as mean ± standard deviation. Statistical analysis was performed using SigmaPlot (release 12.5, Systat Software, Inc). Statistical significance between two groups was determined using the Mann–Whitney Rank Sum Test or the *t*-test. Statistical significance between three groups or more was determined using the Kruskal–Wallis one-way analysis of variance on ranks. Two-way analysis of variance was used when the experimental setups involved two independent variables. For each experiment, the statistical test used is indicated in the figure legend. p-values < 0.05 were considered statistically significant and noted by * on the graphs. Artworks were created using SigmaPlot (release 12.5, Systat Software, Inc).

Results

Antibacterial Assays of Solution of $Ca(OH)_2$

In a preliminary experiment, the efficiency of a saturated solution of $Ca(OH)_2$ (1.7 mg/mL, pH 9) was assessed by broth dilution assays on *E. faecalis* and *F. nucleatum*. Our results confirmed previous studies showing that a saturated solution of $Ca(OH)_2$ could not completely inhibit the growth of *E. faecalis* (Ferreira et al. 2007). In our hands, the inhibition rate was only 58% (±5%). In addition, as previously described (Ferreira et al. 2007), we confirmed that a saturated solution of $Ca(OH)_2$ was able to inhibit 100% (±1%) of *F. nucleatum* growth (Fig. 1).

Antibacterial Activity of Several CgA-Derived Peptides Against *E. faecalis*

We then tested the antibacterial activity of several CgA-derived peptides such as Chromofungin (CHR), Catestatin (CAT) and Cateslytin (L-Ctl or D-Ctl) against *E. faecalis* using broth dilution assays (Fig. 2). All peptides were tested at 200 µg/mL. Our results demonstrated that no natural peptides derived from CgA (CHR, CAT and L-Ctl) display

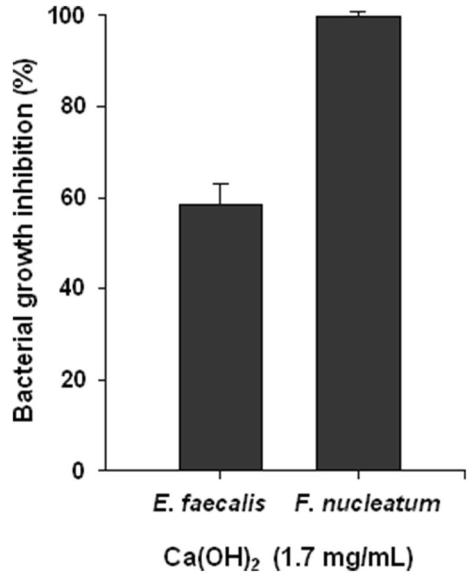
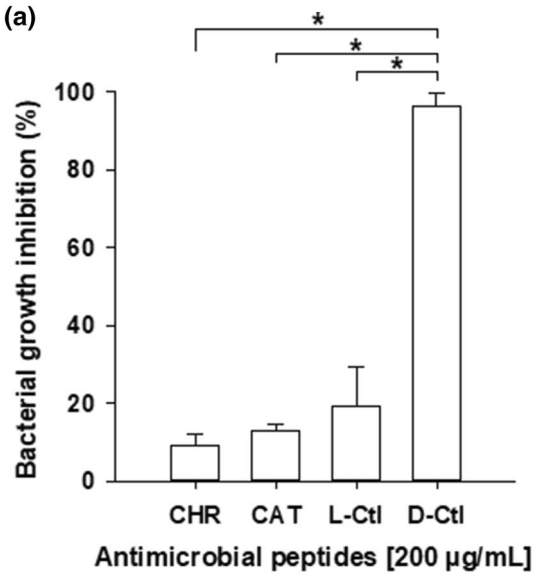


Fig. 1 Inhibition of *E. faecalis* (n = 9) and *F. nucleatum* (n = 3) growth by a saturated solution of $Ca(OH)_2$



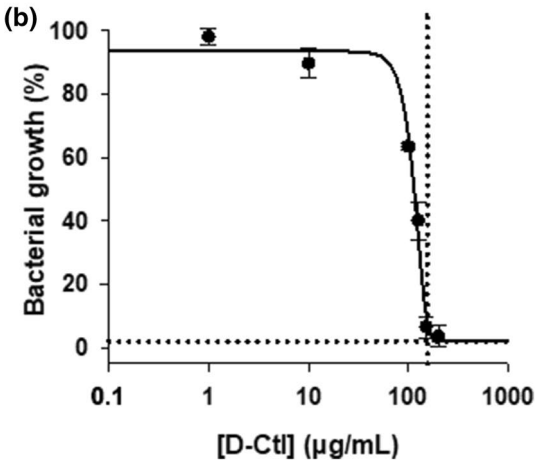


Fig. 2 Activity of antimicrobial CgA-derived peptides against *E. faecalis*. **a** Antimicrobial activity of CgA-derived peptides at 200 µg/mL against *E. faecalis*. For CHR, CAT and L-Ctl n = 3 and for D-Ctl n = 9. Statistical significance was determined by a Kruskal–Wallis one-way analysis of variance on ranks ($p = 0.007$ indicated by "*"). **b** Determination of the MIC (156 µg/mL) of D-Ctl against *E. faecalis* using a modified Gompertz model

antimicrobial activity against *E. faecalis*. In contrast, D-Ctl was able to achieve bacterial growth inhibition (Fig. 2a). Its MIC on *E. faecalis*, determined by using the Gompertz model, was evaluated to 156 µg/mL (Fig. 2b). D-Ctl was therefore chosen as the best candidate to combine with $Ca(OH)_2$.

D-Ctl is Stable in a Saturated Solution of $Ca(OH)_2$

The stability of D-Ctl at the MIC in a saturated solution of $Ca(OH)_2$ (1.7 mg/mL, pH 9) was assessed by HPLC (Fig. 3a). Under our experimental conditions, D-Ctl was eluted after 38 min (Fig. 3a, chromatogram 1). The same peak was identified in a saturated solution of $Ca(OH)_2$, suggesting that D-Ctl at the MIC remains stable under these conditions. The shoulder in the peak corresponds to an oxidized form (oxidation of methionine residue) of D-Ctl. Indeed, the first major peak is the oxidized D-Ctl and the second one to the non-oxidized form (Fig. 3a, chromatogram 2). The areas under the curves were calculated in three independent experiments for each condition. The ratio of areas under the curve of chromatogram 2 to chromatogram 1 was 1.01 ± 0.12.

D-Ctl Remains Stable in the Supernatant of *E. faecalis*

The stability of D-Ctl in the supernatant of *E. faecalis* was assessed by HPLC (Fig. 3b). In our experimental conditions, D-Ctl in milli-Q water was eluted at 38 min (Fig. 3b, chromatogram 1). The same peak was still observed when D-Ctl was incubated with the bacterial supernatant (Fig. 3b, chromatogram 2). The areas under the curves were calculated in three independent experiments for both conditions. The ratio of areas under the curve of chromatogram 2 to chromatogram 1 was 0.97 ± 0.04. The chromatogram 3 represents the bacterial supernatant alone (Fig. 3b, chromatogram 3). Notably, the other peaks on the chromatograms 2 and 3 correspond to proteins included in the media and the proteases secreted by the bacteria. In conclusion, D-Ctl is resistant to the degradation by the virulence factors of *E. faecalis*, allowing a prolonged action of the peptide against this pathogen.

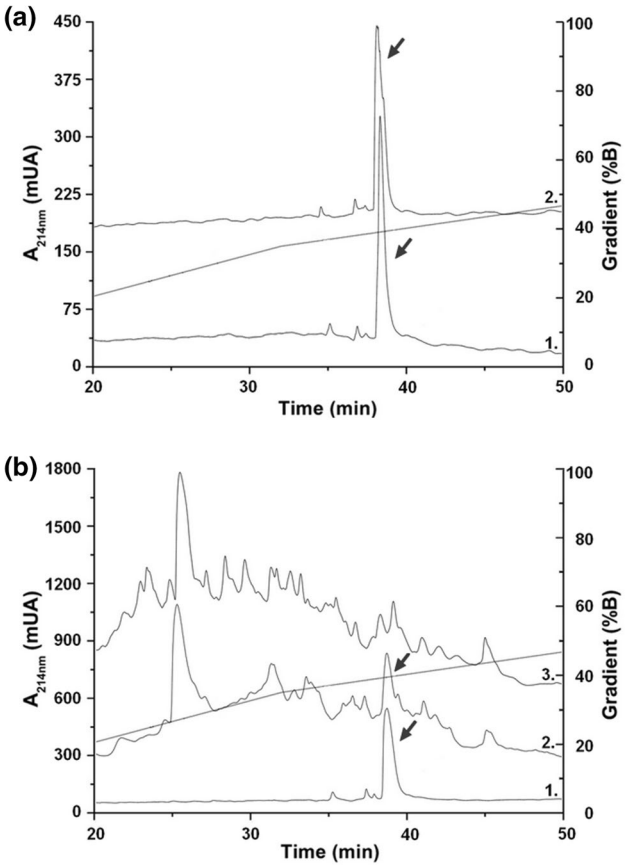


Fig. 3 Stability of D-Ctl in a saturated solution of $Ca(OH)_2$ and in the supernatant of *E. faecalis*. **a** The chromatograms 1 and 2 correspond to D-Ctl diluted in milli-Q water and D-Ctl diluted in a saturated solution of $Ca(OH)_2$ (pH 9), respectively. A black arrow indicates presence of D-Ctl. **b** The chromatograms 1, 2 and 3 correspond to D-Ctl diluted in milli-Q water, D-Ctl diluted in the supernatant of *E. faecalis* and the supernatant of *E. faecalis* alone, respectively. A black arrow indicates presence of D-Ctl

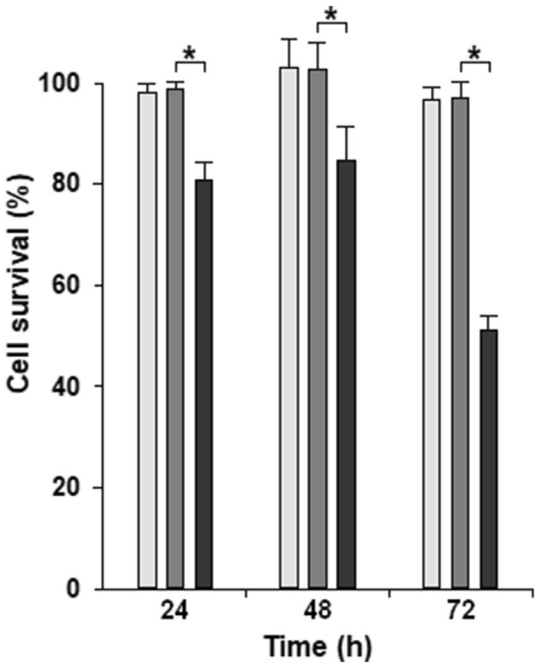


Fig. 4 Cytotoxicity of $Ca(OH)_2$ towards human gingival fibroblasts (n = 5). The bars represent cytoxicity for a solution of $Ca(OH)_2$ at 0.425 mg/mL (light grey bars), 0.85 mg/mL (dark grey bars) and 1.7 mg/ml (black bars). Statistical significance was determined by the Two-Way Analysis of Variance ($p < 0.05$ is indicated by "*")

$Ca(OH)_2$ is Cytotoxic for HGF-1 at High Concentration

Cytotoxicity of solutions of $Ca(OH)_2$ at various concentrations (0.425 mg/mL, 0.85 mg/mL, 1.7 mg/mL) was assessed by MTT assays on HGF-1 grown for 72 h in culture. Cell viability was assessed and expressed as a percentage of control (without $Ca(OH)_2$). $Ca(OH)_2$ at 0.85 mg/mL and 0.425 mg/mL showed no statistically significant toxicity (4% ± 5%), even after 3 days of incubation. Meanwhile, only 50% (± 2%) of the cells were alive after 72 h of incubation with a saturated solution of $Ca(OH)_2$ (1.7 mg/mL) (Fig. 4). Based on these results, diluted solutions of $Ca(OH)_2$ (0.425 mg/mL, 0.85 mg/mL) constituted better choices than a saturated solution for subsequent assays.

$Ca(OH)_2$ Combination of D-Ctl and $Ca(OH)_2$ Inhibits Key Endodontic Pathogens

Since D-Ctl displayed antimicrobial activity against *E. faecalis* and was stable in $Ca(OH)_2$, experiments were conducted to determine the most efficient and the least toxic combination of these antimicrobial agents to eradicate root canal pathogens.

To identify the most efficient combination of $Ca(OH)_2$ and D-Ctl to inhibit the growth of *E. faecalis*, broth dilution assays were performed with different concentrations of $Ca(OH)_2$ and D-Ctl. Specifically, a solution of $Ca(OH)_2$ at non-toxic concentrations (0.85 mg/mL, 0.425 mg/mL) was combined with the MIC, ½ MIC and ¼ MIC of D-Ctl (Fig. 5). As depicted, the combination using the lowest concentration of $Ca(OH)_2$ and D-Ctl able to inhibit *E. faecalis* growth was a solution of $Ca(OH)_2$ at 0.85 mg/mL with ½ MIC of D-Ctl (78 µg/mL) (Fig. 5).

The stability of D-Ctl after 24 h in such a solution was confirmed by HPLC (Fig. 6a). Indeed, D-Ctl eluted at 38 min (Fig. 6a, chromatogram 1) was stable in a solution of $Ca(OH)_2$ at 0.85 mg/mL (pH 9) (Fig. 6a, chromatogram 2). Shoulder on the peaks corresponds to an oxidized form of D-Ctl. The areas under the curves were calculated in three independent experiments for both conditions. The ratio of areas under the curve of chromatogram 2 to chromatogram

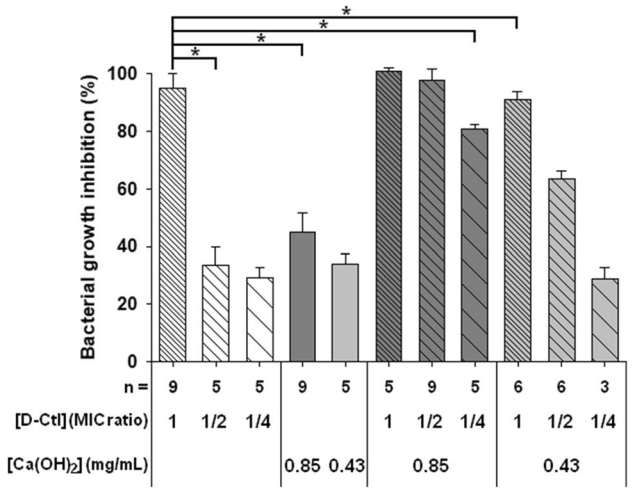


Fig. 5 Inhibition of *E. faecalis* growth by various combinations of $Ca(OH)_2$ and D-Ctl. The combinations tested are indicated on the graph. The number of replicates (n) is indicated on the graph for each combination. Statistical significance was determined by the two-way analysis of variance ($p < 0.05$ is indicated by "*")

1 was 1.075 ± 0.06. We also assessed the cytotoxicity of this combination towards HGF-1 by MTT assays during 72 h. No statistically significant decrease in cell viability was observed (Fig. 6b).

Finally, we verified that this combination was also efficient against the most common endodontic pathogens. To this aim, in addition to *E. faecalis*, we performed broth dilution assays against *P. micra*, *P. intermedia*, *F. nucleatum* and *C. albicans* (Fig. 6c). Our results showed that $Ca(OH)_2$ at 0.85 mg/mL was able to inhibit 87% (± 2%) of *C. albicans* growth, 15% (± 6%) of *P. micra* growth, 77% (± 6%) of *P. intermedia* growth and 30% (± 10%) of *F. nucleatum* growth, whereas the combination inhibited respectively 97% (± 1%), 98% (± 2%), 98% (± 3) and 97% (± 5%) of the pathogen's growth. Therefore, the combination of D-Ctl and $Ca(OH)_2$ is efficient against other key endodontic pathogens like *C. albicans*, *P. micra*, *P. intermedia* and *F. nucleatum*.

Discussion

The use of antimicrobial agents able to eliminate resistant species from root canals and to modulate the periapical inflammatory-immune response may improve the success rate of endodontic treatments (Turner et al. 2004; Lima et al. 2015). In the field of endodontics, AMPs have been studied as irrigants, used during chemomechanical preparation and as intracanal medicament, applied between treatment sessions.

Concerning endodontic irrigants, DJK-5, a cationic peptide, was shown to be effective in improving the

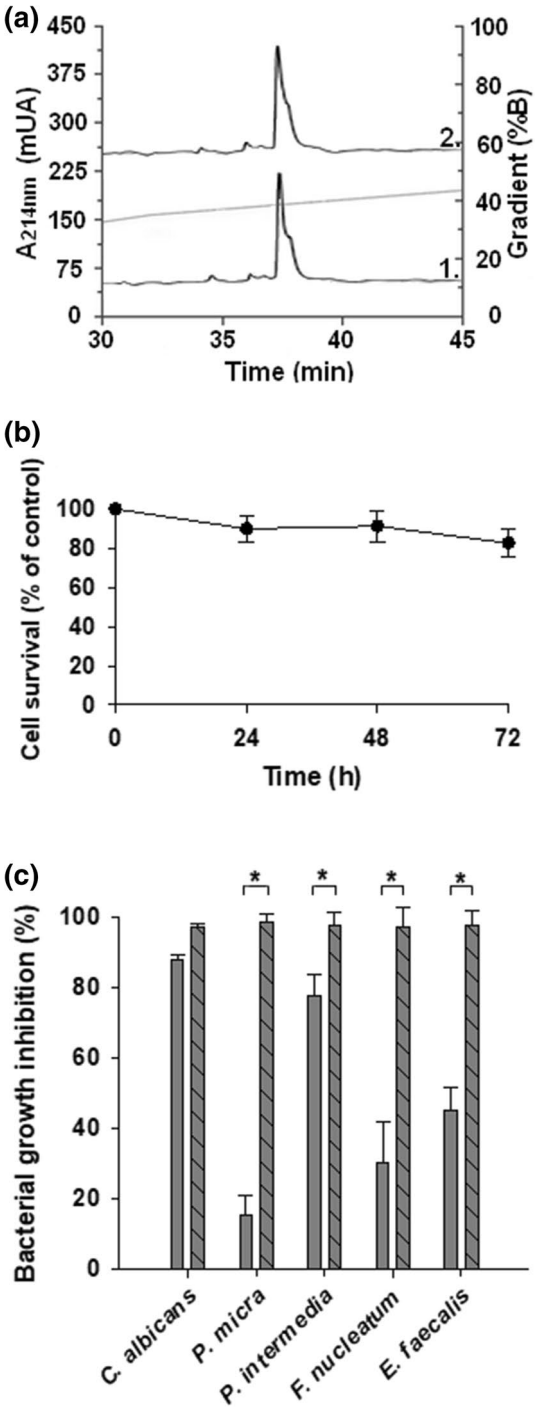


Fig. 6 Stability, cytotoxicity and antimicrobial activity of the combination between D-Ctl and $Ca(OH)_2$. **a** The chromatograms 1 and 2 correspond to D-Ctl diluted in milli-Q water and the combination of $Ca(OH)_2$ at 0.85 mg/mL and ½ MIC of D-Ctl (pH 9) respectively. **b** The cytotoxicity of the combination between $Ca(OH)_2$ at 0.85 mg/mL and ½ MIC of D-Ctl on HGF-1 cells was assessed with MTT assays (n = 4). The Kruskal–Wallis One-Way Analysis of Variance on Ranks showed no statistical significance ($p = 0.239$). **c** The efficacy of the combination between $Ca(OH)_2$ at 0.85 mg/mL and ½ MIC of D-Ctl (striped bars) compared with $Ca(OH)_2$ at 0.85 mg/mL (grey bars) was assessed on four other main endodontic pathogens (n = 3). Statistical significance was determined by t-test ($p < 0.05$ is indicated by "*")

antimicrobial efficacy of a rinse of 6% sodium hypochlorite followed by rinse with ethylenediaminetetraacetic acid (EDTA) (Wang et al. 2018). Furthermore, nisin, an AMP derived from *Lactococcus lactis* (Severina et al. 1998), was demonstrated to improve the antimicrobial efficacy of two conventional endodontic irrigants (Mixture of A Tetracycline Isomer, An Acid and A Detergent (MTAD) and sodium hypochlorite), against some Gram-positive bacteria associated with persistent intracanal infection, including *E. faecalis* (Tong et al. 2012, 2014; Kajwadkar et al. 2017). Therefore, nisin has potential as an alternative to conventional irrigation with sodium hypochlorite.

Another aspect of endodontic disinfection is the use of an intracanal medicament, such as $Ca(OH)_2$, to prevent the multiplication of residual microorganisms in the root canal between treatment sessions. Two peptides have been studied as intracanal medicaments: Human β-defensin-3 (HBD3), an epithelial derived cationic AMP (Harder et al. 2001), widely expressed in the oral cavity and nisin. In recent studies, HBD3 demonstrated better antimicrobial activities against *E. faecalis* biofilms and multispecies biofilms than $Ca(OH)_2$ and chlorhexidine (Lee et al. 2013a, b; Ahn et al. 2017). Nisin has also been shown to be effective in inhibiting *E. faecalis* with a minimum bactericidal concentration (MBC) of 70 mg/mL and *Streptococcus gordonii* with a MBC of 20 mg/mL (Turner et al. 2004). However, nisin alone may not be an appropriate intracanal medicament, because it is less effective against Gram-negative bacteria, which are also present in infected root canals (Turner et al. 2004).

Even though AMPs can seem suitable to improve the root canal distinction, one barrier to their therapeutic applications is that they are expensive to manufacture (Hancock and Sahl 2006). HBD3 for example, is a cationic peptide of 45 residues with a complex tertiary structure that makes its synthesis difficult (Dhople et al. 2006). In this perspective, D-Ctl seemed to be a good candidate for this study as it is short and linear (15 amino acids).

Besides their costs, AMPs also have the drawback to be susceptible to proteolytic degradation. *E. faecalis* can overcome the innate immune system response and trigger persistent infections. One of its mechanisms of resistance is the degradation of AMPs (Schmidtchen et al. 2002; Nešuta et al. 2017). For example, proteases secreted by *E. faecalis* can degrade LL-37 and HYL-20, two AMPs known for their antimicrobial properties (Schmidtchen et al. 2002; Nešuta et al. 2017). In order to counteract an endodontic infection, D-Ctl should not be degraded by the proteases secreted by *E. faecalis.* Our study showed that D-Ctl maintains its integrity in presence of *E. faecalis* (Fig. 3b). Indeed, as D-Ctl strictly consists of D-amino acids, it is not sensitive to bacterial proteases (Zaet et al. 2017). Interestingly, D-Ctl is also stable in $Ca(OH)_2$ and their combination ($Ca(OH)_2$ (0.85 mg/mL) and D-Ctl (½ MIC)) is able to completely inhibit *E. faecalis* growth (Fig. 6). Recent studies, using *Escherichia coli* as a model, have shown that the bacteriolytic activity of D-Ctl is due to the rapid permeabilization of the bacterial cell membrane leading to bacterial death (Zaet et al. 2017). This mechanism is due to the positive charge and amphipathic structure of the AMPs, allowing them to interact with negatively charged phospholipid bilayers, causing the disruption of bacterial cell membranes (Nešuta et al. 2017).

The absence of cytotoxicity is also an important characteristic for an efficient intracanal medicament. From this point of view, $Ca(OH)_2$ and chlorhexidine are the most acceptable intracanal medicaments while others, like phenol and formocresol are highly cytotoxic (Kawashima et al. 2009). In our study, $Ca(OH)_2$ at high concentrations (1.7 mg/mL) was found to be toxic to HGF-1 after 72 h (Fig. 4). Therefore, our work focused on finding a combination including lower concentration of $Ca(OH)_2$ (0.425 mg/mL and 0.85 mg/mL). Interestingly, $Ca(OH)_2$ at 0.85 mg/mL alone or in combination with D-Ctl was not toxic to HGF-1.

Even though *E. faecalis* is strongly correlate with endodontic failure, several other pathogens are involved in endodontic infection. Here, we showed that the combination between D-Ctl and $Ca(OH)_2$ is also efficient against four other endodontic pathogens: *P. micra*, *P. intermedia*, *F. nucleatum* and *C. albicans*. Interestingly, *F. nucleatum* and *P. intermedia* are Gram-negative bacteria, showing that the efficacy of D-Ctl does not only concern Gram-positive species. These encouraging results suggest that this combination therapy could eradicate endodontic biofilms.

Despite promising preliminary results about the antimicrobial efficacy of the combination of D-Ctl and $Ca(OH)_2$, this drug combination was tested in vitro against planktonic bacteria and might not necessarily be effective against multispecies microbial biofilms in vivo. Therefore, further investigations are needed to test the efficiency of this combination. Interestingly, we present a comparison of sequences of Ctl with two others potent anti-biofilm AMPs (Fig. 7) (Pletzer and Hancock 2016). This comparison suggests that Ctl belongs to the same group of amphipathic peptides and could therefore be efficient against biofilms.

Peptide	Sequence	Positive charges	Hydrophobic residues
D-Ctl	R S M R L S F R A R G Y G F R	+5	5
IDR 1018	V R L I V A V R I W - - R R	+4	7
DJK 5	V Q W R - A I R V R V I - - R	+4	6
Consensus sequence	x x H B H x H B x B x H x x B	+4	4

Fig. 7 Comparison sequence of D-Ctl with other amphipatic anti-biofilm peptides. Basic residues are underlined and hydrophobic residues framed. Positive charges and the number of hydrophobic residues are indicated for each AMP. The final line presents a consensus sequence where basic residues (B) and hydrophobic residues (H) are pointed out

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Ahn KB, Kim AR, Kum KY, Yun CH, Han SH (2017) The synthetic human beta-defensin-3 C15 peptide exhibits antimicrobial activity against *Streptococcus mutans*, both alone and in combination with dental disinfectants. J Microbiol 55:830–836. https://doi.org/10.1007/s12275-017-7362-y

Aslam R, Atindehou M, Lavaux T, Haïkel Y, Schneider F, Metz-Boutigue MH (2012) Chromogranin A-derived peptides are involved in innate immunity. Curr Med Chem 19:4115–4123. https://doi.org/10.2174/092986712802430063

Aslam R, Marban C, Corazzol C et al (2013) Cateslytin, a chromogranin a derived peptide is active against *Staphylococcus aureus* and resistant to degradation by its proteases. PLoS ONE 8:e68993. https://doi.org/10.1371/journal.pone.0068993

Briolat J, Wu SD, Mahata SK et al (2005) New antimicrobial activity for the catecholamine release-inhibitory peptide from chromogranin A. Cell Mol Life Sci 62:377–385. https://doi.org/10.1007/s00018-004-4461-9

Byström A, Sundqvist G (1981) Bacteriologic evaluation of the efficacy of mechanical root canal instrumentation in endodontic therapy. Scand J Dent Res 89:321–328. https://doi.org/10.1111/j.1600-0722.1981.tb01689.x

Caplan DJ, Chasen JB, Krall EA et al (2006) Lesions of endodontic origin and risk of coronary heart disease. J Dent Res 85:996–1000. https://doi.org/10.1177/154405910608501104

Caplan DJ, Pankow JS, Cai J, Offenbacher S, Beck JD (2009) The relationship between self-reported history of endodontic therapy and coronary heart disease in the atherosclerosis risk in communities study. J Am Dent Assoc 140:1004–1012. https://doi.org/10.14219/jada.archive.2009.0311

Dartevelle P, Ehlinger C, Zaet A et al (2018) D-Cateslytin: a new antifungal agent for the treatment of oral *Candida albicans* associated infections. Sci Rep 8:9235. https://doi.org/10.1038/s41598-018-27417-x

Dhople V, Krukemeyer A, Ramamoorthy A (2006) The human beta-defensin-3, an antibacterial peptide with multiple biological functions. Biochim Biophys Acta 1758:1499–1512. https://doi.org/10.1016/j.bbamem.2006.07.007

El Karim I, Kennedy J, Hussey D (2007) The antimicrobial effects of root canal irrigation and medication. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 103:560–569. https://doi.org/10.1016/j.tripleo.2006.10.004

Evans M, Davies JK, Sundqvist G, Figdor D (2002) Mechanisms involved in the resistance of *Enterococcus faecalis* to calcium hydroxide. Int Endod J 35:221–228. https://doi.org/10.1046/j.1365-2591.2002.00504.x

Fava LR, Saunders WP (1999) Calcium hydroxide pastes: classification and clinical indications. Int Endod J 32:257–282. https://doi.org/10.1046/j.1365-2591.1999.00232.x

Ferreira FB, Torres SA, Rosa OP, Ferreira CM, Garcia RB, Marcucci MC, Gomes BP (2007) Antimicrobial effect of propolis and other substances against selected endodontic pathogens. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 104:709–716. https://doi.org/10.1016/j.tripleo.2007.05.019

Figdor D, Davies JK, Sundqvist G (2003) Starvation survival, growth and recovery of *Enterococcus faecalis* in human serum. Oral Microbiol Immunol 18:234–239. https://doi.org/10.1034/j.1399-302X.2003.00072.x

Gupta SP, Bhati M, Jhajharia K, Patel H, Paliwal A, Franklin S (2015) Evaluation of antimicrobial and antifungal efficacy of inter appointment intracanal medicaments against *Enterococcus* and *Candida albicans*: an in vitro study. J Int Oral Health 7:97–102

Haapasalo M, Endal U, Zandi H, Coil JM (2005) Eradication of endodontic infection by instrumentation and irrigation solutions. Endod Top 10:77–102. https://doi.org/10.1111/j.1601-1546.2005.00135.x

Hancock REW, Sahl H (2006) Antimicrobial and host-defense peptides as new antiinfective therapeutic strategies. Nat Biotechnol 24:1551–1557. https://doi.org/10.1038/nbt1267

Harder J, Bartels J, Christophers E, Schroder JM (2001) Isolation and characterization of human beta -defensin-3, a novel human inducible peptide antibiotic. J Biol Chem 276:5707–5713. https://doi.org/10.1074/jbc.M008557200

Joshipura KJ, Pitiphat W, Hung HC, Willett WC, Colditz GA, Douglass CW (2006) Pulpal inflammation and incidence of coronary

heart disease. J Endod 32:99–103. https://doi.org/10.1016/j.joen.2005.10.039
Kajwadkar R, Shin JM, Lin GH, Fenno JC, Rickard AH, Kapila YL (2017) High-purity nisin alone or in combination with sodium hypochlorite is effective against planktonic and biofilm populations of *Enterococcus faecalis*. J Endod 43:989–994. https://doi.org/10.1016/j.joen.2017.01.034
Kawashima N, Wadachi R, Suda H, Yeng T, Parashos P (2009) Root canal medicaments. Int Dent J 59:5–11. https://doi.org/10.1922/IDJ_2060Kawashima07
Lambert RJ, Pearson J (2000) Susceptibility testing: accurate and reproducible minimum inhibitory concentration (MIC) and non-inhibitory concentration (NIC) values. J Appl Microbiol 88:784–790. https://doi.org/10.1046/j.1365-2672.2000.01017.x
Law A, Messer H (2004) An evidence-based analysis of the antibacterial effectiveness of intracanal medicaments. J Endod 30:689–694. https://doi.org/10.1097/01.don.0000129959.20011.ee
Lee JK, Chang SW, Perinpanayagam H et al (2013a) Antibacterial efficacy of a human β-defensin-3 peptide on multispecies biofilms. J Endod 39:1625–1629. https://doi.org/10.1016/j.joen.2013.07.035
Lee JK, Park YJ, Kum KY et al (2013b) Antimicrobial efficacy of a human β-defensin-3 peptide using an *Enterococcus faecalis* dentine infection model. Int Endod J 46:406–412. https://doi.org/10.1111/iej.12002
Lima SMF, de Pádua GM, Sousa MGDC, Freire MS, Franco OL, Rezende TMB (2015) Antimicrobial peptide-based treatment for endodontic infections—biotechnological innovation in endodontics. Biotechnol Adv 33:203–213. https://doi.org/10.1016/j.biotechadv.2014.10.013
Love RM (2001) *Enterococcus faecalis*—a mechanism for its role in endodontic failure. Int Endod J 34:399–405. https://doi.org/10.1046/j.1365-2591.2001.00437.x
Lugardon K, Chasserot-Golaz S, Kieffer AE et al (2001) Structural and biological characterization of chromofungin, the antifungal chromogranin A-(47-66)-derived peptide. J Biol Chem 276:35875–35882. https://doi.org/10.1074/jbc.M104670200
McHugh CP, Zhang P, Michalek S, Eleazer PD (2004) pH required to kill *Enterococcus faecalis* in vitro. J Endod 30:218–219. https://doi.org/10.1097/00004770-200404000-00008
Mohammadi Z, Dummer PM (2011) Properties and applications of calcium hydroxide in endodontics and dental traumatology. Int Endod J 44:697–730. https://doi.org/10.1111/j.1365-2591.2011.01886.x
Mohammadi Z, Shalavi S, Yazdizadeh M (2012) Antimicrobial activity of calcium hydroxide in endodontics: a review. Chonnam Med J 48:133–140. https://doi.org/10.4068/cmj.2012.48.3.133
Nair PN (2006) On the causes of persistent apical periodontitis: a review. Int Endod J 39:249–281. https://doi.org/10.1111/j.1365-2591.2006.01099.x
Nešuta O, Budešínský M, Hadravová R, Monincová L, Humpolicková J, Cerovský V (2017) How proteases from *Enterococcus faecalis* contribute to its resistance to short α-helical antimicrobial peptides. Pathog Dis 75:15. https://doi.org/10.1093/femspd/ftx091
Peciuliene V, Maneliene R, Balcikonyte E, Drukteinis S, Rutkunas V (2008) Microorganisms in root canal infections: a review. Stomatologija 10:4–9
Persoon IF, Özok AR (2017) Definitions and epidemiology of endodontic infections. Curr Oral Health Rep 4:278–285. https://doi.org/10.1007/s40496-017-0161-z
Pletzer D, Hancock RE (2016) Antibiofilm peptides: potential as broad-spectrum agents. J Bacteriol 198:2572–2578. https://doi.org/10.1128/JB.00017-16
Radek KA, Lopez-Garcia B, Hupe M et al (2008) The neuroendocrine peptide catestatin is a cutaneous antimicrobial and induced in the skin after injury. J Invest Dermatol 128:1525–1534. https://doi.org/10.1038/sj.jid.5701225
Ricucci D, Siqueira JF (2010) Biofilms and apical periodontitis: study of prevalence and association with clinical and histopathologic findings. J Endod 36:1277–1288. https://doi.org/10.1016/j.joen.2010.04.007
Rocas IN, Siqueira JF, Santos KRN (2004) Association of *Enterococcus faecalis* with different forms of periradicular diseases. J Endod 30:315–320. https://doi.org/10.1097/00004770-200405000-00004
Schmidtchen A, Frick IM, Andersson E, Tapper H, Bjorck L (2002) Proteinases of common pathogenic bacteria degrade and inactivate the antibacterial peptide LL-37. Mol Microbiol 46:157–168. https://doi.org/10.1046/j.1365-2958.2002.03146.x
Severina E, Severin A, Tomasz A (1998) Antibacterial efficacy of nisin against multidrugresistant gram-positive pathogens. J Antimicrob Chemother 41:341–347. https://doi.org/10.1093/jac/41.3.341
Siqueira JF, Rocas IN (2009) Diversity of endodontic microbiota revisited. J Dent Res 88:969–981. https://doi.org/10.1177/0022034509346549
Sjogren U, Figdor D, Persson S, Sundqvist G (1997) Influence of infection at the time of root filling on the outcome of endodontic treatment of teeth with apical periodontitis. Int Endod J 30:297–306. https://doi.org/10.1046/j.1365-2591.1997.00092.x
Stuart CH, Schwartz SA, Beeson TJ, Owatz CB (2006) *Enterococcus faecalis*: its role in root canal treatment failure and current concepts in retreatment. J Endod 32:93–98. https://doi.org/10.1016/j.joen.2005.10.049
Tong Z, Zhou L, Kuang R, Lv H, Qu T, Ni L (2012) *In vitro* evaluation of MTAD and nisin in combination against common pathogens associated with root canal infection. J Endod 38:490–494. https://doi.org/10.1016/j.joen.2011.11.015
Tong Z, Ni L, Ling J (2014) Antibacterial peptide nisin: a potential role in the inhibition of oral pathogenic bacteria. Peptides 60:32–40. https://doi.org/10.1016/j.peptides.2014.07.020
Trope M, Bergenholtz G (2002) Microbiological basis for endodontic treatment: can a maximal outcome be achieved in one visit? Endod Top 1:40–53. https://doi.org/10.1034/j.1601-1546.2002.10104.x
Turner SR, Love RM, Lyons KM (2004) An in vitro investigation of the antibacterial effect of nisin in root canals and canal wall radicular dentine. Int Endod J 37:664–671. https://doi.org/10.1111/j.1365-2591.2004.00846.x
Wang D, Shen Y, Hancock REW, Ma J, Haapasalo M (2018) Antimicrobial effect of peptide DJK-5 used alone or mixed with EDTA on mono- and multispecies biofilms in dentin canals. J Endod 44:1709–1713. https://doi.org/10.1016/j.joen.2018.07.018
Zaet A, Dartevelle P, Daouad F et al (2017) D-Cateslytin, a new antimicrobial peptide with therapeutic potential. Sci Rep 7:15199. https://doi.org/10.1038/s41598-017-15436-z