ASSESSMENT OF IN VITRO EFFICACY OF THE NOVEL ANTIMICROBIAL PEPTIDE CECT7121 AGAINST HUMAN GRAM POSITIVE BACTERIA FROM SERIOUS INFECTIONS REFRACTORY TO TREATMENT.

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Running Title: EFFICACY OF THE PEPTIDE CECT7121 AGAINST RESILIENT HUMAN BACTERIA

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Abstract

Background: Resistant Gram positive bacteria are causing increasing concern in clinical practice. This work investigated the efficacy of AP-CECT7121 (an antimicrobial peptide isolated from an environmental strain of *Enterococcus faecalis* CECT7121) against various pathogenic Gram positive bacteria. ***Methods:*** Strains were isolated from intensive care unit patients unresponsive to standard antibiotic treatments. Inhibitory activity of AP-CECT7121 was assessed using the well-diffusion agar method. The most resistant isolates from each species screened (*Enterococcus faecium*, *E. faecalis, Staphylococcus aureus*, *Streptococcus pneumoniae*, *Strep. pyogenes*, *Clostridium perfringens*, *C. difficile*) were further examined in time-killing curve (KC) studies.

Results: These bactericidal kinetic experiments demonstrated a rapid killing effect with no viable bacteria being detected within 30 and 90 minutes for enterococcal and streptococcal strains and 180 min for Ca-MRSA and *C. perfringens*: viable counts for *C. difficile* were decreased threefold after 90 min. ***Conclusions:*** AP-CECT7121 may provide a novel strategy for treating potentially fatal clinical infections in hospitalized patients.

Keywords: AP-CECT7121; Efficacy; Resistance; Gram positives; Human strains.

1.INTRODUCTION

The rapid emergence of multi-resistant Gram positive and Gram negative bacteria, is now causing serious therapeutic concern in clinical practice [1, 2, 3, 4]. The pharmaceutical industry has responded by making concerted efforts to develop new molecules as therapeutic alternatives.

One comparatively recent therapeutic approach is the biological control of infectious diseases, which is only now attracting worldwide interest, despite the fact that the potential use of bactericidal peptides (bacteriocins) in clinical practice was first suggested in 1950. However, research in this area has been eclipsed since, for decades, the focus of the pharmaceutical industry has been on the development of new synthetic drugs and it is only in the last few years that there has been a resurgence of interest in the potential clinical application of bacteriocins.

Currently, our laboratory is studying the inhibitory properties of specific strains of *Enterococcus* isolated from natural corn silage, particularly in relation to the biological control of infection [5,6,7,8,9]. One of these strains, *Enterococcus faecalis* CECT7121 (deposited at the Spanish Collection of Typical Cultures, Burjasot, Valencia, Spain, with European patent, EP1816190) was shown to produce a novel antimicrobial peptide (AP) with a broad inhibitory spectrum against different Gram-positive bacteria in mastitic dairy cattle [10]. Assessment of the physico-chemical properties of AP-CECT7121 revealed it to be a low molecular weight (5 kDa), lipophilic compound, stable over a wide pH range (4.0-8.0) (data stated in European patent, EP1816190). These properties are

consistent with the assignment of AP-CECT7121 into Group II of the master bacteriocin classification reported by Hechard and Sahl [11].

Moreover, in evaluating *E. faecalis* CECT7121 as a “biological controller” it demonstrated several interesting biological properties (e.g. immunomodulation, antiinfective action and food bio-preservative activity) of potential use in humans [7, 8, 9].

The main aim of the work described in this paper was to assess the *in vitro* inhibition spectrum and bactericidal action of the AP-CECT7121 against a range of Gram positive aerobic and anaerobic bacterial strains isolated from patients with severe infections, some of which were recalcitrant to standardized *in vivo* treatment regimes and demonstrated high levels of resistance to the antimicrobials used.

2. MATERIAL AND METHODS

2.1. Isolation and characterization of bacterial strains from patients.

In a preliminary screening study of bacterial sensitivity to the AP-CECT7121 was assessed a panel of ATCC reference strains (Manassas, VA, USA) and also a variety of strains isolated from patients with severe bacterial infections and hospitalized at the intensive unit care (IUC) of the R. Santamarina Hospital, Tandil, Argentina; from 2005 to 2007 (see Table 1). The source of the clinical strains tested were as follows: *Streptococcus pyogenes* (HRS3107, HRS3410, HRS3114; blood culture), *Enterococcus faecium* (HRS808, HRS950, HRS856, HRS807; blood culture), ampicillin-resistant (HRS905, HRS1005, HRS1036; blood culture) and vancomycin-resistant *Enterococcus faecalis* (HRS1084; blood culture), penicillin-resistant *Streptococcus pneumoniae* (HRS2672, HRS2984; blood culture) and (HRS2563; pleural fluid), community-acquired methicillin-resistant *Staphylocccus aureus* (Ca-MRSA) HRS45 and HRS47 (pleural fluid and blood culture respectively), hospital acquired methicillin-resistant *S aureus* (H-MRSA) HRS162, HRS23 (blood culture), HRS95 (cerebrospinal fluid), *Clostridium perfringens* HRS37, HRS64 (cellulitis aspiration fluid) and HRS71, HRS73, HRS148 (necrotising fasciitis biopsy), *Clostridium difficile* HRS21 (faeces from patient diagnosed with pseudomembranous colitis). All the above isolates were of clinical significance, since most of source patients were suffering from infections which were recalcitrant to conventional treatment, and in many cases resulted in fatality. For each species screened, strains with lowest inhibition zone (see Table 1) were subsequently subjected to a time-killing curve study (KC) in order to evaluate the *in vitro* efficacy of the antimicrobial peptide AP-CECT7121.

Phenotypic characterisation of the clinical *Staphylococcus* strains was based on the confirmation of a) catalase and coagulase production, b) the ability to hydrolyze mannitol using mannitol salt agar (Lab. Britania, Argentina), c) the presence of DNAse (DNase Test Agar, Difco) and d) pyrrylidonylarylamidase (PYR-A-ENT, Lab. Britania, Argentina) and e) production of acetoin from pyruvate by the Voges-Proskauer test. Identity of the clinical *Streptococcus* strains was confirmed according to protocols described by Holt et al., [12] and Ruoff et al., [13]. The presence of β-hemolysis (sheep blood) was also evaluated and β-hemolytic *Streptococcus* strains were further characterized using a latex agglutination test (Slidex Strepto-Kit, BioMérieux) to detect group-specific polysaccharide antigens (A, B, C, D, F and G). The clinical *Enterococcus* strains, were confirmed by: esculin hydrolysis in the presence of 40% bile (agar bile-esculin agar, Difco), leucine arylamidase activity (LAP, Lab. Britania, Argentina), hemolysis in anaerobically incubated agar-base Columbia (Oxoid) containing 5% (v/v) sheep blood blood, resistance to vancomycin (30 µg), acetoine production, mobility, pigment production, pyruvate utilisation, tolerance to 0.04% potassium telurite, arginine hydrolysis, acidification of 1% methyl-alpha-d-glucopyranoside (Sigma). Fermentation studies were also undertaken with carbohydrates such as: glucose, melibiose, adonitol, D-rafinose, lactose, melezitose, ramnose, mannitol, sorbitol, L-arabinose, L-sorbose, trehalose, D-xylose, ribose, sacarose and D-arabitol [14, 15]. Strains were maintained in brain heart infusion (BHI) broth with glycerol 20% (v/v) at -70ºC. For testing, all isolates were thawed and transferred at least twice on BHI agar-5% defibrinated sheep blood for *Streptococcus* and BHI agar for *Enterococcus* and *S. aureus* strains.

Clinical *Clostridium* strains were confirmed by standard methods [16]. Briefly, this included confirmation of the presence of catalase and urease, lipase and lecitinase activity on egg yolk agar (Lab. Britania, Argentina), morphologic characteristics, haemolytic activity on sheep blood agar and sugar fermentation (glucose, maltose, lactose, inulin, dulcitol, mannitol, inositol and salicin). The inoculated sugar media were incubated anaerobically at 37ºC for 24h and examined for acid and gas production. In addition,, the presence of toxin A of *C. difficile* was determined (*Clostridium difficile* Tox A, OXOID). The strains were maintained in skimmed milk at -70°C. For testing, isolates were thawed and transferred at least twice on brucella agar supplemented with hemin, vitamin K1, and 5% sheep blood (brucella blood agar [BBA]), to ensure purity and optimal growth (Anaerobe Systems, OXOID, Cambridge, UK).

An *in vitro* diffusion test was used to confirm the antibiotic resistance according to the policy established by the Clinical and Laboratory Standards Institute (CLSI). Minimum inhibitory concentration (MIC) tests were undertaken using a micro-dilution method for penicillin on *Str. pneumoniae* strains and for vancomycin and teicoplanin on *Enterococcus* strains [17, 18].

The inhibitory activity of the AP-CECT7121 was screened using the agar-well diffusion method previously described by Sparo et al. [6]. Briefly, plates containing 13mL of Mueller Hinton agar (Merck, Darmstadt, Germany) were inoculated with the indicator microorganism (approx. 10^7 CFU mL^{-1}) in 7mL of the appropriate medium, with soft agar (7g L^{-1}). For *Str. pyogenes* and *Str. pneumoniae* Mueller Hinton agar supplemented with 5% defibrinated sheep

blood was used. *Enterococcus* and *Staphylococcus* were tested using BHI agar whilst, for *Clostridium*, Reinforced Clostridium Medium (RCM) agar was used. Each well in the agar (diameter 6mm) contained 50µl purified AP-CECT7121 (powder dissolved in 1mL 50mM^{-1} sodium phosphate buffer, pH7).

A strain of known sensitivity, *Listeria monocytogenes* CEB101, was used as positive control in screening tests to assess the inhibitory activity of AP-CECT7121 [10].

2.2. Time-killing curve (KC) studies

For each bacterial species initially screened, bactericidal activity of the AP-CECT7121 against the strains which gave the smallest inhibition zone was further examined in time-killing curve (KC) studies using a previously described method [19]. Isolates were incubated in Mueller–Hinton broth, either unsupplemented (*S. aureus* and *Enterococcus* spp.*)* or supplemented with 5% lysed horse blood (*Str. pneumoniae* and *Str. pyogenes*). Briefly, 18h cultures of each selected strain were diluted in series and spread on two to four agar plates in the appropriate broth (at a final viable count of approximately 10^5 CFU mL $^{-1}$) and then purified AP-CECT7121 was added to give a final concentration of 400 AU mL $^{-1}$. *Note:* This was equivalent to an actual peptide concentration of 12 µg mL $^{-1}$ and constituted a 1:1 dilution of the material obtained following HPLC purification - see section 2.3). The limit of detection (LOD) was 10 CFU/mL. Drug carryover was assessed by visual inspection of the distribution of colonies on the plates.

Sub-samples were removed from this mixture every 30 min over a total incubation period of 180 min. Viable counts in these sub-samples were estimated after incubation in BHI agar at 35 ºC for 24 h for *Enterococcus* and *S. aureus* strains. For *Streptococcus* strains, viable counts were determined after incubation in agar base Columbia (Lab. Britania , Argentina) supplemented with 5% sheep blood, in a 5% CO_2 atmosphere, at 35 ºC for 48 h . For each strain, cultures incubated in parallel in absence of the AP-CECT7121 acted as controls.

KC studies for *Clostridium* species were performed in reduced brucella broth supplemented with vitamin K1 and hemin. The AP-CECT7121 and test strains (final inoculum, approximately 10^5 UFC mL^{-1}) were added to tubes, which were then sealed with Hungate-type caps (Bellco Inc., NY, USA), placed in an anaerobic chamber and then incubated at 35ºC. Aliquots were removed with a tuberculin syringe at 0, 30, 60, 90, 120 and 180 min and transferred to Brucella agar plates supplemented with vitamin K1 and hemin. After incubation for 24 h at 35ºC colonies were quantitated at appropriate dilutions.

For each strain tested cultures incubated and processed in a similar fashion, but in the absence of the AP-CECT7121, acted as controls.

For all strains studied, bactericidal activity was defined as a minimum of 3-Log_{10} reduction in viable bacterial counts. Three separate KC experiments for each species were carried out.

2.3. Isolation and purification of AP-CECT7121

AP-CECT7121 was isolated and purified from 18h (at 35ºC) cultures of *E. faecalis* CECT7121 in BHI broth. This culture was inoculated into 4 L of BHI broth and incubated for 9 h at 35ºC. Cells were then removed by centrifugation (15.000 x *g*, 4ºC, 20 min), after which the supernatant was adjusted to pH 7 and precipitated according to the method described by Dawson (1969) [20]. After centrifugation (20,000 x *g*, 4ºC, 20 min), the resulting sediment was suspended in 40mL 50mM^{-1} sodium phosphate buffer, pH7. This suspension was stored in two aliquots at -70 ºC until analysis by Reverse Phase High Performance Liquid Chromatography (RP-HPLC).

The AP-CECT 7121 was isolated by physico-chemical extraction using Sep-Pak C_{18} cartridges (Sep-Pak®, Waters Co, USA). Briefly, 5mL of the *E. faecalis* extract was loaded onto the cartridge which was washed with acetonitrile in trifluoroacetic acid (TFA; 0.1%, v/v) and then eluted with a mixture of acetonitrile (60% v/v) in TFA (0.1% v/v). The eluant was concentrated to dryness using a vacuum centrifuge (ThermoSavant Instruments, NY, USA). The resulting residue was then re-suspended in PBS (250µL) and analyzed for inhibitory activity by the agar-well diffusion method, using *L. monocytogenes* CEB101 as the marker strain, as described above. Samples with demonstrable inhibitory activity were combined and stored at -70 ºC.

Twenty µL of the sample obtained from the physico-chemical extraction was injected onto the RP-HPLC system, and separated on a 5µm Nucleosil® C_{18} column (100 x 2.1mm; Pharmacia). The mobile phase consisted of Buffered

Solution A: (TFA 0.1% v/v) and Buffered Solution B (acetonitrile 95% v/v in TFA 0.1% v/v). The AP-CECT7121 was eluted using a linear gradient (95% A / 5% B to 15% A / 85% B) at a flow rate of 0.2mL/min. The eluant was monitored using UV detection at 210 nm. Fractions were collected at regular intervals, evaporated to dryness and again re-suspended in 50mM phosphate buffer, pH 7 for the subsequent evaluation of inhibitory activity, again using *L. monocytogenes* CEB101 as the marker strain, as described above.

Fractions with high AP-CECT7121 activity were mixed and re-chromatographed on the reverse phase column to obtain chromatographically pure peptide. Molecular weight (MW) was determined by mass spectrometry (Finnigan TSQ Quantum, Thermo Scientific, Waltham, USA) and the protein concentration of pure AP-CECT7121 was determined by the Coomassie protein assay reagent as described the manufacturer (Pierce Rockford, ILL., USA). Finally, the AP-CECT7121 was lyophilized until further analysis.

2.4. SDS-PAGE studies

To confirm the identity of AP-CECT7121 in the final HPLC eluate, chemical features including MW and protein nature, were assessed by SDS-PAGE electrophoresis (21). Briefly, native and trypsinized AP-CECT7121 were analysed by Tricine SDS-PAGE (4% T, 3% C stacking and 16.5% T, 6% C running 0.75mm thick gel) using a vertical slab gel apparatus operating at 10 mA for 7 h was used. For trypsin treatment, pure AP-CECT7121 diluted in100µL $50mM^{-1}$ sodium phosphate buffer, pH7 was mixed with equal volume of the

same buffer containing 20 µg of trypsin. The mixture was incubated for 1h at 37ºC. After electrophoresis, the gel containing native and trypsin-treated AP-CECT7121 and pre-stained molecular weight markers (Bio-Rad, Hercules, CA, USA) was fixed for 2h at room temperature in 15% (v/v) isopropanol and 10% (v/v) glacial acetic acid, and then exhaustively washed in deionized water for 12h. Finally the gel was placed on a sterile Petri dish with 15mL of BHI agar and overlaid with 7mL BHI soft agar previously inoculated with an overnight culture of *L. monocytogenes* CEB101 containing approx. 10^7 CFU mL^{-1}. The plate was incubated for 18h at 30ºC and examined for inhibition areas on the indicator strain.

2.5. Transmission Electron Microscopy

Morphological changes in *S. aureus* HRS47 and *E. faecalis* CECT7121 were assessed by transmission electron microscopy (TEM) after incubation with pure AP-CECT7121 diluted in 100 µL 50 mM^{-1} sodium phosphate buffer, pH7

Logarithmic phase cultures were suspended in 50mM sodium phosphate buffer, pH7, at an OD620 value of 0.1, and mixed with the AP-CECT7121. After incubation for 3h at 35ºC, samples were fixed in 2% (v/v) glutaraldehyde for 3h and embedded in Spurr's resin. Embedded cells were examined using a 100 CXII electron microscope (JEOL, Tokyo, Japan) operating at an accelerating voltage of 80 kV [22]. For each bacterium, cultures incubated in the absence of AP-CECT7121 acted as controls. .

RESULTS AND DISCUSSION

Reverse phase high performance liquid chromatography (RP-HPLC) was repeated twice and resulted in a single absorbance peak which corresponded with the antimicrobial activity against *L. monocytogenes* CEB101. At the end of this procedure the peptide concentration was 24 µg mL^{-1} . The identity of the peptide was confirmed by testing for inhibitory activity against *L. monocytogenes* CEB101 following SDS-PAGE electrophoresis. The presence of inhibitory activity corresponded to a MW of approximately 5000 Da according to standard MW markers run in adjacent lanes on the SDS-PAGE gel. In contrast, no inhibitory activity for *L. monocytogenes* CEB101 was associated with trypsin-treated AP-CECT7121.

The identity of a range of bacterial isolates from hospitalized patients suffering recalcitrant infections was confirmed by a panel of tests described in the Materials and Methods section and their *in vitro* antimicrobial resistance was then assessed. Data on the inhibitory activity of AP-CECT7121 against the various clinical species and isolated strains, a panel of ATCC reference strains and the peptide source strain (*E. faecalis* CECT7121), obtained using the agar-well diffusion method are summarized in Table 1. The AP-CECT7121 had no inhibitory effect (i.e. inhibition zone = 0mm) on *E. faecalis* CECT7121. In contrast, the peptide had a marked inhibitory effect on all the ATCC reference strains and clinical isolates tested, although there were quantitative differences in sensitivity between both bacterial species and individual strains. The

Streptococcus and *S. aureus* strains were the least sensitive with inhibition zones ranging from 9.1 to 10.7 mm and 9.5 to 11.7 mm, respectively. Inhibition zones for the *Enterococcus* strains (with the exception of *E. faecalis* CECT7121) and the Gram positive anaerobes (*C. perfringens, C. difficile*) were generally larger (11.1 to 16.9 mm and 10.3 to 15.8 mm respectively) indicating a greater sensitivity to peptide inhibition. Despite the quantitative inter- and intra-species differences observed, the inhibitory effect of the AP-CECT7121 was statistically significant *($p < 0.001$)* for all the isolates tested according to the Tukey-Kramer multiple comparison test (see Tables 1 and 2).

Transmission electron microscopy, as illustrated by the representative image obtained from experiments on *S. aureus* HRS47 (Figure 1a), demonstrated that the bactericidal mode of action of the AP-CECT7121 involved the destruction of the bacterial cell wall resulting in the escape of intracellular contents. In contrast, as shown in Figure 1 b), the peptide had no visible effect on its source bacterium, *E. faecalis* CECT7121.

The efficacies of the AP-CECT7121 (as determined by KC studies) against several human bacterial strains, selected on the basis of their relatively low sensitivity to the peptide, are illustrated in Figures 2 a), b), c) and d) for Gram positive aerobes, and in Figure 3 a) and b) for Gram positive anaerobes. All the strains were assessed over an incubation period of 180 min in contact with the AP-CECT7121. For all the bacterial strains tested, no viable bacterial counts were detected after 120 min, with the exception of *C. difficile* for which there was a threefold reduction in bacterial counts (CFU Log 10 mL^{-1}**)** at 90 min post incubation.

E. faecalis CECT7121, is a non-pathogenic, naturally-occurring strain isolated from additive-free silage corn, and this may be an important factor in comparing its efficacy with other commercial biological controllers of infection, which are primarily derived from mammalian sources [7,8,9]. As previously reported by Hechard and Sahl [11], the range of inhibitory action of antimicrobial peptides derived from Gram positive bacteria varies from narrow to broad according their origin, and their main mode of action depends on initiating the formation of pores in the cell walls of susceptible bacteria. This effect is illustrated in Figure 1 a) which shows transmission electron micrographs of *S. aureus* before and after incubation in the presence of the AP-CECT7121. Interestingly, an equivalent micrograph (see Figure 1 b) confirmed that the peptide caused no visible changes in the morphology of the source bacterial strain, *E. faecalis* CECT7121, and this correlated with the absence of inhibitory activity of the peptide against this strain (see Table 1).

Evolution of bacteria towards resistance has been increased by the selective pressure exerted by over-prescription of antimicrobials in clinical settings and their use as growth promoters for farm animals [23]. This concept is consistent with the estimated data provided by Wise et al., [24] who reported that 20 to 50% of human and 40 to 80% of agricultural antimicrobials use is highly questionable.

The issue of antibiotic resistance has recently been reviewed by Beovic [25], who concluded that the intensive use of antimicrobials and possibility of cross-

infections make modern hospitals a hostile environment, in which MRSA is now probably the most prevalent resistant bacteria worldwide. In contrast to hospital-acquired MRSA (H-MRSA), community-acquired MRSA (Ca-MRSA) strains are isolated from healthy people in the community. Clinical syndromes caused by these Ca-MRSA isolates have ranged from skin and soft tissue infections (SSTIs) to necrotising pneumonia, severe sepsis and necrotising fasciitis [26, 27]. All MRSA isolates carry the Staphylococcal cassette chromosome mec (SCCmec) [27] and Ca-MRSA isolates usually specifically carry SCCmec type IV.

In the present study it has been demonstrated that the novel bacteriocin, AP-CECT7121, has an interesting *in vitro* sensitivity and activity (see Table 1, and Figures 2 and 3) against a range of both aerobic and anaerobic Gram positive bacteria. Of particular note was the observation that the AP-CECT7121 was effective in inhibiting a variety of virulent strains, including H-MRSA and Ca-MRSA, isolated from clinical patients. The time-killing curve obtained for the AP-CECT7121 when tested against the most resistant of these MRSA isolates demonstrated that the peptide still had a high efficacy (viable bacterial counts were not detected) within the relatively short incubation period incubation period of 180 min (Figure 2 a).

KC studies on the most resistant bacterial strains, as identified by the agar well-diffusion experiments, confirmed that the AP-CECT7121 was effective in killing all the aerobic strains tested, although the kinetics differed between species. For *E. faecium* (Figure 2 e) and a vancomycin-resistant *E. faecalis* strain (Figure 2 d), viable bacterial counts were not detected within 30 minutes. The

effect was less immediate with the penicillin-resistant *Str. pneumoniae* (Figure 2 b) and *Str. pyogenes* (Figure 2 c) strains although for both the viable bacterial counts decreases under the baseline of detection within 90 min. For community acquired MRSA (Ca-MRSA; Figure 2 a)) killing was even more gradual but, the absence of detection of viable bacteria was achieved within the 180 min incubation period; a comparable killing-curve was obtained for the anaerobic *C. perfringens* isolate tested (Figure 3a). Only the *C. difficile* strain, demonstrated a slower decrease of viable bacterial counts during the incubation with the peptide, and by the end of the 90 min incubation period achieved the bactericidal activity by reducing threefold the log10 CFU mL^{-1} ?of viable counts. Moreover, after overnight incubation (18h) no viable *C. difficile* cells were detected in the same assay (data not shown).

It is important to note that evaluation the primary activity of a novel peptide should not be based solely the results of agar diffusion test studies, but should also include complementary KC studies. Using this combined approach it was interesting to note in the present study that the AP-CECT7121 demonstrated rapid bactericidal activity in KC experiments on strains (*Streptococcus pyogenes* HRS3410, *Str. pneumoniae* HRS2563 and *S.aureus* HRS47) with low sensitivity to the peptide according to the agar well diffusion method. Other authors [28] have reported a similar discordance using Lacticin 3147.

Clearly the results presented in this paper have potentially major implications for the future treatment of drug-resistant, clinically relevant bacteria. AP-CECT7121 could provide a realistic therapeutic alternative to standard, broad spectrum

antimicrobial therapy, particularly with the emergence of new resistant pathogenic strains such as vancomycin-resistant in *S. aureus* [29] and enterococci [30, 31], and the appearance of vancomycin [32], metronidazole-resistant [32, 33] strains of *C. difficile*, as well as *S. pneumoniae* Telithromycin resistant strains [34], and *E. coli* ciprofloxacin resistant [35] which are now causing great clinical concern worldwide.

In general terms, bacteriocins are associated more with traditional narrow spectrum rather than broad spectrum antimicrobials. However, the studies presented here suggest that the AP-CECT7121, like the Nisin A [11, 36] and Mutacin B-Ny266 [37, 38] peptides, has a broad spectrum of activity. Whilst this *in vitro* evidence is encouraging, substantial additional research is required to explore the practical potential of peptide therapy. For example, the production of bacteriocins is related to the change from the log phase to the stationary phase of the bacterial growth curve, and this could have important consequences for the development of the production-scale cultures of *E. faecalis* CECT7121 which would be required to obtain the sufficiently large quantities of the AP-CECT7121 needed initially to conduct *in vivo* trials in small animal models, and subsequently to facilitate commercial production of the peptide.

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FIGURE CAPTIONS

Figure 1: Transmission electron micrographs showing the effects of the AP-CECT7121 on bacterial cell morphology of a) a pathogenic isolate (HRS47) of *S. aureus* in the presence (right) or absence (left) of the peptide and b*)* the source strain, *E. faecalis* CECT7121.

Figure 2: *In vitro* efficacy of the AP-CECT7121 against Gram positive aerobic bacterial strains from hospitalized patients: a) Ca-MRSA HRS47; b) and c): penicillin-resistant *Str. peumoniae* HRS2563 and *Str. pyogenes* HRS3410 respectively; d) and e), vancomycin-resistant *E. faecalis* HRS1084 and *E. faecium* HRS808 respectively. The figure summarizes data from 3 separate KC experiments for each species, and shown are the mean (of $\log_{10}$ viable bacterial counts (expressed as colony forming units, CFU mL^{-1}) plotted against incubation time for individual isolates cultured in the presence or absence (Control) of the AP-CECT7121.

Figure 3: In vitro efficacy of the AP-CECT7121 against Gram positive anaerobic bacterial strains from hospitalized patients: a) *C perfringens* HRS148 and b) *C. difficile* HRS21. The figure summarizes data from 3 separate KC experiments for each species, and shown are the mean (of log10 viable bacterial counts (expressed as colony forming units, CFU mL^{-1}) plotted against incubation time for individual isolates cultured in the presence or absence (Control) of the AP-CECT7121.

Table 1: Bacterial Inhibition spectrum of the AP-CECT7121 against selected ATCC reference strains and clinical isolates. Clinical (HRS) strains in emboldened text were those subjected to further Killing Curve studies.

Bacterial Strain	**Inhibition zone (mm)**	**Bacterial Strain**	**Inhibition zone (mm)**
S. pneumoniae	**Mean ± SD**	***E. faecalis***	**Mean ± SD**
ATCC 10015	9.50 ± 0.30	ATCC 29212	16.9 ± 0.40
HRS2672, 2984	9.80 ± 0.20	33186, 33550	15.8 ± 0.30
HRS2563* **	**9.40 ± 0.50**	HRS905, 1005,1036	14.3± 0.40
S. pyogenes		**HRS1084**	**12.5 ± 0.70**
ATCC 49117	10.7 ± 0.30	**CECT7121**	0.00 ± 0.00
HRS3107, 3114	10.3 ± 0.40	***C. difficile***	
HRS3410	**9.10 ± 0.20**	ATCC 43255	11,2 ± 0,40
S. aureus		**HRS21**	**10,3 ± 0,20**
ATCC 25923, 29213	11.7 ± 0.80	***C.perfringens***	
HRS** 162, 23, 95	11.3 ± 0.30	ATCC 13124	12,9 ± 0,60
HRS*45	11.9 ± 0.20	**HRS148**	**11,2 ± 0,60**
HRS*47	**9.50 ± 0.60**	HRS37, 64, 71, 73	15,8 ± 0,90
E. faecium		***L. monocytogenes****	19.8 ± 0.10
HRS808	**11,1 ± 0,20**		
HRS950, 856, 807	14,7 ± 0,50		

Data were obtained using the Agar Well Diffusion Method. Inhibition zone results are the Mean + SD of 3 experiments for each strain. *Ca-MSRA, **H-MSRA; *** penicillin- resistant. HRS¨= R. Santamarina Hospital.

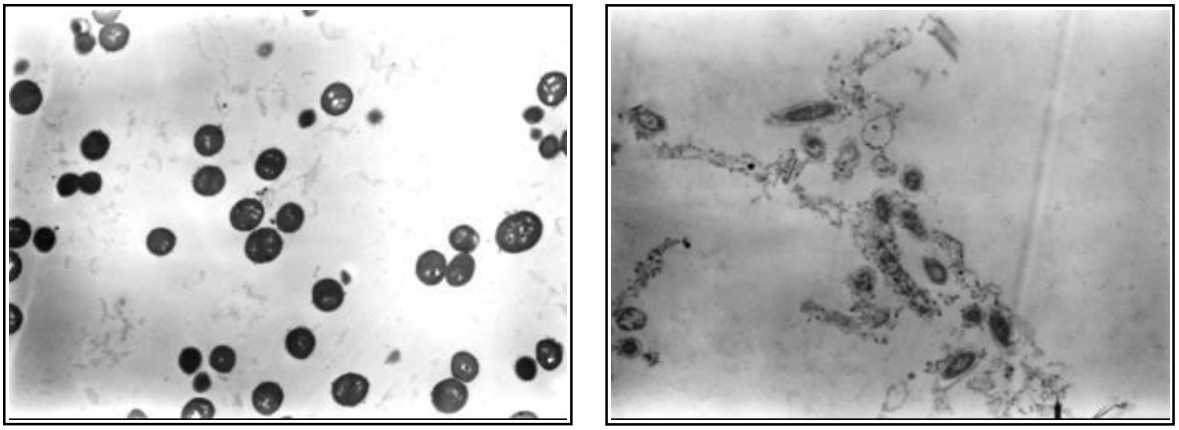
Table 2: Statistical comparison by Tukey-Kramer multiple comparison test of mean ± SD inhibition zones given by the peptide AP-CECT7121 against the whole clinical isolates examined.

WHOLE ISOLATE STRAINS						
S. pneumoniae	***S. pyogenes***	***S. aureus***	***E. faecium***	***E. faecalis***	***C. difficile***	***C. perfringens***
9.62 ± 0.20 ^{a} *(n=4)*	10.1 ± 0.69 ^{a,b} *(n=4)*	11.2 ± 0.80 ^{a,b,c} *(n=7)*	13.8 ± 1.84 ^{b,c,d,f} *(n=4)*	14.8 ± 1.40 ^{b,c,d,e,f} *(n=7)*	10.7 ± 0. 50 ^{b,c,d,f,g} *(n=4)*	14.3 ± 2.13 ^{b,c,d,e,f} *(n=6)*

Different superscripts indicate statistical differences among the pathogen strains at P< 0.05. Inhibition zones of all the isolated strains were significantly different (P<0.01) to the marker strain, L. monocytogenes.

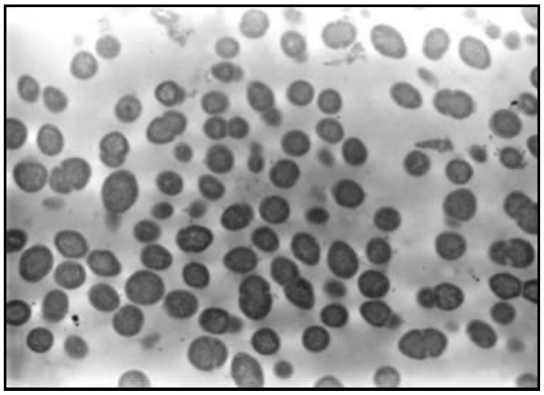
Figure 1

a) *S. aureus* HRS47



Without AP-CECT7121 **With AP-CECT7121**

b) *E. faecalis* CECT7121

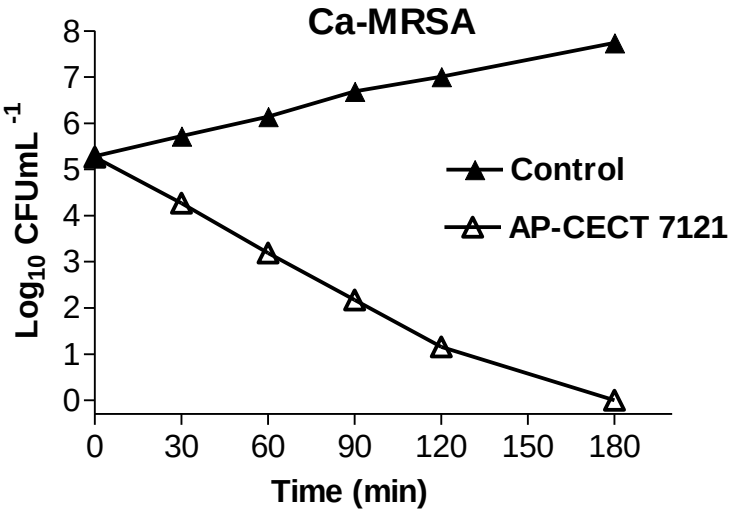


With AP-CECT7121

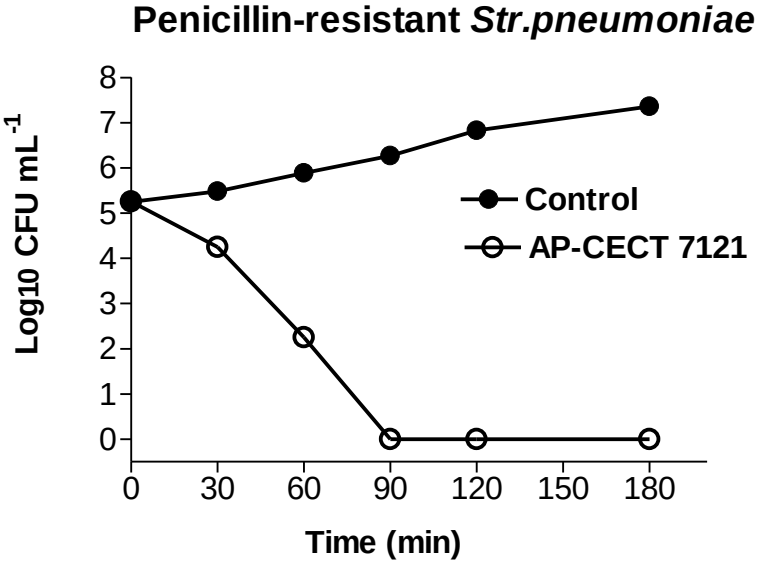
Figure 2

GRAM POSITIVE AEROBES

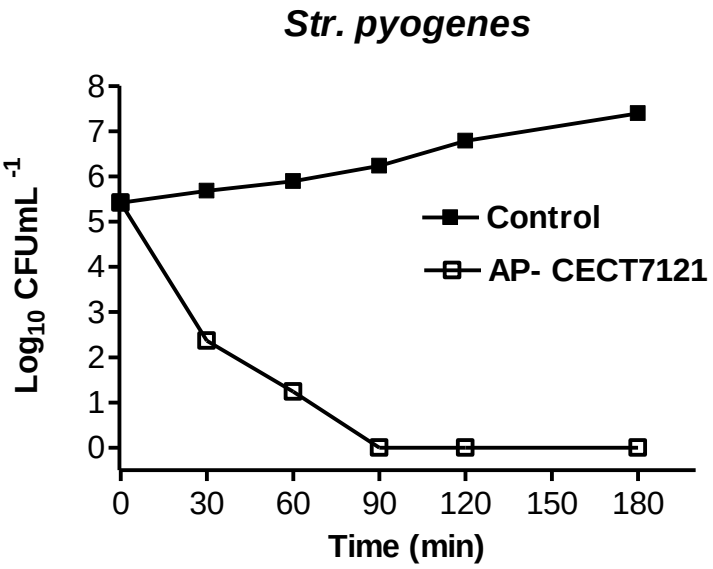
a)



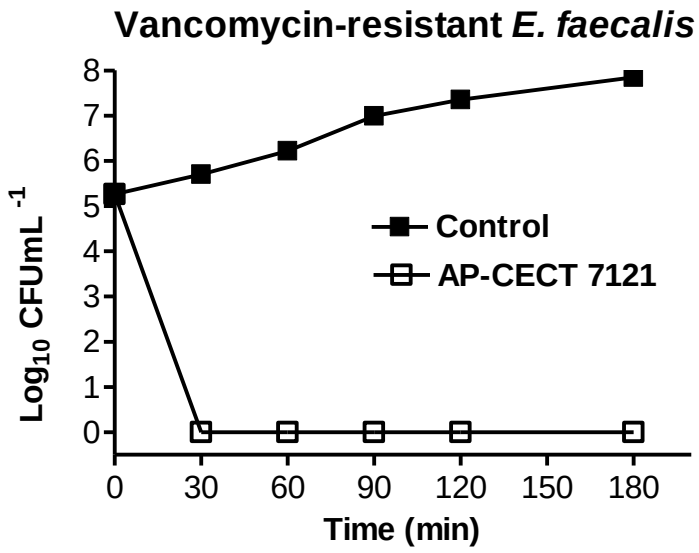
b)



c)


Str. pyogenes
Log10 CFUmL-1
Control
AP- CECT7121
Time (min)
0
30
60
90
120
150
180

d)


Vancomycin-resistant E. faecalis
Log10 CFUmL-1
Control
AP-CECT 7121
Time (min)
0
30
60
90
120
150
180

e)

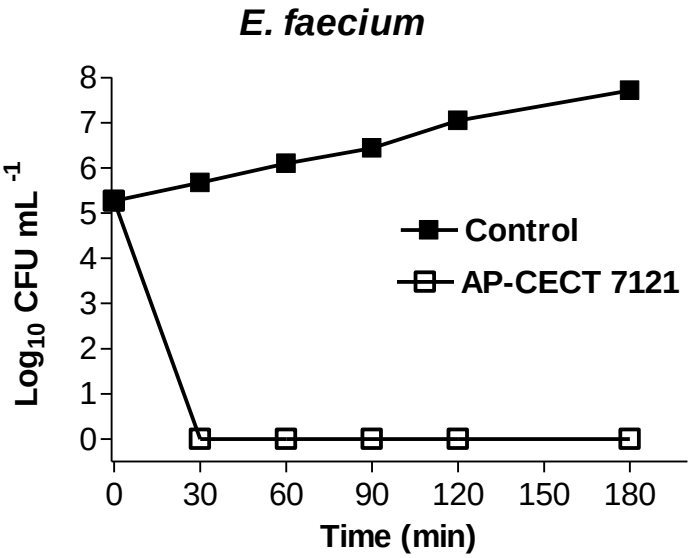
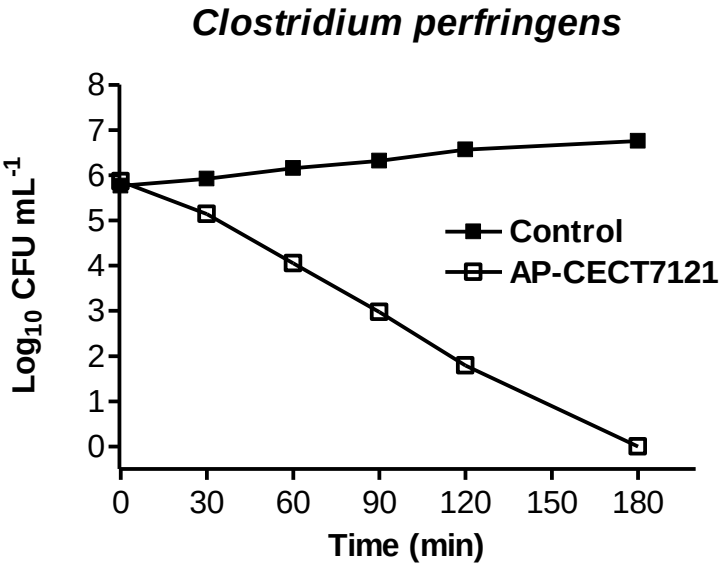

E. faecium
Log10 CFU mL-1
8
7
6
5
4
3
2
1
0
0
30
60
90
120
150
180
Time (min)
Control
AP-CECT 7121

Figure 3

GRAM POSITIVE ANAEROBES

a)



b)