Novel immune-pharmacological approaches for the treatment of bacterial invasive infections.

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The emergency of multiresistant bacterial strains is, likely, one of the main concerns related with the clinical practice’s misuse of antimicrobial (ATM) drugs in human and veterinary medicine. Some of these bacteria are mentioned as follow: community multiresistant Staphylococcus aureus (Ca-MRSA) and, hospitalary multiresistant Staphylococcus aureus (H-MRSA), Enterococcus spp. vancomycin resistant genotype vanA, Streptococcus pneumoniae beta lactams resistant and food transmission quinolones resistant Salmonella spp. The re-emergency of pathogens with difficulties to be treated by conventional ATM therapy, i.e Listeria monocytogenes, must also be having into account. The complexity of this situation in clinical practice is reflected in a therapeutic failure with poor patient response and likelihood of death risk. For this reason, new alternatives for helping the conventional ATM therapy will be described in this chapter. The actions of a novel antimicrobial peptide (AP-CECT7121) and a natural immunomodulator, both obtained from a saprophytic environmental strain, Enterococcus faecalis CECT7121 (characterized as probiotic), will here be described as new model of therapeutic strategy for helping the control of some invasive infections.

Keywords: Peptides, Immunomodulation, Antimicrobials, Efficacy, Resistance

1. Introduction

Infectious diseases constitute a relevant concern in Human and Veterinary Medicine worldwide. The antimicrobial (ATM) used to fight off it have largely contributed to the development of bacterial resistance against these chemical agents. Development resistance was rapidly evidenced after 6 years after Penicilin G commercialization (1941), where the frequency of Staphylococcus aureus resistant strains increased 50% at the UK hospitals. The increase of methicillin-resistant Staph. aureus strains (H-MRSA), turned it into the more prevalent multiresistant pathogen of hospitalary infections. Other strains of Staph. aureus appeared on the community (Ca-MRSA) being resistant only to oxacicillin [1]. Also, the use of fluoroquinolones in food-animals contributed to the bacterial resistance development in different microorganisms, being Salmonella recognized as the main genus with trend to cross-resistance against these ATM agents [2]. Even more it is unknown the amount of ATM agents used in livestock and agriculture, although in some countries could represent the 50% of the ATM drugs consumed [3]. Other re-emergent pathogen, which affects animals and humans, is Listeria monocytogenes. This bacteria, has some concerns with its ATM susceptibility by which the association of two ATM agents such as, ampicillín and gentamicin, need to be combined to attain bactericide activity in invasive infections like meningitis and sepsis [4]. The ATM resistance involves to other Gram positives bacterial strains like the multiresistant Streptococcus pneumoniae [5] and Enterococcus faecium with high level resistance to vancomycin (VRE), being the later epidemiologically relevant since it is horizontally transferible (gen vanA) to other species and also Staph. aureus (http://www.rivm.nl/earss/result/Monitoring_reports).

The modern ATM therapy involves the strengthen interaction among Microbiology, Pharmacology and Immunology, as well as the severity of the infectious diseases. Thus, when the physician set up a rational therapy (good medical practice) must have into account some limitations, which go through to an unsuccessful therapy: a) infectious disease with different etiological agents, b) pharmacokinetics and pharmacodynamics interactions in combined therapy c) pharmacological variations among patients d) the variety of ATM available on the market) development of bacterial resistance to ATM commonly used. The increase and rapid widespread of multiresistant-pathogen forced the consideration of alternative methods to fight against infections [6,7]. Seeking new alternatives to the prevention and treatment of invasive diseases some reports demonstrated the utility of the probiotics, bacteriocins, and bacterial extracts as antimicrobial immunodulators [8-12].

A new enterocin (bacteriocin), the peptide AP-CECT7121, has recently been reported by Sparo et al. [11,12] This peptide was produced by an environmental probiotic strain namely Enterococcus faecalis CECT7121; deposited at the Spanish Collection of Typical Cultures, Burjasot, Valencia, Spain, with European patent EP1816190 and United States Patent Application 20080063666 Kind Code: A1. Assessment of the physical-chemical properties of AP-CECT7121 characterized an hydrophobic and stable peptide (over a wide pH range 4–8) with a low molecular weight. This peptide,
demonstrated immunomodulation; antibacterial activity against aerobic and anaerobic Gram positive species, and some Gran negative strains; antiparasitic action and biopreservation in animal derived food [8-14]. The actions of a novel antimicrobial peptide (AP-CECT7121) and a natural immunomodulator, both obtained from a saprophytic environmental strain, Enterococcus faecalis CECT7121 (characterized as probiotic), will be in this chapter described as new model of therapeutic strategy for helping the control of some invasive infections.


The absence of pathogenicity is essential when selecting bacteria are postulated as immunomodulator. Sepsis induced by the intraperitoneal inoculation in mice is a good approach to test this concern. The systemic immune response can be evaluated by measuring the unspecific response of peritoneal macrophages and by lymphocyte proliferation assays. Also, it is important to determine the implantation capacity and survival of E. faecalis CECT7121 in the intestinal epithelium, as well as its influence on the preexisting enterobacteria microbiota. Also, it is important to point out the feasibility of intestinal translocation and consequently systemic infections.

2.1 Pathogenicity assay

The probiotic strain, E. faecalis CECT712, was evaluated using BALB/c mice (n: 8 / group) injected intraperitoneally (IP) with 0.5 ml of the following suspensions of E. faecalis CECT7121 (group A: 1.0x10⁷ CFU ml⁻¹, group B:1.0x10⁹ CFU ml⁻¹, and group C: 1.0x10¹¹ CFU ml⁻¹). As controls, the animals were inoculated either with: a) 0.5 ml sterile saline, b) non-pathogenic E. faecalis JH2SS and E. faecalis JH2-2 (Gilmore Laboratory, USA), and c) pathogenic E. faecalis DS16 (Gilmore Lab.) and E. faecalis ATCC 51299. The inoculations with saprophytic and pathogenic control strains were performed in the same concentrations used for E. faecalis CECT7121. The animals were observed during one week and evaluated for changes in behavior, coat, diarrhea, and death.

E. faecalis CECT7121 was grown in brain heart infusion (BHI) broth at 35 °C for 18 h. After incubation, the culture was harvested by centrifugation and extensively washed with sterile saline solution. To prepare E. faecalis CECT7121 suspensions, standard growth curves were produced by plotting the OD₆₂₀ versus agar plate counts of serially diluted cultures. Bacterial suspensions for implantation and pathogenicity assays were prepared in sterile saline, and in RPMI plus 10% fetal calf serum when used in cell cultures. Previous to dilution in RPMI, microorganisms were killed by heating at 70 °C for 20 min. Samples were plated onto BHI agar to corroborate bacterial death.

The IP administration of 1.0 x10⁷ CFU ml⁻¹ and 1.0x10⁹ CFU ml⁻¹. E. faecalis CECT7121 was innocuous for all of the animals tested. The highest concentration (10¹¹ CFU ml⁻¹) of E. faecalis CECT7121 killed 100% of the animals after 72 h post- inoculation. The pathogenic strains (DS16 and ATCC 51299) were lethal after 24 h at all the concentrations tested.

Since enterococci have a closer relationship with human infections in susceptible patients (immunosuppressed and prolonged hospitalization patients), it have great importance to have demonstrated in a murine model of sepsis the innocuousness of E. faecalis CECT7121; before being employed as an immunomodulator.

2.2. Implantation assay

To evaluate the optimal concentration of E. faecalis CECT7121that is implanted in the intestine, groups of 8 animals were administered intragastrically (IG) with 0.2 ml of an E. faecalis CECT7121 suspension (group A: 3.0x10¹⁰ CFU ml⁻¹, group B: 3.0x10⁹ CFU ml⁻¹, group C: 3.0x10⁸ CFU ml⁻¹, and group D: 3.0x10⁷ CFU ml⁻¹). The administration was performed daily during 3 consecutive days using a stainless-steel gavage needle. A control group (n: 8) was inoculated with the same volume of sterile saline solution. The persistence of E. faecalis CECT7121 in each portion of the intestinal mucosa (small and large intestine and Peyer’s patches) was analyzed employing the optimum concentration of E. faecalis CECT7121. The putative translocation of E. faecalis CECT7121 was evaluated by blood, spleen, liver and kidneys cultures after IG administration of an optimal bacterial concentration in BALB/c mice (n: 3). Groups of 4 animals treated with the different concentrations of E. faecalis CECT7121 were sacrificed on days 3 and 6 after the last inoculation. Working under sterile conditions, the whole intestines were weighed and blended in phosphate buffer pH 7.0 for 5 min. Tenfold dilutions of each sample were plated in selective media (bile esculin azide agar) for viable counts. Once the optimum concentration of E. faecalis CECT7121 was determined, the experiment was repeated but in this case, the small and large intestine and the Peyer’s patches were obtained on days 6, 11, 15 and 18 after the last administration. For translocation assays, blood, spleen, liver and kidney cultures were performed 3 days after the last administration of E. faecalis CECT7121 employed at the optimum concentration.

Gram-positive colonies were in all cases E. faecalis and they were identified as CECT7121 by RAPD-PCR assays [10]. E. faecalis CECT7121 was isolated from the whole intestines after 3 days of the last inoculation in all treated animals (Fig. 1 a). After 6 days of inoculation, E. faecalis CECT7121 was isolated only in the group treated with
concentrations >10^7 CFU ml^-1 (Fig. 1b). Viable counts obtained on days 3 and 6 post-inoculation did not differ significantly. Viable counts of enterobacteria in experimental groups were similar to those obtained in control groups. A lower value of enterobacteria in intestines on day 3 post-inoculation was found only in the group treated with the highest dose of *E. faecalis* CECT7121 (3x10^10 CFU ml^-1, p<0.05). Therefore, the concentration of *E. faecalis* CECT7121 of 3x10^8 CFU ml^-1 was used in all the studies of implantation on small and large intestine and in Peyer’s patches. Even though viable counts decreased over time, the presence of *E. faecalis* CECT7121 was detected in all samples 18 days after the last administration (Fig. 2). After 3 days of the last inoculation of *E. faecalis* CECT7121, no bacteria were detected in, neither blood, spleen, kidneys, nor liver.

**Fig. 1.** Implantation and persistence of *E. faecalis* CECT7121 in BALB/c mice a) at 3 days after last administration and b) at 6 days after 3 follow days administration *p=0,05, ANOVA.

**Fig. 2.** Implantation and persistence of *E. faecalis* CECT7121 in BALB/c mice in different portion of intestine over 18 days post-administration. *p<0,05, **p<0,01, ANOVA.

A possible risk of the administration of probiotic bacteria is the translocation phenomena. Using the optimal suspension of *E. faecalis* CECT7121 (3x10^8 CFUml^-1) was not detected the presence of the bacteria on none studied samples (blood, spleen, kidney and liver), indicating no translocation.

### 2.3 Protection assay

*Salmonella* serotype Enteritidis. Isolation of this bacteria (obtained from hemoculture of a patient at the Hospital), was grown in BHI broth at 35 °C for 18 h. After incubation, the culture was harvested by centrifugation, washed and adjusted to 5.0 x 10^4 CFU ml^-1 in BHI broth.

In survival studies, two groups of 6-weeks-old BALB/c mice (n=10) were inoculated IG during 5 consecutive days with 200 µl of *E. faecalis* CECT7121 (3.0x10^8 CFU ml^-1). The control group was inoculated with sterile saline. On days 7 and 8, animals belonging to the study group were challenged IG with 200 µl of a mixture of *Salmonella* serotype Enteritidis (5.0x10^4 CFU ml^-1) and *E. faecalis* CECT7121. The control group was challenged with *Salmonella* serotype Enteritidis only. To perform microbiological studies, two groups of BALB/c mice (n=9) were challenged in the same manner.

On day 14 after challenge, all animals treated with *E. faecalis* CECT7121 were alive, whereas the mortality percentage was 100% in the control group. The first deaths were registered on day 9 and 17 post-challenge in control and treated groups respectively (Fig. 3A). At the end of the experiment (day 30 post-challenge) a 50% of the animals...
treated with *E. faecalis* CECT7121 had survived. *Salmonella* viable counts in the intestine of animals treated with *E. faecalis* CECT7121 were lower than those of controls on all the time points studied (days 2, 5 and 8 post-challenge). *Salmonella* was detected earlier in the spleen of control animals (day 5) post-challenge vs. day 8 post-challenge for animals treated with *E. faecalis* CECT7121, *p*<0.001. Only control animals had *Salmonella* in their livers.

### 2.4 Immunological evaluation

In order to study the effect of *E. faecalis* CECT7121 on the cytokine production, BALB/c mice (*n=10*) were inoculated IG with 0.2 ml *E. faecalis* CECT7121 (3.0x10⁸ CFU ml⁻¹) for 3 consecutive days. One day after the last inoculation, peritoneal macrophages were obtained by the IP injection of 5 ml of sterile saline collecting the peritoneal exudate after 10 min. Cells were adjusted to 5.0x10⁵ cells ml⁻¹ in RPMI plus 5% FCS. Cell suspensions were plated onto 24-well plates and incubated at 37 °C in an atmosphere containing 5% CO₂ for 2 h. Non-adherent cells were washed off with RPMI. Cell cultures from untreated controls were stimulated with 3 different suspensions of heat-killed *E. faecalis* CECT7121 (5.0x10⁵, 5.0x10⁶ and 5.0x10⁷ CFU ml⁻¹). The concentrations of IL-6, TNF, IL-10 and IL-12 p40 in culture supernatants of macrophages were measured by ELISA.

When peritoneal macrophages were stimulated with = 5.0x10⁶ CFU ml⁻¹ of heat-killed *E. faecalis* CECT7121, the synthesis of IL-6, TNFα and IL-12 was induced in a concentration-dependent fashion (A-C; *p*<0.001) whereas the synthesis of IL-10 was induced only by the highest concentration of *E. faecalis* CECT7121 (D, *p*<0.001). The IG treatment of animals with *E. faecalis* CECT7121 modified the cytokine profile produced by the peritoneal macrophages, i.e. similar levels of IL-12 and TNFα and lower levels of IL-6 were detected, whereas IL-10 was not detected. Considering that during the multiplication of *E. faecalis* CECT7121 in the gastrointestinal tract there was a controlled increase in the population without any alterations in the endogenous flora, it could be hypothesized that an equilibrium between both exogenous and endogenous bacteria is reached. It would be hypothesized that a low level of spontaneous translocation of *E. faecalis* CECT7121 to the mesenteric lymphs nodes might actually be occurring, being this phenomenon beneficial to the host by stimulating the immune system to respond more rapidly and more effectively to pathogenic exogenous microorganisms. Indeed, peritoneal macrophages stimulated in vitro with *E. faecalis* CECT7121 responded with an inflammatory cytokine profile (IL-12, TNFα, and IL-6) (Fig 3). Although inflammation serves as protective function in controlling infections, it can also cause tissue damage and disease, therefore it is important to achieve a balance in the profiles of pro and anti-inflammatory cytokines. This concept would be in line with the synthesis of IL-10 (anti-inflammatory) found upon stimulation of peritoneal macrophages with a high concentration of *E. faecalis* CECT7121. These results indicate that *E. faecalis* CECT7121 would be able to either suppress or stimulate the development of inflammatory responses.

![Fig. 3. Peritoneal macrophage cytokines in vitro stimulated by 24 h with heat-killed strain of heated *E. faecalis* CECT7121 *p*<0.05, **p<0.01, ***p<0.001 (ANOVA).](image-url)
In other hand, peritoneal macrophages from animals treated with *E. faecalis* CECT7121 and stimulated with *Salmonella* were able to respond releasing high levels of inflammatory cytokines, indicating that these animals would have a higher ability to respond to certain infections than untreated animals.

### 3. Pharmacological evaluation of the antibacterial activity of the peptide AP-CECT7121 isolated from *E. faecalis* CECT7121.

#### 3.1. Purification, isolation and characterization by RP-HPLC.

Many different strategies to find new antimicrobial agents are proposed at present and the area of antibacterial peptides is under intensive investigation. Among the most promising antibacterial peptides are bacteriocins. Enterococci-derived bacteriocins can be easily isolated from many fermented foods and are featured by their strong biological activity against *Listeria* spp. As a rule, they are heat resistant, remaining active over a wide range of pH and have a wide inhibitory spectrum on Gram positive bacteria.

AP-CECT7121 was isolated and purified from 18-hour (at 35 °C) cultures of *E. faecalis* CECT7121 in BHI broth. This culture was inoculated into 4 liters of BHI broth and incubated for 9 h at 35 °C. Cells were then removed by centrifugation (15,000 g, 4 °C, 20 min), after which the supernatant was adjusted to pH 7 and precipitated. After centrifugation (20,000 g, 4 °C, 20 min), the resulting sediment was suspended in 40 ml of 50 mM sodium phosphate buffer, pH 7. This suspension was stored in two aliquots at –70 °C until analyzed by reverse-phase (RP) HPLC. AP-CECT7121 was isolated by physicochemical extraction using Sep-Pak C 18 cartridges (Waters, Milford, Mass., USA). The resulting residue was then resuspended in PBS (250 µl) and analyzed for inhibitory activity by the agar-well diffusion method, using *L. monocytogenes* CEB101 as the marker strain [11,12]. Fractions with high AP-CECT7121 activity were mixed and rechromatographed on the RP column to obtain chromatographically pure peptide. Molecular weight (MW) was determined by mass spectrometry (Finnigan TSQ Quantum, Thermo Scientific, Waltham, Mass., USA) and the protein concentration of pure APCECT7121 was determined using the Coomassie protein assay reagent. Finally, AP-CECT7121 was lyophilized until further analysis.

#### 3.2. Isolation and Characterization of Bacterial Strains from Human Patients

In a preliminary screening study, bacterial sensitivity to AP-CECT7121 was assessed using a panel of ATCC reference strains (Manassas, Va., USA) as well as a variety of strains isolated from patients with severe bacterial infections hospitalized at the intensive care unit of the Hospital R. Santamarina (HRS), Tandil, Argentina, from 2005 to 2007 (Table 1). The source of the clinical strains tested were as follows: *Streptococcus pyogenes* (HRS 3107, HRS3410 and HRS3114: blood culture), *Enterococcus faecium* (HRS808, HRS950, HRS856 and HRS807: blood culture), ampicillin-resistant (HRS905, HRS1005 and HRS1036: blood culture) and vancomycin-resistant *E. faecalis* (HRS1084: blood culture), penicillin-resistant *Streptococcus pneumoniae* (HRS2672 and HRS2984: blood culture, and HRS2563: pleural fluid), community- acquired methicillin-resistant *Staphylococcus aureus* (Ca- MRSA; HRS45, and HRS47: pleural fluid and blood culture, respectively), hospital-acquired methicillin-resistant *Staphylococcus aureus* (H-MRSA; HRS162 and HRS23: blood culture, and HRS95: cerebrospinal fluid), *Clostridium perfringens* (HRS37 and HRS64: cellulitis aspiration fluid, and HRS71, HRS73 and HRS148: necrotizing fasciitis biopsy), *Clostridium difficile* HRS21 (feces from a patient diagnosed with pseudomembranous colitis). All the above isolates were of clinical significance, since most of the 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 (Table 1) were subsequently subjected to a time-killing curve (KC) study in order to evaluate the in vitro efficacy of the antimicrobial peptide AP-CECT7121.

The limit of detection was 10 CFU ml⁻¹. Drug carryover was assessed by visual inspection of the distribution of colonies on the plates. Subsamples were removed from this mixture every 30 min over a total incubation period of 180 min. For each strain tested, cultures incubated and processed in a similar fashion, but in the absence of AP-CECT7121, acted as controls. For all strains studied, bactericidal activity was defined as a reduction in viable bacterial counts ≥ 3 log₁₀.

Data on the inhibitory activity of APCECT7121 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. AP-CECT7121 had no inhibitory effect (i.e. inhibition zone = 0 mm) 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 *Staph. aureus* strains were the least sensitive with inhibition zones ranging from 9.1 to 10.7 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* and *C. difficile*) were generally larger (11.1–16.9 and 10.3–15.8 mm, respectively), indicating a greater sensitivity to peptide inhibition. 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. 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 Fig. 4 a), b), c).

Table 1: Bacterial Inhibition spectrum of the AP-CECT7121 against selected ATCC reference strains and clinical isolates.

<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>Inhibition Halo (mm)</th>
<th>Bacterial Strain</th>
<th>Inhibition Halo (mm)</th>
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<tr>
<td><em>S. pneumoniae</em></td>
<td></td>
<td><em>E. faecalis</em></td>
<td></td>
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<tr>
<td>ATCC 10015</td>
<td>9.50 ± 0.30</td>
<td>ATCC 29212</td>
<td>16.9 ± 0.40</td>
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<tr>
<td>HRS2672, 2984</td>
<td>9.80 ± 0.20</td>
<td>33186, 33550</td>
<td>15.8 ± 0.30</td>
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<td>HRS2563***</td>
<td>9.40 ± 0.50</td>
<td>HRS905, 1005, 1036</td>
<td>14.3 ± 0.40</td>
</tr>
<tr>
<td><em>S. pyogenes</em></td>
<td></td>
<td>HRS1084</td>
<td>12.5 ± 0.70</td>
</tr>
<tr>
<td>ATCC 49117</td>
<td>10.7 ± 0.30</td>
<td>CECT7121</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>HRS3107, 3114</td>
<td>10.3 ± 0.40</td>
<td><em>C. difficile</em></td>
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<tr>
<td>HRS3410</td>
<td>9.10 ± 0.20</td>
<td></td>
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<td><em>Staph. aureus</em></td>
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<tr>
<td>ATCC 25923, 29213</td>
<td>11.7 ± 0.80</td>
<td><em>C. perfringens</em></td>
<td></td>
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<tr>
<td>HRS** 162, 23, 95</td>
<td>11.3 ± 0.30</td>
<td>ATCC 13124</td>
<td>12.9 ± 0.60</td>
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<tr>
<td>HRS* 45</td>
<td>11.9 ± 0.20</td>
<td>HRS148</td>
<td>11.2 ± 0.60</td>
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<tr>
<td>HRS* 47</td>
<td>9.50 ± 0.60</td>
<td>HRS37, 64, 71, 73</td>
<td>15.8 ± 0.90</td>
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<td><em>E. faecium</em></td>
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<td><em>L. monocytogenes</em></td>
<td>19.8 ± 0.10</td>
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<tr>
<td>HRS808</td>
<td>11.1 ± 0.20</td>
<td></td>
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<tr>
<td>HRS950, 856, 807</td>
<td>14.7 ± 0.50</td>
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</table>

Data were obtained using the Agar Well Diffusion Method. Inhibition halo results are the Mean + SD of 3 experiments for each strain. *Ca-MSRA, **H-MSRA; *** penicillin- resistant. HRS¨= R. Santamarina Hospital.
**3.3 Efficacy of AP-.CECT7121 against bacterial strains isolated from mastitis dairy cattle.**

In terms of economic loss, inflammation of the mammary gland, usually associated with bacterial infection, is certainly the most costly disease of dairy cattle. Mastitis impacts negatively on animal production, animal welfare and on the quality of milk produced. Currently, treatment and control of mastitis relies primarily on antimicrobial therapy. Mastitic cattle are usually treated with conventional antimicrobial formulations administered by the intramammary route in lactation periods or parenterally during dry periods. Selection of the most appropriate antimicrobials for rational therapy depends on the susceptibility of the specific causal bacteria and also the pharmacological properties of the bactericidal antimicrobials used. The irrational use of these drugs may generate unwanted drug residues in milk and/or promote the development of bacterial resistance. Gram-positive, *Staphylococcus* and *Streptococcus*, are the bacteria most commonly recovered from mastitic dairy cattle although co-infections with Gram-negative species have also been described. The evaluation of the *in vitro* bactericidal activity of the novel antimicrobial peptide AP-CECT7121 against Gram-positive bacteria from mastitic dairy cattle was carried out.

A total of 15 *Staph. aureus*, 10 *Streptococcus dysgalactiae*, 7 *Streptococcus uberis*, 1 *Streptococcus agalactiae* strains were isolated from 33 different mastitic dairy cattle, sourced from two dairies in Tandil-Argentina. Isolates from each of the bacterial species screened which developed the lowest inhibition zones in response to the peptide, were further evaluated in a series of time-killing curve studies. No survivors were detected in whole strains (from the three *Streptococcal* species isolated) within 120 min of incubation in presence of the peptide. The *Staph. aureus* isolates were less sensitive but, nevertheless, the dropped of viable counts below the detection limit was achieved for all the test strains by the final post-incubation sampling point at 180 min.

In conclusion both studies demonstrated the *in vitro* efficacy and rapid bactericidal activity, using the KC method, of the AP-. CECT7121 against a variety strains of Gram-positives isolated from Human patient recalcitrants to conventional ATM therapy and mastitic dairy cattle.

**4. Transmission Electron Microscopy (TEM):**

TEM was undertaken with a 100 CXII electron microscope (JEOL, Tokyo, Japan), following the technique of Sparo et al., [15] to assess potential changes in morphology of selected bacterial strains which showed sensitivity to the ATM peptide AP-CECT7121. TEM, as illustrated by the representative image obtained from experiments on *Staph. aureus* (Fig. 5), demonstrated that pharmaco-dynamically the bactericidal mode of action of the ATM peptide 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.

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**Fig. 4:** *In vitro* efficacy of the ATM peptide CECT7121 against Gram positive aerobic bacterial strains from hospitalized patients: a) Ca-MRSA HRS47; b) vancomycin-resistant *E. faecium* HRS808 and C) penicillin-resistant *Str. pneumoniae* HRS2563. 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) plotted against incubation time for individual isolates cultured in the presence or absence (Control) of the peptide (6µg/ml).

**Fig. 5.** Transmission electron micrographs: Right: lytic effect of the ATM peptide AP-CECT7121 on a pathogenic strain *Staph. aureus* HRS47. Left: *Staph. aureus* HRS47 without the peptide
Conclusions

The intestinal microbiota has a marked influence on the host’s biochemistry, physiology and immune responses [16,17]. Even though germ-free mice can survive in the absence of the gut microflora, they do have important biochemical and physiological differences and are more sensitive to infections than wild type animals [18]. *E. faecalis* CECT7121, an environmental strain, behaves as an immunomodulator strain. *E. faecalis* CECT7121 has antibacterial activity, does not have virulence determinants (gelE, cylA, esp, asa1, asa373, efaA) and does not present antibiotic multiresistance. Since enterococci have a closer relationship with human infections than other lactic bacteria [19], we considered of great importance to have demonstrated the innocuousness of *E. faecalis* CECT7121 before being employed as an immunomodulator. To this end, a murine model of sepsis was employed. On demonstrated that *E. faecalis* CECT7121, at 3x10^7 – 3x10^10 CFU ml^-1 can implant in the intestinal epithelium without changing the pre-existing enterobacteria microflora. A transient displacement of the flora was observed only with the highest concentration of *E. faecalis* CECT7121 without inducing diarrhea. These results are in line with the absence of virulence determinants of this strain. These results indicate that *E. faecalis* CECT7121 (3x10^6 CFU ml^-1) implants and persists at least for 18 days in the small and large intestine and in Peyer’s patches. Considering that during the multiplication of *E. faecalis* CECT7121 in the gastrointestinal tract there was a controlled increase in the population without any alterations in the endogenous flora, it could be hypothesized that an equilibrium between both exogenous and endogenous bacteria is reached. Although the microbiological studies did not allow detecting *E. faecalis* CECT7121 in extraintestinal sites, there is a great deal of evidence indicating that in mice that are challenged with intestinal doses of commensal bacteria, a small number these microorganisms penetrate the epithelial cell layer and survive within dendritic cells (DCs) confined within the mucosal immune system in the mesenteric lymph nodes (MLNs), a site were the intestinal and peripheral immune systems intersect [20]. Therefore, it can be hypothesized that a low level of spontaneous translocation of *E. faecalis* CECT7121 to the MLNs might actually be occurring, being this phenomenon beneficial to the host by stimulating the immune system to respond more rapidly and more effectively to pathogenic exogenous microorganisms. Indeed, peritoneal macrophages stimulated in vitro with *E. faecalis* CECT7121 responded with an inflammatory cytokine profile (IL 10). Therefore, it can be speculated that the treatment with *E. faecalis* CECT7121 might be beneficial for other non-enteric pathological conditions, e.g. antitumor immune responses, defense mechanisms against pathogenic microorganisms and regulation of atopic reactions. *E. faecalis* CECT7121 is a non-pathogenic strain that can attach and remain in the intestinal epithelium of BALB/c mice, can hamper the colonization by pathogenic strains and modulates the systemic innate immune response by inducing the synthesis of IL-12 and IL-10, two pivotal cytokines for the maintenance of the host’s immune homeostasis.

It has been demonstrated that the novel enterocin (bacteriocin), AP-CECT7121, has an interesting in vitro sensitivity and activity against a range of both aerobic and anaerobic Gram-positive bacteria. Of particular note was the observation that AP-CECT7121 was effective in inhibiting a variety of virulent strains, including H-MRSA and Ca-MRSA, isolated from cl patients with invasive infections. When tested against the most resistant of these MRSA isolates, KC studies for AP-CECT7121 demonstrated that the peptide still had a high efficacy (viable bacterial counts were not detected) within the relatively short incubation period of 180 min. Killing curves studies on the most resistant bacterial strains, as identified by the agar-well diffusion experiments, confirmed that AP-CECT7121 was effective in killing all the aerobic strains tested, although the kinetics differed between species. For *E. faecium* and the vancomycin resistant *E. faecalis* strain, viable bacterial counts were not detected within 30 min. The effect was less immediate with the penicillin-resistant *S. pneumoniae* and *S. pyogenes* strains although for both the viable bacterial counts decreased under the detection limit within 90 min. For Ca-MRSA, killing was even more gradual, but the absence of detection of viable bacteria was achieved within the 180 min incubation period. These models will answer to the challenge of contributing to the development of new strategies for the treatment of invasive infections in either, Human and Veterinary Medicine. The combination of a novel therapy using conventional or non conventional ATM agents with immunomodulators is proposed, and it is now being evaluated at our laboratory.

References


