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Bactericidal Activity and Synergy Studies of Peptide AP-CECT7121 Against Multi-resistant Bacteria Isolated from Human and Animal Soft Tissue Infections

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Abstract AP-CECT7121 is an antimicrobial peptide, produced by *Enterococcus faecalis* CECT7121, with bactericidal activity against Gram-positive bacteria. The aim of this study was to evaluate the bactericidal activity of AP-CECT7121, alone and with gentamicin, against multi-resistant bacteria isolated from human and animals with soft tissue infections. During the period 2014–2015, bacterial strains producing human and animal soft tissue infections were studied. Samples from patients attended at a general hospital and cattle from four dairies in the Province of Buenos Aires (Argentina) were included. Twenty-two methicillin-resistant *Staphylococcus aureus* (11, human blood samples; 11, cow milk) and five vancomycin-resistant *Ent. faecium* strains isolated from four mastitic dairy cows were tested. AP-CECT7121 (12 mg/L) potency was assessed by time-kill curves alone or with subinhibitory concentrations of gentamicin. All staphylococcal strains were susceptible to gentamicin; enterococci did not show high-level gentamicin resistance. Colony counts were carried out at 0, 2, 4, 8, and 24 h of incubation. AP-CECT7121 showed bactericidal activity against all the enterococcal strains. In addition, AP-CECT7121 had a bactericidal effect on most staphylococci (16/22). Early AP-CECT7121/gentamicin synergy (4–8 h) for all staphylococci was detected. At 24 h, synergy (19/22) and indifference (3/22) were observed. Synergy with gentamicin was detected for staphylococci. AP-CECT7121 constitutes an attractive candidate for its use as a natural therapeutic tool for the treatment of infections produced by multi-resistant *Staph. aureus* and vancomycin-resistant *Ent. faecium* isolated from humans and animals.

Keywords AP-CECT7121 · Bactericidal activity · *Enterococcus faecium* · *Staphylococcus aureus* · Skin and soft tissue

Introduction

Bovine mastitis is an intramammary infection caused by a wide spectrum of microorganisms. It has a significant relevance for milk industry with a negative impact on costs, quality, and volume of dairy production [1].

Gram-positive bacteria such as *Staphylococcus aureus* are among the most prevalent etiological agents of this infectious disease. However, *Enterococcus faecium* and *Ent. faecalis* are associated with environmental bovine mastitis due to inadequate hygiene practices [2].

Nowadays, antimicrobial multi-resistant bacteria are emerging and spreading globally, threatening the possibility to treat common infectious diseases. According to the Healthcare Infection Control Practices Advisory Committee from the Centers for Disease Control and Pevention (HICPAC-CDC), bacteria expressing resistance to more than one class of antimicrobial agents are defined as multi-resistant oganisms (https://www.cdc.gov/hicpac/mdro/mdro_2.html).

In human medicine, the use of antimicrobials exerts a high selective pressure upon prevalent pathogens, as methicillin-resistant *Staph. aureus* (MRSA), which expresses resistance to beta-lactams. Selection of multi-resistant bacteria leads to the existence of few remaining therapeutic options [3].

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Community-associated MRSA differ in certain aspects with healthcare associated multi-resistant staphylococci, such as the target population, clinical syndromes (e.g., severe sepsis, necrotizing fasciitis, and necrotizing pneumonia), susceptibility patterns (SCC*mec* cassettes), and infections in healthcare environment as well as in the community [4, 5].

Resistance to beta-lactams has also been detected in *Staph. aureus* isolated from farm animal, e.g., cattle, currently known as livestock-associated MRSA (LA-MRSA). Besides occupational contact, private farm visits or contact with persons who are directly exposed to livestock may also increase the risk for LA-MRSA acquisition [6].

For more than 20 years, vancomycin-resistant *Ent. faecium* have been acknowledged as etiological agents of human infectious diseases. However, the same variants of the *vanA* gene cluster (*Tn1546*) encoding vancomycin resistance can be detected in enterococci of both human and animal origin [7].

Gentamicin is widely used together with cell-wall active agents (beta-lactams, vancomycin) for the treatment of severe infectious diseases caused by vancomycin-resistant *Ent. faecium* VRE and MRSA [8, 9].

Soft tissue infections are an important morbidity factor among hospitalized patients and represent a therapeutic challenge for physicians. Selection of appropriate antimicrobials is one of the most important keys for a successful treatment. Gram-positive bacteria are the predominantly isolated organisms from patients with these severe infections [10].

Nowadays, the absence of new pharmacological options for the treatment of infectious diseases led to the search of new therapeutic strategies that can be a complement for conventional antimicrobials. One possibility is the antagonism mediated by bacterial proteins. Bacteriocins are antimicrobial peptides with bactericidal activity [11].

Peptide AP-CECT712 is an enterocin produced by the probiotic *Ent. faecalis* CECT7121, a non-hemolytic, gelatinase negative strain recovered from a natural corn silage in Tandil District, Argentina. Moreover, it does not show antimicrobial multiresistance (minimum inhibitory concentration $[MIC]_{vancomycin}$ 0.12 μg/mL, $MIC_{ampicillin}$ 0.5 μg/mL, $MIC_{gentamicin}$ < 500 μg/mL, $MIC_{streptomycin}$ < 1000 μg/mL) [12, 13].

Physicochemical studies showed that AP-CECT7121 presents heat-stability (1 h, 75 °C). Also, it is sensitive to proteolytic enzymes, detergents, and chelants; in addition, it is stable against the activity of enzymes such as DNase, RNase, amilase, glucuronidase, and lipase. Furthermore, presents inhibitory activity among a wide range of pH values (4.0–8.0). AP-CECT7121 is a cationic hydrophobic peptide, with a low molecular weight (3000 Da) included in class II bacteriocins. The mechanism of action is mediated by formation of pores in bacterial membranes, followed by osmotic shock and cell lysis [13].

The aim of this study was to evaluate the bactericidal activity of AP-CECT7121 alone and with gentamicin against multi-resistant bacteria isolated from human and animal soft tissue infections.

Materials and Methods

Bacterial Strains

During the period of January 2014–December 2015, multi-resistant bacterial strains producing human and animal soft tissue infections were included.

Methicillin-resistant *Staph. aureus* and vancomycin-resistant *Ent. faecium* were recovered from mastitic dairy cows. Milk samples were obtained from *n*: 4 dairy farms (DF01-DF04), with a mean number of 700 dairy cows, located at the District of Tandil, Argentina.

Human MRSA were isolated from blood samples of patients with severe soft tissue infections attending at Ramón Santamarina Hospital's Emergency Room (Tandil, Argentina), under Medical prescription. One bacterial strain per patient was included.

Phenotypic Characterization

Phenotypic characterization of *Ent. faecium* and *Staph. aureus* were carried out according to Sparo et al. [12] and Winn et al. [14], respectively. VITEK 2® Compact automated system (bioMérieux, Buenos Aires, Argentina) was used for validation of phenotypic characterization.

Antimicrobial Susceptbility Testing

Preliminary antimicrobial susceptibility studies (disk diffusion) for staphylococcal and enterococcal isolates were done. Multi-resistant *Staph. aureus* (resistant to beta-lactams, erythromycin, and tetracycline) and *Ent. faecium* isolates (resistant to vancomycin, ampicillin, and tetracyciline) were selected.

MRSA (*n*: 11) and VRE (*n*: 5) from 16 different mastitic dairy cows were studied. MRSA were recovered from dairy farms DF01 (5/11), DF02 (1/11), DF03 (3/11), and DF04 (2/11). VRE were isolated from animals at dairy farms DF02 (2/5), DF03 (2/5), and DF04 (1/5).

MIC was determined by the agar dilution method, according to the Clinical and Laboratory Standards Institute's recommendations, CLSI [15]. The following antimicrobials were tested: cefoxitin (MRSA), gentamicin (MRSA, VRE), vancomycin (VRE), and teicoplanin (VRE). *Ent. faecalis* ATCC 29212, *Ent. faecalis* ATCC 51299, and *Staph. aureus* ATCC 29213 were used for quality control.

Isolation of AP-CECT7121

Isolation of AP-CECT7121 was carried out according to the protocol of Sparo et al. [16]. Probiotic strain *Ent. faecalis* CECT7121 (deposited at the Spanish Collection of Type Cultures, CECT, Burjasot, Valencia, Spain) was incubated in brain-heart infusión (BHI) broth (Laboratorio Britania, Buenos Aires, Argentina) at 35 ± 2 °C for 18 h. This culture was inoculated in 4 L of BHI broth and incubated at 35 ± 2 °C for 9 h. Then, it was centrifuged at 15,000*g*, 4 °C, for 20 min. Supernatant was adjusted to pH: 7.0 and precipitated according to Dawson et al. [17]. After centrifugation at 20,000*g*, 4 °C, for 20 min, the pellet was re-suspended in 40 mL of phosphate buffer saline (PBS), pH: 7.0 (50 mM).

AP-CECT7121 was isolated by physicochemical extraction employing Sep-Pak™ C18 cartridges (Waters, Milford, MS, USA). *Ent. faecalis* extract (5.0 mL) was loaded into a cartridge, previously washed with acetonitrile in trifluoroacetic acid (TFA, 0.1%), and it was eluted with acetonitrile (60%)-TFA (0.1%). Eluate was concentrated to dryness using a vacuum centrifuge (ThermoSavant Instuments, Hollbrook, NY, USA). The obtained pellet was re-suspended in PBS (250 μL).

Aliquotes (20 μL) of the suspensión were injected in a reverse-phase HPLC system (Shimadzu, Kyoto, Japan) and separated in a Nucleosil C18 (5 μm, Pharmacia, Uppsala, Sweden) column. Mobile phase: buffer A (TFA 0.1%) and buffer B (acetonitrile 95% in TFA 0.1%). AP-CECT7121 was eluted using a linear gradient (95% A/5% B to 15% A/85% B), with a flow rate of 0.2 mL/min, controlling elution with a UV detector. Fractions were collected at regular time period. Then, the active fraction was evaporated to dryness and re-suspended in phosphate buffer (50 mM, pH: 7.0). Biological activity of AP-CECT7121 was detected in the eluate fractions after 30 min of the sample injection, when it was ca. 40% of buffer B.

Time-Kill Curves

Efficacy of in vitro bactericidal activity of AP-CECT7121 alone or combined with gentamicin, for assessing a synergistic effect, was studied carrying out time-kill curves. Three time-kill curves were done for each isolate.

Fresh cultured bacterial cells were washed, suspended, and diluted in PBS, 50 mM, pH: 7.0, for reaching a 10^6 CFU inoculum. Samples (100 μL) of each bacterial suspension were obtained at 0, 4, 8, and 24 h of incubation (35 ± 2 °C). Viable colony counts were performed in brain-heart infusión agar, after incubation at 35 ± 2 °C for 24 h. AP-CECT7121 concentration used 12 mg/L. A viable cell count in the same experimental conditions, with PBS, was performed as quality control [16].

For assessing a synergistic effect, viable cell counts were carried out with AP-CECT7121 combined with sub-inhibitory concentrations of gentamicin (MIC/4) and with gentamicin alone.

Bactericidal effect was defined as a decrease in viable counts with AP-CET7121 alone ($\Delta \geq -3$ $\log_{10}$). Sinergy: an increase in lethality ≥1 log at 4–8 h (early synergy, $\Delta \geq -1$ $\log_{10}$) and ≥ -2 $\log_{10}$ at 24 h of incubation (late synergy, $\Delta \geq -2$ $\log_{10}$) for AP-CECT7121-gentamicin combination compared with AP-CECT7121 alone. When synergy was not achieved after 24 h, it was considered as indifference between AP-CECT7121 and the antimicrobial [18].

Ethical Aspects

Milk samples from mastitic dairy cows were obtained with the cooperation of each dairy farm Veterinarian. For the obtention of human blood samples, briefings with patients or their relatives were performed. Ethical legislation (Helsinki's Declaration, Argentinian Bill for Personal Data Protection) was applied.

Results

Bacterial Strains and Antimicrobial Resistance

All the animal and human *Staph. aureus* isolates included in this study were resistant to cefoxitin (MIC ≥8 μg/mL). Therefore, these staphylococci were characterized as human MRSA and livestock-associated MRSA. In addition, 100% of MRSA isolates showed no resistance to gentamicin (MIC ≤4 μg/mL).

Human MRSA (*n*: 11) were recovered from blood samples, from different patients at an Emergency Room, later admitted to the hospital. Severe soft tissue infections were acquired in the community; patients did not have previous records of MRSA colonization nor infection, no history of hospitalization, surgery, dialysis, or admission to a nursing home on the previous year; no permanent catheters or medical devices that passed through the skin. A wide age range among the patients was observed (30–60 years old). Isolates were recovered from female (6/11) and male (5/11) patients.

Ent. faecium isolated from mastitic dairy cows expressed vancomycin (MIC ≥32 μg/mL) and teicoplanin (MIC ≥32 μg/mL) resistance. This pattern was compatible with a glycopeptide-resistant VanA phenotype, and, hence, enterococci were characterized as VRE. High-level gentamicin resistance was not detected (MIC <500 μg/mL).

Bactericidal activity of AP-CECT7121 and synergy with gentamicin in MRSA isolated from mastitic cows.

AP-CECT7121 was bactericidal against 7/11 of the bovine methicillin-resistant staphylococci (−3.7 to −5.0 $\log_{10}$ CFU/

mL). Therefore, a 63.6% killing efficacy frequency was observed (Table 1).

Early synergy, at 4–8 h, between APCECT7121 and gentamicin was detected in all the assayed mastitic staphylococci (−1.0 to −1.6 $\log_{10}$ CFU/mL). At 24 h, in 8/11 isolates (72.7%), synergy was observed (−2.3 to −5.0 $\log_{10}$ CFU/mL), while indifference 3/11 (27.3%) MRSA showed indifference (−1.1 to −1.2 $\log_{10}$ CFU/mL).

Bactericidal activity of AP-CECT7121 and synergy with gentamicin in VRE isolated from mastitic cows.

Table 1 Variation of cell counts ($\Delta\log_{10}$CFU/mL) after AP-CECT7121 sinergy experiments with gentamicin in methicillin-resistant *Staphylococcus aureus* from mastitic cows

Strain[a]	Time (h)	Non ATM[b] ($\Delta\log_{10}$CFU/mL)[c]	AP-CECT7121 ($\Delta\log_{10}$CFU/mL)	GEN[c] ($\Delta\log_{10}$CFU/mL)	AP-CECT7121 + GEN ($\Delta\log_{10}$CFU/mL)[c]
DF01–120	0	0	0	0	0
	4	1.6	−1.9	1.4	−3.1
	8	1.9	−3.1	1.8	−4.1
	24	2.1	*−5.0*	2.1	ND[d]
DF02–138	0	0	0	0	0
	4	1.4	−1.9	1.6	−2.9
	8	1.9	−3.0	2.2	−3.2
	24	2.2	*−3.7*	2.3	ND
DF01–142	0	0	0	0	0
	4	1.3	−2.0	1.7	−3.2
	8	1.9	−3.2	2.0	−3.4
	24	2.1	*−4.4*	2.2	ND
DF04–145	0	0	0	0	0
	4	1.3	−1.9	1.7	−3.2
	8	1.7	−3.1	2.0	−3.4
	24	2.1	*−3.9*	2.1	ND
DF04–153	0	0	0	0	0
	4	1.2	−2.0	1.4	−3.0
	8	1.7	−3.1	1.8	−4.1
	24	2.3	*−4.3*	2.0	ND
DF03–157	0	0	0	0	0
	4	1.4	−1.7	1.5	−2.8
	8	1.9	−2.6	2.0	−3.7
	24	2.1	*−4.1*	2.2	ND
DF03–163	0	0	0	0	0
	4	1.7	−1.5	1.7	−2.6
	8	1.9	−2.7	2.0	−3.8
	24	2.0	*−4.1*	2.0	ND
DF01–167	0	0	0	0	0
	4	1.6	−0.8	1.7	−1.8
	8	2.0	−1.3	2.1	−2.6
	24	2.2	−2.4	2.2	ND
DF01–172	0	0	0	0	0
	4	1.6	−0.5	1.4	−2.1
	8	2.0	−1.3	1.8	−2.5
	24	2.3	−2.6	2.0	−3.7
DF03–178	0	0	0	0	0
	4	1.7	−1.1	1.3	−2.2
	8	2.1	−1.9	1.9	−2.9
	24	2.2	−2.5	2.2	−3.6
DF01–179	0	0	0	0	0
	4	1.4	−0.9	1.4	−2.1
	8	2.0	−1.6	2.0	−2.7
	24	2.1	−2.3	2.0	−3.5

Italicized item states the decrease of CFU was higher than 3log, defined as bactericidal effect of AP-CECT7121

[a] Strain code: establishment + identification number

[b] Antimicrobial

[c] Gentamicin

[d] Not detected

Bactericidal activity of AP.CECT7121 against VRE was detected (Table 2). Higher decrease in enterococcal counts were observed, when there were compared with staphylococcal counts (−4.3 to −6.2 $\log_{10}$ CFU/mL).

However, the activity of AP-CECT7121 with gentamicin was slightly enhanced against enterococci. In 4/5 isolates, there was not proved early synergy (−0.2 to −0.9 $\log_{10}$ CFU/mL). Nevertheless, early synergy was observed in 1/5 VRE (−1.3 $\log_{10}$ CFU/mL) at 4–8 h, and synergy was detected in 2/5 VRE at 24 h (−4.3 $\log_{10}$ CFU/mL).

Bactericidal activity of AP-CECT7121 and synergy with gentamicin in human community-origin MRSA.

Bactericidal activity of AP-CECT7121 was observed in 9/11 (81.8%) multi-resistant community-origin MRSA (−3.1 to −3.8 $\log_{10}$ UFC/mL) recovered from human patients (Table 3).

In all the staphylococcal isolates, early synergy was detected, at 4–8 h (−1.0 to −1.3 $\log_{10}$ UFC/mL). However, indifference was observed in 100% of the human MRSA (≤−1.1 $\log_{10}$ UFC/mL).

Discussion

Bactericidal activity of the antimicrobial peptide AP-CECT7121 and a synergistic effect with gentamicin were assayed against human and animal multi-resistant bacteria. MRSA and VRE strains were selected based on their epidemiological relevance.

Isolation of VRE from mastitic cows represents a relevant concern for human health. Previous studies showed the horizontal transfer of *van* genes between enterococci from different origin, as well as between vancomycin-resistant *Ent. faecium* and *Staph. aureus* [19, 20]. Previously, in the same studied region, VRE were recovered from artisanal cow cheese, strengthening the possibility of antimicrobial resistance transfer to humans through the food chain [21].

Emergence of community-associated MRSA has been linked with an increase in beta-lactam resistant staphylococci. A meta-analysis showed high pooled prevalence rates of community-associated MRSA infections in Asia (23.1%), Europe (37.4%), and North America (47.4%). These findings highlighted the epidemiological relevance of community-

Table 2 Variation of cell counts ($\Delta\log_{10}$CFU/mL) after AP-CECT7121 sinergy experiments with gentamicin in vancomycin-resistant *Enterococcus faecium* from mastitic cows

Strain[a]	Time (h)	Non ATM[b] ($\Delta\log_{10}$CFU/mL)	AP-CECT7121 ($\Delta\log_{10}$CFU/mL)	GEN[c] ($\Delta\log_{10}$CFU/mL)	AP-CECT7121 + GEN ($\Delta\log_{10}$CFU/mL)
DF02–043	0	0	0	0	0
	4	1.6	−1.8	1.5	−2.7
	8	2.1	−3.7	2.3	−3.5
	24	2.3	*−4.3*	2.5	ND[d]
DF04–056	0	0	0	0	0
	4	1.5	−1.9	1.5	−2.6
	8	2.2	−3.3	2.1	−4.1
	24	2.5	*−4.3*	2.3	ND
DF02–065	0	0	0	0	0
	4	1.7	−2.2	1.7	−2.8
	8	2.2	−3.3	2.2	−4.1
	24	2.5	*ND*	2.4	ND
DF03–072	0	0	0	0	0
	4	1.6	−2.1	1.3	−2.8
	8	2.0	−3.2	1.9	−3.6
	24	2.3	*ND*	2.3	ND
DF03–078	0	0	0	0	0
	4	1.5	−2.3	1.4	−3.6
	8	1.9	−4.2	2.0	−4.2
	24	2.1	*ND*	2.1	ND

Italicized item states the decrease of CFU was higher than 3log, defined as bactericidal effect of AP-CECT7121

[a] Strain code: establishment + identification number

[b] Antimicrobial

[c] Gentamicin

[d] Not detected

Table 3 Variation of cell counts (Δlog_{10}CFU/mL) after AP-CECT7121 sinergy experiments with gentamicin in methicillin-resistant *Staphylococcus aureus* from human skin and soft-tissue human infections

Strain[a]	Time (h)	Non ATM[b] (Δlog_{10}CFU/mL)	AP-CECT7121 (Δlog_{10}CFU/mL)	GEN[c] (Δlog_{10}CFU/mL)	APCECT+ GEN (Δlog_{10}CFU/mL)
HS-224	0	0	0	0	0
	4	1.8	−0.7	−0.9	−0.9
	8	2.7	−1.4	−1.5	−2.7
	24	3.9	*−3.8*	−2.8	−3.7
HS-228	0	0	0	0	0
	4	1.6	−0.8	−0.7	−0.7
	8	2.5	−1.7	−1.7	−2.7
	24	3.8	*−3.8*	−2.9	−3.9
HS-232	0	0	0	0	0
	4	1.3	−0.8	−0.8	−0.6
	8	2.6	−1.6	−1.7	−2.8
	24	3.7	*−3.5*	−3.0	−3.6
HS-235	0	0	0	0	0
	4	1.8	−0.7	−0.7	−0.9
	8	2.4	−1.1	−1.5	−2.2
	24	3.5	*−3.2*	−2.7	−3.2
HS-242	0	0	0	0	0
	4	1.6	−0.5	−0.9	−0.9
	8	2.1	−1.2	−1.5	−2.2
	24	3.4	−2.6	−2.7	−2.5
HS-243	0	0	0	0	0
	4	1.4	−0.8	−0.4	−0.7
	8	2.0	−1.6	−1.4	−2.8
	24	3.5	*−3.3*	−2.9	−3.3
HS-247	0	0	0	0	0
	4	1.5	−0.7	−0.7	−0.8
	8	2.3	−1.5	−1.5	−2.7
	24	3.2	*−3.1*	−3.0	−3.2
HS-253	0	0	0	0	0
	4	1.3	−0.9	−0.5	−0.6
	8	2.5	−1.4	−1.2	−2.5
	24	3.8	*−3.6*	−2.8	−3.5
HS-256	0	0	0	0	0
	4	1.6	−0.4	−0.5	−0.4
	8	2.7	−1.1	−1.0	−2.2
	24	3.8	−2.4	−2.2	−2.3
HS-257	0	0	0	0	0
	4	1.1	−0.7	−0.6	−0.8
	8	1.7	−1.4	−1.0	−2.6
	24	3.4	*−3.1*	−2.7	−3.2
HS-262	0	0	0	0	0
	4	1.3	−1.0	−0.6	−0.8
	8	2.5	−1.7	−1.1	−2.7
	24	3.6	*−3.4*	−3.0	−3.5

Italicized item states the decrease of CFU was higher than 3log, defined as bactericidal effect of AP-CECT7121

[a] HS: Ramón Sanatamarina hospital

[b] Antimicrobial

[c] Gentamicin

associated MRSA as an etiological agent of human invasive infectious diseases [22].

In this study, the effectiveness of enterocin AP-CECT7121 as antimicrobial peptide on human and animal strains was investigated. Other authors focused on *bacteriocins* synthesized by other genera such as *Bacillus* spp., *Staphylococcus* spp., *Streptococcus* spp., and *Lactococcus* spp. [23].

So far, research for development of bacteriocins for therapeutic purposes has focused on antimicrobial peptides such as nisin, lacticin, and lysostaphin. Interestingly, is needed to highlight that AP-CECT7121 is produced by a probiotic strain, with proved no toxic or nocive effects [13, 24, 25].

AP-CECT7121 showed bactericidal activity against MRSA isolated from mastitic cows (63.6%). A similar inhibition activity (60%) against MRSA strains was observed for a fusion peptide, pheromonicin. However, nisin therapy had a lower success rate (54.5%), when assayed against mastitic *Staph. aureus* [24, 25]. Furthermore, the inhibitory activity of seven staphylococcal bacteriocins was tested against 165 *Staph. aureus* isolated from mastitic cows in Brazil and Argentina. Inhibitory activity of staphylococcins was detected against Brazilian (0–91.5%) and Argentinian (0–100%) *Staph. aureus,* a potential pathogenic bacterial species [26].

AP-CECT7121 showed a bactericidal effect against mastitic MRSA. In a previous study, bactericidal activity of three different bacteriocins against planktonic MRSA cells was assessed. Nisin A and lacticin Q had a bactericidal mode of action, in less than 24 h after the administration of the bacteriocins [27].

Bovine VRE isolates recovered from mastitic cows were killed by AP-CECT7121, with a viable cells decrease over 4.0 $\log_{10}$ UFC/mL. Similar results were reported for nisin with clinical enterococci. After 4 h of exposure to nisin, all the VRE isolates showed a 10^4 fold loss of viability [28]. However, pumilicin 4 showed a mild inhibition effect against vancomycin-resistant enterococci. After 6 h of incubation, viable cell counts decreased ca. 2.0 $\log_{10}$ UFC/mL and were over 7.0 $\log_{10}$ UFC/mL after 24 h [29].

In vitro efficacy of AP-CECT7121 was assayed against human multi-resistant *Staph. aureus*. Bactericidal activity of the enterocin was detected in 81.8% of MRSA isolates. Pumilicin 4 showed a strong inhibition effect and a significant decrease in MRSA viability. Nevertheless, a faster bactericidal effect was observed for nisin against MRSA isolates, since a 10^4 fold decrease was observed after 6 h [28, 29].

The existence of a synergistic effect between AP-CECT7121 and gentamicin was investigated. Early synergy with AP-CECT7121 was observed for all the bovine and human MRSA, while late synergy was detected for bovine strains. Recently, two enterocins produced by *Ent. faecalis*, alone and combined with kanamycin, were assayed against a MRSA strain recovered from a human patient. When early and late synergistic effects were studied, enterocins altogether with kanamycin exerted those effects [30].

In a cow VRE strain, early synergy AP-CECT71/gentamicin was detected. In a previous study, it was investigated the bactericidal activity of three bacteriocins alone and in combination. When two bacteriocins were assayed together, a more effective bactericidal effect was observed against enterococci. A combination of all the bacteriocins was the most effective against VRE [31].

Approximately 7 to 10% of hospitalized patients are affected by skin and soft tissue infections, such as necrotizing fasciitis. Depending on the severity of the infection, the affected area may become dysfunctional [32, 33].

Traditionally, staphylococcal infections were treated with commonly used antimicrobials, but emergence of drug-resistant bacteria is nowadays a major therapeutic concern. Community MRSA can cause severe or fatal diseases, and antimicrobial therapy requires second-line drugs or vancomycin. There are major issues with the utilization of vancomycin, including problems with prolonged bacteremia during therapy, high rates of clinical failure, and nephrotoxicity [34].

AP-CECT7121 showed homogenous bactericidal activity against most human MRSA isolates. However, there is scarce available information about the effectiveness of bacteriocins for the treatment of skin and soft tissue infections. Other authors found that although nisin seemed to be an effective option for the treatment of human mastitis, reductions in the staphylococcal counts of nisin-treated strains were under the limit for being considered as a bactericidal effect [35]. In another study, activity of nisin and lacticin 3147, against antimicrobial-resistant human staphylococci and enterococci, was investigated. Unlike AP-CECT7121, nisin was more effective against MRSA isolates, while lacticin 3147 showed a greater potency against human VRE [36].

In conclusión, AP-CECT7121 showed bactericidal activity alone against glycopeptide-resistant enterococci and methicillin-resistant staphylococci recovered from mastitic dairy cows and human patients with invasive soft tissue infections. Synergy with gentamicin was detected against bovine staphylococci, while indifference was observed for human *S. aureus*. AP-CECT7121 constitutes an attractive candidate for its use as a natural therapeutic tool for the treatment of bovine mastitis produced by animal multi-resistant bacteria, such as *Staph.aureus* and vancomycin-resistant *Ent. faecium* as well as for infectious diseases caused by MRSA.

Compliance with Ethical Standards

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

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