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Antimicrobial activity of prophage endolysins against critical *Enterobacteriaceae* antibiotic-resistant bacteria

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ABSTRACT

Enterobacteriaceae species are part of the 2017 World Health Organization antibiotic-resistant priority pathogens list for development of novel medicines. Multidrug-resistant *Klebsiella pneumoniae* is an increasing threat to public health and has become a relevant human pathogen involved in life-threatening infections. Phage therapy involves the use of phages or their lytic endolysins as bioagents for the treatment of bacterial infectious diseases. Gram-negative bacteria have an outer membrane, making difficult the access of endolysins to the peptidoglycan. Here, three endolysins from prophages infecting three distinct *Enterobacterales* species, Kp2948-Lys from *K. pneumoniae*, Ps3418-Lys from *Providencia stuartii*, and Kaer26608-Lys from *Klebsiella aerogenes*, were purified and exhibited antibacterial activity against their specific bacterium species verified by zymogram assays. These three endolysins were successfully associated to liposomes composed of dimyristoyl phosphatidyl choline (DMPC), dioleoyl phosphatidyl ethanolamine (DOPE) and cholesteryl hemisuccinate (CHEMS) at a molar ratio (4:4:2), with an encapsulation efficiency ranging from 24 to 27%. Endolysins encapsulated in liposomes resulted in higher antibacterial activity compared to the respective endolysin in the free form, suggesting that the liposome-mediated delivery system enhances fusion with outer membrane and delivery of endolysins to the target peptidoglycan. Obtained results suggest that Kp2948-Lys appears to be specific for *K. pneumoniae*, while Ps3418-Lys and Kaer26608-Lys appear to have a broader antibacterial spectrum. Endolysins incorporated in liposomes constitute a promising weapon, applicable in the several dimensions (human, animals and environment) of the One Health approach, against multidrug-resistant *Enterobacteriaceae*.

1. Introduction

Antibiotics are one of the most important and successful scientific discoveries and continue to be the leading resource for the management of infectious diseases (Durão et al., 2018; Huemer et al., 2020). However, shortly after antibiotic discovery it also became evident that its use could led to development of resistant bacterial strains thus compromising the treatment of these infections (Munita and Arias, 2016).

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Infections by antibiotic-resistant bacteria are responsible for about 700,000 deaths worldwide, and are estimated to reach 10 million per year in 2050 (Huemer et al., 2020), being however challenging to quantify the future associated excess of mortality. With the rapid emergence and increase of resistant bacteria strains, the World Health Organization (WHO) released in 2017 a global priority pathogen list of antibiotic resistant bacteria with the intuit of prioritization the research and development of new and effective treatments (World Health Organization, 2017). Among the list of priority pathogens, several species from *Enterobacteriaceae,* which is a ubiquitous family of Gram-negative bacteria, are included in the critical priority group. Within this family, we highlight *Klebsiella pneumoniae* species, an opportunistic and commensal pathogen that can cause life-threatening infections (Jenkins et al., 2017). *K. pneumoniae* has been classified as an ESKAPE organism (*Enterococcus faecium*; *Staphylococcus aureus*, *K. pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species) and it is responsible for hospital-acquired infections, such as pneumonia and bloodstream infections, with an increased mortality rate in new-borns and intensive-care unit patients (Wyres and Holt, 2016; Herridge et al., 2020). *K. pneumoniae* is one of the most important multidrug-resistant (MDR) pathogen, presenting resistance to several antibiotic classes, such as fluoroquinolones, aminoglycosides and beta-lactams (penicillins, cephalosporins, and carbapenems) (Moolchandani, 2017; Pendleton et al., 2013).

Carbapenem antibiotics (imipenem, ertapenem and meropenem) are one of the last resort antibiotics to treat severe bacterial infections, so the emergence of carbapenem-resistant *Enterobacteriaceae* (CRE) is considered a major concern (World Health Organization, 2017; Tacconelli et al., 2018). Resistance of K. pneumoniae to carbapenem antibiotics has spread to all regions of the world, where carbapenem antibiotics do not work in more than half of the patients treated for K. pneumoniae infections (Grundmann et al., 2017; Lan et al., 2021).

Bacteriophages (phages) are viruses that infect bacteria. Phage therapy uses phages and their lytic endolysins as a potential alternative to the current antibacterial therapies (Dorval Courchesne et al., 2009; Sulakvelidze et al., 2001). Given the increase in the number of MDR infections caused by Gram-negative bacteria such as *K. pneumoniae*, the use of phages or their specific gene products are increasingly becoming more relevant (Dorval Courchesne et al., 2009; Cisek et al., 2017; Burrowes et al., 2011; Lin et al., 2017; São-José et al., 2022).

Endolysins are phage-encoded enzymes produced at the end of the phage replication cycle. They are responsible for the degradation of the peptidoglycan wall, enzymatically cleaving specific peptidoglycan bonds leading to the release of phage progenies (Chang, 2020; Lai et al., 2020). Endolysins have certain advantages over antibiotics, where they can practically kill bacteria upon contact. Additionally, endolysins can also be specific to the target pathogen thus preserving the microbiota (Cheng et al., 2017).

The use of endolysins from without in Gram-positive bacteria, easily reach the peptidoglycan due to the lack of an outer membrane (Schuch et al., 2017; Vacek et al., 2020). However, Gram-negative bacteria contain an outer membrane that prevents the endolysin access to the peptidoglycan (Lukacik et al., 2012). Nevertheless, there are reports of phage endolysins that are naturally effective against Gram-negative bacteria (Antonova et al., 2019; Wang et al., 2020). To overcome the limitation of endolysin use in the Gram-negative bacteria, some approaches rely on: i) the use of outer membrane permeabilizers, such as ethylenediamine tetra acetic acid (EDTA) (Antonova et al., 2019; Morais et al., 2022; Briers et al., 2011; Guo et al., 2017; Lim et al., 2014); ii) artilysins, i.e., the fusion of an endolysin to an outer membrane-permeabilizing peptide (Briers et al., 2014); iii) or the incorporation of endolysins in liposomes, aiming to maximize the delivery of loaded endolysin to the peptidoglycan layer (Morais et al., 2022; Ferreira et al., 2021; Daraee et al., 2016). Liposomes are considered the most-well known and versatile lipid-based system enclosing one or various aqueous compartments separated by lipid bilayers being able to incorporate hydrophilic or hydrophobic compounds (Ferreira et al., 2021; Ribeiro et al., 2022; Juszkiewicz et al., 2020; Ferreira et al., 2021). Liposome effectiveness is dependent on its lipid composition, size, surface charge, pH and surrounding environment. When developing liposomal formulations for therapeutic purposes one of the main objectives is to promote the release of loaded material at affected tissues or organs. In particular, liposomes with pH sensitive properties, depending on the chosen lipid composition, have been widely described and reviewed in literature (Ferreira et al., 2021; Simões, 2004). One example is the inclusion in the lipid composition, for liposomes preparation, the lipids DOPE and CHEMS that originate stable bilayers at neutral pH while at slightly acidic microenvironments give rise to the destruction of the liposomal bilayer organization and payload release. Furthermore, the combination of DOPE and CHEMS in nanoliposomal formulations has demonstrated to potentiate the antibacterial effect of loaded antibiotics when compared to liposomes that do not include these two lipid components (Ferreira et al., 2021; Nicolosi et al., 2010).

It is also possible to change the membrane permeability of the liposomes by altering the lipid composition and introducing other components, such as cholesterol that will be located in the lipid bilayer (Nakhaei et al., 2021). Incorporation of drugs within liposomes can help to overcome the resistance mechanisms of bacteria, possibly attributed to the ability of the liposomal phospholipid bilayer to fuse with bacterial cell membranes, enabling a high delivery of loaded drug within bacteria as well as to provide protection, derived by encapsulation, against enzymatic deactivation (Ferreira et al., 2021; Wang et al., 2020).

Here, we study the antibacterial activity of putative endolysins encoded by genes from prophages infecting *Enterobacterales* species. To accomplish this aim, each endolysin encoding gene was cloned and expressed, producing a recombinant endolysin. These endolysins incorporated in liposomes allowing the endolysin-mediated lysis of multidrug resistant *K. pneumoniae* strains, constitute an important alternative strategy for answering to a major societal challenge recognized by WHO.

2. Materials and methods

2.1. Bacterial species and endolysins

The *in vitro* efficacy of endolysins was tested using four multidrug-resistant extended-spectrum β-lactamases (ESBL)-producing *K. pneumoniae* strains, one of them co-producing a carbapenemase, identified in samples collected from patients attending an Hospital in Lisboa (Table 1) (Perdigão et al., 2019; Pourgholi et al., 2022; Kpoda et al., 2018). These four strains were isolated from urine (Kp4859 and Kp4885), catheter (Kp4855), and blood (Kp4867), and showed distinct phenotype and genotype characteristics, belonging to different clonal groups: sequence type (ST)15, ST37, ST423, and ST147. The Capsular locus (KL) and antigen O (O_locus) were also distinct in all of them. Analysis of the resistome revealed antibiotic-resistance genes producing the ESBLs CTX-M−15 (Kp4867, Kp4855), CTX-M−32 (Kp4859), and BEL-1 (Kp4885); and co-occurrence of KPC-3 and GES-5 carbapenemases (in Kp4885). Regarding virulence genes, all strains showed a profile of virulence-associated determinants, specifically fimbrial adhesins (Type I and Type 3); and siderophores *entB* (enterobactin), *iroE* (salmochein), *iutA* (aerobactin) genes. The siderophore yersiniabactin cluster, contained 11 genes, was identified in Kp 4867 ST15 genome.

The endolysins used in this work included i) Kp2948Endo-Lys identified in *K. pneumoniae* 2948 prophage; ii) Ps3418-Lys from *Providencia stuartii* 3418 prophage; and iii) Kaer26608-Lys from *Klebsiella aerogenes* 26,608 prophage (Table 2). Endolysins were identified taking in consideration gene annotation, three-dimensional structure and sequence variability of the putative endolysins as previously described (Morais et al., 2022; Marques et al., 2021). Molecular weight, isoelectric point and sequence length were determined using ProtParam bioinformatics tool on the ExPASY server [67].

Table 1
Characterization of *K. pneumoniae* strains collected from patients attending a Hospital in Lisboa.

Strain	Biological product	ST	K_locus	O_locus	ESBL	Carbapenemases	Other β-lactamases
4867	blood	ST15	KL112	O1v1	CTX-M−15	–	SHV-28, TEM-1, OXA-1
4855	catheter	ST37	KL15	O4	CTX-M−15	–	SHV-11, TEM-1, OXA-1
4859	urine	ST423	KL8	O2v2	CTX-M−32	–	SHV-11
4885	urine	ST147	KL64	O2v1	BEL-1	GES-5; KPC-3	SHV-11, TEM-1, OXA-9

ST, Sequence type; KL, Capsular locus; O_locus, antigen O and ESBL, Extended Spectrum β-Lactamases.

Table 2
Physical and chemical parameters for selected endolysins determined through the ProtParam bioinformatics tool on the ExPASY server (Gasteiger et al., 2005).

Endolysin	Classification	Molecular Weight (kDa)	Theoretical pI	Number of Aminoacids
Kp2948-Lys	Glycoside hydrolase family 108 protein	19.89	6.14	181
Kaer26608-Lys	Glycoside hydrolase family protein	16.88	9.30	156
Ps3418-Lys	D-alanyl-D-alanine carboxypeptidase family protein / endopeptidase	15.10	9.91	130

pI, isoelectric point.

For the *in vitro* assays testing endolysins the following species were used: *K. pneumoniae* (strains 4859, 4867, 4885, and 29186); *Micrococcus luteus*; *P. stuartii* (strain 3418); *K. aerogenes* (strain 32891); *P. aeruginosa* (strain ATCC 27853).

2.2. Lysin genes cloning

The three putative endolysin genes were codon-optimized for expression in *Escherichia coli* BL21 (DE3), synthesized and cloned on the commercial vector pET15b (Synbio Technologies, Aurora, CO, USA), with a resistance mark for ampicillin, and a N-terminal (His)6-tag. Competent *E. coli* BL21 (DE3) cells were transformed with 0.06 μg of construct by heat-shock procedure, as described previously (Froger and Hall, 2007). The constructs were confirmed by colony PCR (Bergkessel and Guthrie, 2013) using GoTaq® DNA Polymerase (Promega, Madison, WI, USA) and T7 promoter (5′- TAATACGACTCACTATAGGG −3′) and T7 terminator primers (3′- GCTAGTTATTGCTCAGCGG −5′) (STABVida, Lisbon, Portugal), with standard PCR program, followed by Sanger sequencing (STABVida).

2.3. Protein expression and purification

Single colonies of *E. coli* BL21(DE3) transformed with the pET15b-cloned putative endolysin sequence were grown overnight at 37 °C in LB broth supplemented with 100 μg/mL ampicillin (Sigma-Aldrich, St. Louis, MO, USA), under agitation, and used as pre-inoculum in new LB broth for expression of the putative endolysins. The bacterial cultures were grown in 500 mL of LB broth supplemented with 100 μg/mL ampicillin (Sigma-Aldrich) at 37 °C under agitation, up to an OD600nm ≈ 0.6 and the expression of the putative endolysins was induced with 0.1 to 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 3 h. Induced cultures were ice-cooled and then centrifuged (3220 × g, 10 min, 4 °C). The pellet was washed with Tris-HCl 20 mM pH 8.0 and centrifuged again under the same conditions. The supernatant was removed, and the pellets were frozen at − 20 °C for short term processing or − 80 °C for long term storage. Induction confirmation was performed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Gallagher, 2012) and western blot (Yang and Mahmood, 2012).

Small scale preliminary purifications were performed using Ni-NTA Spin kit columns (Qiagen, Hilden, Germany) following manufacturer's instructions. Buffer exchange was performed using Vivaspin 500 centrifugal concentrators (Sigma-Aldrich), with a 5000 Da molecular weight cut-off, following manufacturer's instructions or using PD-10 columns (Cytiva, Marlborough, MA, USA), following manufacturer's instructions, using PBS 1/15X with Trehalose (for a final osmolarity of 100 mOsm, PBS-Trehalose) as final buffer. In up-scaled purifications, the Ni-NTA agarose resin purification (Qiagen), or n-small scale purifications were performed following manufacturer's instructions. Buffer exchange using PBS-Trehalose was done using PD-10 columns (Cytiva, Marlborough, MA, USA).

The purified endolysins were quantified in Qubit fluorometer (Invitrogen, Carlsbad, CA, USA), and confirmation of protein purification and size was done by 12 % SDS-PAGE. Additionally, to confirm the purification of the desired protein, a western blot was done using as primary antibody the His-tag (CT) rabbit polyclonal antibody (Bioss, Woburn, MA, USA) and the Goat Anti-Rabbit IgG (H + L)-HRP Conjugated antibody (Bio-Rad, Berkeley, CA, USA) in PBS-Tween + 1 % BSA, as secondary antibody.

2.4. Zymogram analysis

Zymogram assay for detection of bacteriolytic activity of the putative endolysins were performed as previously described (Morais et al., 2022). Briefly, for cell preparation, *K. pneumoniae* 4855, *K. pneumoniae* 29186, *P. stuartii* 3418 and *K. aerogenes* 32,891 were grown in LB Broth, at 37 °C, until exhaustion for two days. Cells were centrifugated (4000x g, 10 min), washed in water, and then autoclaved. The autoclaved cells were centrifuged (12000x g, 20 min) and the pellet was dried for approximately 90 min at least at 37 °C and then resuspended in water to 2 % (dry weight/v) final concentration.

For the Gram-negative bacteria *K. pneumoniae* 4855, *K. pneumoniae* 29186, *P. stuartii* 3418 and *K. aerogenes* 32,891 (0.6 %, 0.2 %, 0.2 % and 0.6 %, respectively) cell preparations were incorporated on the 12 % polyacrylamide gel. After electrophoresis, the zymogram gels were incubated in a renaturation buffer (25 mM Tris-HCl pH 7.5 and 1 % Triton X-100) overnight at 37 °C, with mild agitation. The gels were stained for 90 min in zymogram staining solution (0.5 % Methylene Blue and 0.01 % KOH) and distained in distilled water. Lysozyme of egg white (1 μg) (Sigma-Aldrich, St. Louis, MO, USA) and BSA (5 μg) (NZYTech, Lisbon, Portugal) were used as positive and negative control, respectively. For migration control, a regular SDS-PAGE was performed in parallel and stained with BlueSafe (NZYTech, Lisbon, Portugal).

2.5. Antimicrobial activity of endolysins

For preliminary endolysin activity tests, Gram-positive *M. luteus* bacteria were used, to quickly evaluate if the endolysins have activity (Morais et al., 2022). A *M. luteus* suspension was plated in LB-agar plates by flooding. Half-centimeter or one-centimeter round wells (respectively, with 75 μL and 200 μL of capacity) were made in the plates and used as application spots for 30 μg of the putative endolysins. Loaded plates were incubated at 37 °C for 48 h. Bacteriolytic effect was qualitatively assessed by the presence or absence of inhibition growth halos around the wells. Pre-obtained active endolysins and protein elution buffers were included as positive and negative controls, respectively.

2.6. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) determination

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the up-scale purified putative endolysins were assessed by serial dilutions method (Rodríguez-Tudela et al., 2003). The first well of a 12-well plate contained 200 μg/mL of the tested putative endolysin and the following wells were made by 1/2 serial dilutions in LB broth. Each well was then inoculated with 50 μL of a *M. luteus* suspension (OD600nm = 1). Positive and negative controls were included. After 24 h of incubation, at 37 °C with agitation, the optical density (OD600nm) of each well suspension was measured in Varioskan LUX Multimode Microplate Reader (Thermo Scientific, Waltham, MA, USA). The MIC was the lowest putative endolysin concentration at which the *M. luteus* visible growth was inhibited. After, to determine the MBC, 5 μL of each well suspension were spotted in LB agar plates and incubated overnight at 37 °C. The MBC was the lowest putative endolysin concentration at which no growth was observed in the solid medium.

2.7. Encapsulation of endolysins in liposomes

The lipid composition of the liposomes used in this work was dimyristoyl phosphatidyl choline (DMPC), dioleoyl phosphatidyl ethanolamine (DOPE) and cholesteryl hemisuccinate (CHEMS) at a molar ratio (4:4:2), respectively. Liposomal formulations were prepared following the dehydration-rehydration (DRV) method, which consists in the lyophilization of liposomal suspension containing the selected endolysin, followed by rehydration and extrusion to reduce and homogenize liposome mean size (Morais et al., 2022; Gaspar et al., 2015).

Briefly, lipid constituents were weighted to obtain the desired concentrations (30 μmol/mL), (corresponding to 180 μmol of total lipid for each batch of endolysins – 3 mL) and placed in round-bottomed balloons, and dissolved in chloroform. The organic solvent was removed by evaporation in Buchi R-200 rotary evaporator (Flawil, Switzerland) to form a lipid film that was then hydrated with 6 mL of water and 3 mL of the endolysins in PBS-Trehalose (15 x diluted and trehalose 75 mM) buffer. The initial concentration of the endolysins used ranged from 500 to 800 μg/mL. The liposomal solution was distributed by 3 freeze-drying vials (3 mL / vial), then frozen at −70 °C and subsequently lyophilized (Freeze-dryer, Edwards, CO, USA) overnight. The rehydration step was done by adding 1 mL of a solution constituted by 2/3 of PBS pH 7.4 and 1/3 of H_2O MiliQ, to each vial and mixed well, followed by incubation for 30 min at 30 °C in order to be at temperature higher than the phase transition temperature of the main phospholipid, DMPC.

After, 2 mL of PBS pH 7.4 were added and the suspension was then incubated during 30 min at the same temperature (30 °C). The next step was the extrusion of the liposomal suspension using an extruder device, Lipex Biomembranes Inc. (Vancouver, BC, Canada). This extrusion step was also performed at a temperature of 30 °C. The suspensions were filtered through polycarbonate membranes with 0.4 and 0.2 μm pore sizes, under a nitrogen pressure (10–500 lb./in^2) until reaching a mean size below 0.2 μm and a poly dispersion index < 0.1. The separation of non-incorporated endolysin was performed by ultracentrifugation (25000x g, 2 h, 15 °C) in a Beckman LM-80 ultracentrifuge (Beckman Instruments, Inc, USA). The pellet was then suspended in 500 μL of PBS pH 7.4. Lastly, liposomal formulation was sterilized by filtrating through a 0.22 μm pore filter (Cytiva Whatman™, Marlborough, MA, USA).

2.8. Physicochemical characterization endolysin liposomal formulations

Liposomes mean size was determined by dynamic light scattering using Zetasizer Nano S (Malvern Instruments, United Kingdom) at a standard laser wavelength of 663 nm, also reporting a polydispersity index, as a measure of particle size distribution. The zeta potential was determined using Zetasizer Nano Z (Malvern Instruments) by laser Doppler spectroscopy.

The protein concentration was determined by using the Lowry-Folin method (Lowry et al., 1951) and the Lipid content was determined using an enzyme-linked colorimetric method, Phospholipids Choline oxidase-Peroxidase (Spinreact, Spain). The endolysins liposomal formulations were characterized by initial and final endolysin to lipid ratios, ($^{Protein}/_{Lipid}$)i and ($^{Protein}/_{Lipid}$)f, respectively, in μg/μmol, and encapsulation efficiency (E.E), in %, was determined according to the following equation:

$$E.E.(\%) = \frac{\left(\frac{Protein}{Lipid}\right)f}{\left(\frac{Protein}{Lipid}\right)i} * 100$$

2.9. Antimicrobial activity of encapsulated endolysins

To evaluate the bactericidal effect of specific endolysins, a cellular metabolic assay (MTT assay) (Grela et al., 2018) was performed using *K. pneumoniae* 4855 strain. The MTT assay was performed because liposomes exhibit high initial turbidity, which limits the use of OD at 600 nm to assess microbial growth. Briefly, serial dilutions (1:1) of the different endolysins to test were made in 24-well plates to a final volume of 1 mL/well of PBS-Trehalose and starting with a concentration of 12.5 μg/mL. An overnight pre-inoculum of *K. pneumoniae* 4855 strain was inoculated in LB broth to an OD600nm ≈ 0.2 and let to grow up to an OD600nm ≈ 1.0 at 37 °C, under strong agitation. The grown culture was centrifuged (3200x g, 10 min), and the supernatant discarded. Briefly, as previously described, the pelleted bacteria were resuspended in the same volume of PBS-Trehalose, and 50 μL of this suspension were used for inoculation in the previously prepared 24-well plates. The inoculated plates were incubated at 37 °C, with agitation, up to 72 h. Every 24 h, 100 μL of each well's suspension were transferred to 96-well plates, centrifuged (3220x g, 15 min), and resuspended in 100 μL of PBS-Trehalose. Following, 10 μL of MTT Cell Viability Assay Kit (Biotium, Fremont, CA, USA) was added to each well and the plate was incubated for 3 h, at 37 °C, with agitation (Morais et al., 2022). After the incubation, 200 μL of dimethyl sulfoxide (DMSO) (Sigma-Aldrich) were added to each well to dissolve the formazan crystals and the absorbance was measured using Varioskan LUX Multimode Microplate Reader (Thermo Fisher Scientific) at 570 nm, with background at 630 nm, according to manufacturer instructions.

3. Results

3.1. Expression, purification and antibacterial activity of phage endolysins

The insertion of the desired putative endolysin coding gene into the expression vector was confirmed by Sanger sequencing. The induced endolysins were successfully purified, presenting the expected molecular weights. Western blot targeting the (His)6-tag tail in each endolysin confirmed the purification of the desired protein (Fig. 1).

The direct hydrolytic activity of endolysins was determined with a Gram-positive bacterium *M. luteus*, in a halo formation assay. All endolysins showed activity against the bacteria and produced growth inhibition halos (Fig. 2). The hydrolytic activity of the endolysins was tested using a zymogram in which autoclaved cells of *K. pneumoniae* 4855, *K. pneumoniae* 29186, *P. stuartii* 3418 and *K. aerogenes* 32,891 served as substrate. Kaer26608-Lys showed a clear lysis zone in the zymogram pointing to a promising activity against all tested strains. Kp2948-Lys showed activity against *K. pneumoniae* 4855, *K. pneumoniae* 29,186 and *P. stuartii* 3418, but has not tested on *K. aerogenes* 32891. Ps3418-Lys showed activity against *P. stuartii* 3418 and *K. aerogenes* 32,891 (Fig. 3, Supplementary Fig. 1, Table 3).

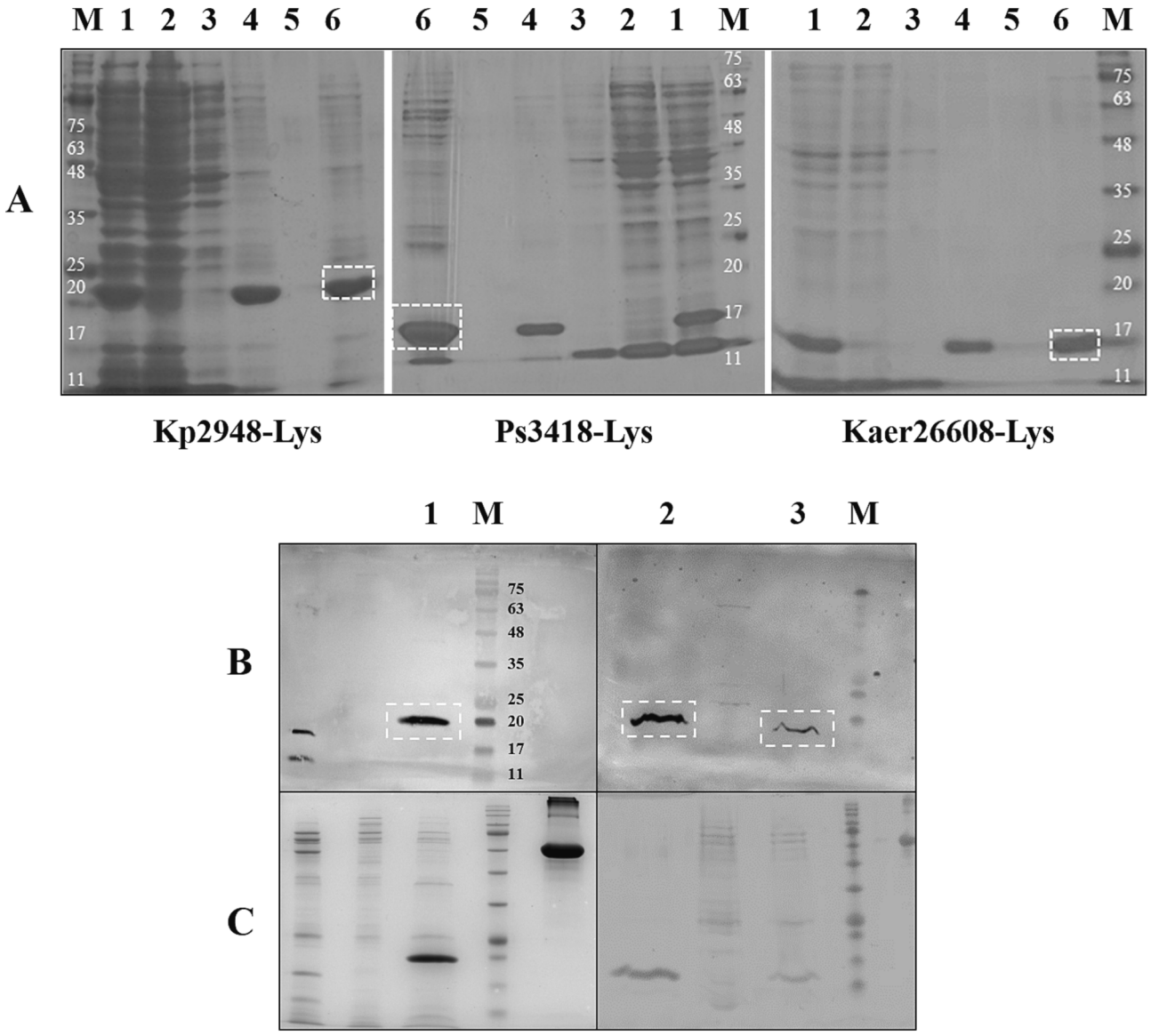

Fig. 1. (A) SDS-PAGE of the small-scale purification for endolysin Kp2948-Lys, Ps3418-Lys and Kaer26608-Lys. Bands shown for: 1 - soluble fraction proteins upon cell lysis; 2 - first wash; 3 - second wash; 4 - protein elution with imidazole; 5 - PD-10 column flowthrough; 6 - protein after buffer exchange. (B) Western-blot and (C) parallel SDS-PAGE under western-blot conditions of the purified endolysins. 1 - Kp2948-Lys; 2 - Kaer26608-Lys; 3 - Ps3418-Lys. M - NZYColour Protein Marker II (NZYTech). Final purified endolysin in PBS-Trehalose is highlighted (dotted box) with an expected molecular weight for Kp2948-Lys (19.9 kDa), Kaer26608-Lys (16.8 kDa) and Ps3418-Lys (15.0 kDa).

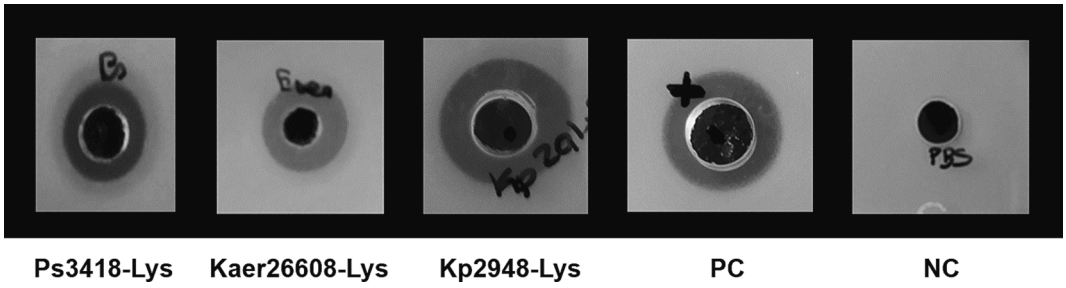

Fig. 2. Halo-formation assays on *M. luteus*. Small scale purified of endolysin bacteriolytic activity is shown as an inhibition halo around the application well, assessed against *M. luteus* in LB agar plates. All the halo assays done using 30 μg of each lysin in 80 μL volume. Ps3418-Lys - endolysin not encapsulated; Kaer26608-Lys - endolysin not encapsulated; Kp2948-Lys - endolysin not encapsulated; PC - positive control; NC - PBS 1x as negative control.

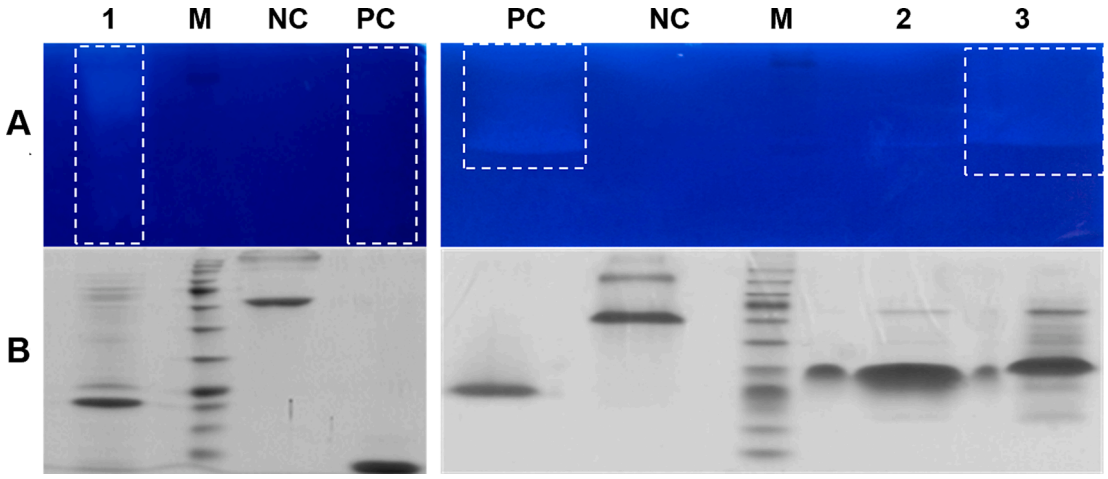

Fig. 3. (A) Zymogram analysis using *K. pneumoniae* 4855 biomass and (B) parallel SDS-PAGE under zymogram conditions of the purified endolysins. 1 - Kp2948-Lys; 2 - Ps3418-Lys; 3 - Kaer26608-Lys; PC - lysozyme as positive control; NC - bovine serum albumin (BSA) as negative control; M - NZYColour Protein Marker II (NZYTech). Lytic activity is observed as a clear hydrolyzed zone of peptidoglycan in the dark background (dotted boxes).

Table 3
Activity of endolysins in Gram-negative bacteria using a zymogram assay.

Endolysins	*K. pneumoniae* 4855	*K. pneumoniae* 29,186	*P. stuartii 3418*	*K. aerogenes* 32,891
Kp2948-Lys	+	+	+	n.d
Ps3418-Lys	–	–	+	+
Kaer26608-Lys	+	+	+	+

+, with activity; -, without activity; n.d, not done.

3.2. Minimal inhibitory concentrations (MIC) and minimal bactericidal concentration (MBC)

The determined values of MICs and MBCs refer only to the action of endolysins against the Gram-positive bacterium *M. luteus* (Table 4, Fig. 4). Ps3418-Lys and Kaer26608-Lys showed MIC 1.56 μg/mL and MBC 3.13 μg/mL. Kp2948-Lys showed MIC 6.25 μg/mL and MBC 12.5 μg/mL. MICs were done in triplicate and the results obtained were consistent during all assays. Therefore, the results obtained should be considered as a reference start point values to be used with encapsulated endolysins.

3.3. Liposomes composition and endolysins encapsulation

Endolysins incorporation in liposomes has already been demonstrated to be highly effective due to their ability to interact and internalize within bacteria allowing the release of loaded compounds (Morais et al., 2022). In view of this statement, the three endolysins that exhibited activity in the zymogram assay in their respective target bacteria (Kp2948-Lys, Ps3418-Lys and Kaer26608-Lys) were selected to be encapsulated in a liposomal formulation of DMPC:DOPE:CHEMS (at a molar ratio of 4:4:2). The lipid composition determined was chosen based on their fusogenic properties (Nicolosi et al., 2010; Nicolosi et al., 2015). All endolysins were successfully incorporated in liposomes. Physicochemical properties of the developed endolysins liposomal formulations are shown in Table 5.

Table 4
Minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) assessed for the endolysins in *M. luteus* cultures.

Endolysins	MICs(μg/mL)	MBCs(μg/mL)
Kp2948-Lys	6.25	12.50
Ps3418-Lys	1.56	3.13
Kaer26608-Lys	1.56	3.13

Endolysins Kp2948-Lys, Ps3418-Lys and Kaer26608-Lys had an encapsulation efficiency of 24.2 %, 27.2 % and 25.5 %, respectively. Endolysin liposomal formulations had a mean size < 150 nm with high homogeneity as demonstrated by the low polydispersity index (<0.1) (Supplementary Fig. 2). All nanoformulations had a negative surface charge, due to the presence of CHEMS in the lipid composition.

3.4. Antimicrobial activity of endolysins encapsulated in liposomes

The efficiency of the liposomal formulations in delivering the incorporated endolysins was tested against *K. pneumoniae* 4855 strain using an *in vitro* MTT assay to assess bacterial cell viability (Grela et al., 2018). The bactericidal effect of the free and encapsulated endolysins upon application against *K. pneumoniae* was evaluated at several time-points. Cellular viability values were much lower for liposome formulations of endolysins Kp2948-Lys, Ps3418-Lys and Kaer26608-Lys at a concentration of 3.13 μg/mL, while the free form did not show activity (Fig. 5). The observed MIC value (3.13 μg/mL) of the encapsulated endolysins in the Gram-negative *K. pneumoniae* was twice the MIC value (1.56 μg/mL) determined against the Gram-positive *M. luteus* using only free endolysins forms. These results show that the lipid formulation chosen was suitable to the delivery of endolysins to *K. pneumoniae* 4855 after 48 h. Empty liposomes showed some toxic impact on the bacterium at 48 h, probably occurring due to membrane destabilization, in line with a similar description using *P. aeruginosa* (Morais et al., 2022). In the conducted experiments, endolysins, either in free or in liposomal forms, retained their activity when stored at 4 °C for a minimum duration of one month.

3.5. Specificity of the encapsulated endolysins

To evaluate the specificity of the encapsulated endolysins, a similar MTT assay was done using different *K. pneumoniae* strains, as well other Gram-negative and Gram-positive bacterial genera/species, for the effective concentration observed (3.13 μg/mL) for the liposome incorporated endolysins. Bacterial metabolic activity was assessed for the target bacteria comparing between the free and encapsulated endolysin. Results and the most effective points and concentrations are shown (Supplementary Fig. 3, Table 6). Encapsulated endolysins showed activity against all *K. pneumoniae* strains tested (*K. pneumoniae* 4859, *K. pneumoniae* 4867 and *K. pneumoniae* 4885). Ps3418-Lys and Kaer26608-Lys showed activity only against *P. aeruginosa* ATCC 27853. Endolysin Kp2948-Lys seems to be specific to the species *K. pneumoniae,*

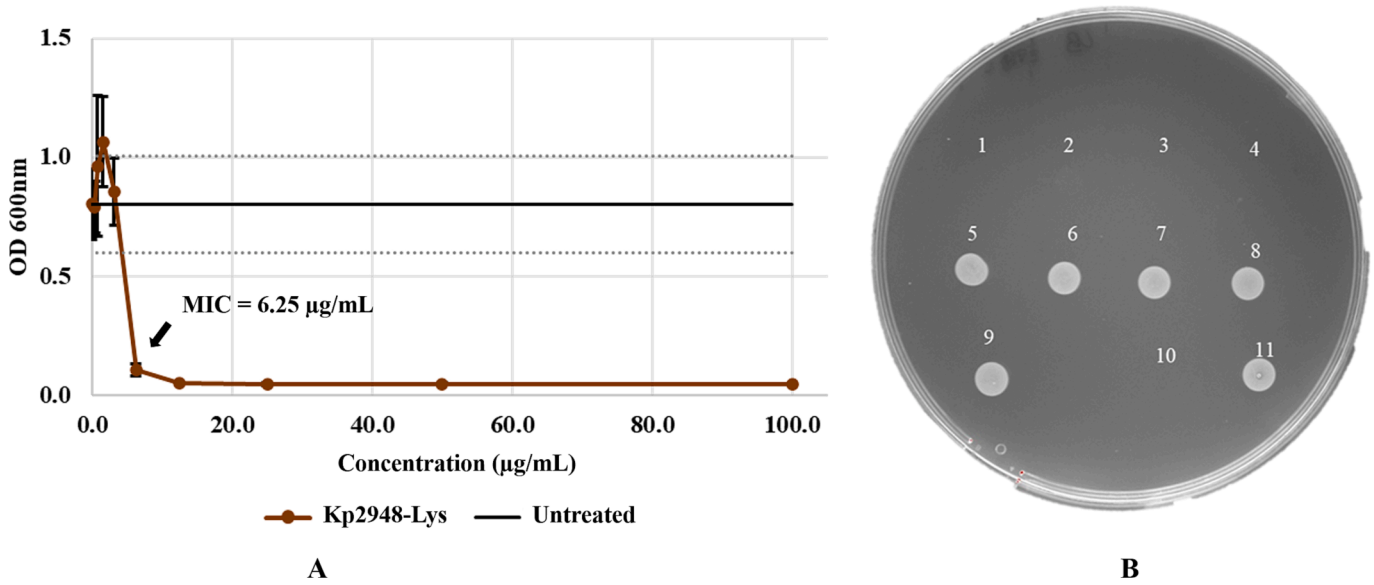

Fig. 4. Bacteriolytic effect of endolysin Kp2948-Lys against *M. luteus* culture for MIC and MBC assessment. Serial dilutions were made in LB medium and inoculated with *M. luteus* to determine (A) MIC and (B) and MBC. MIC is the lowest endolysin concentration at which the *M. luteus* visible growth is inhibited, whereas MBC is the lowest endolysin concentration at which no growth is observed in the solid medium. Untreated control (bacterial suspension without endolysin) is represented as a solid black line, with the respective 95 % confidence interval represented as dashed black lines; 1–100 µg/mL; 2–50 µg/mL; 3–25 µg/mL; 4–12.5 µg/mL; 5–6.25 µg/mL; 6–3.13 µg/mL; 7–1.56 µg/mL; 8–0.78 µg/mL; 9–0.39 µg/mL; 10 - negative control (suspension without bacteria); 11 - Positive control (suspension without endolysin). Assay performed in triplicate.

Table 5
Physicochemical characterization of the endolysin liposomal formulations. Liposomes were characterized in terms of the mean size, polydispersity index (P.I.), surface charge, and incorporation parameters.

Formulation	Lipid Composition (molar ratio)	(Prot /Lip)i (µg/µmol)	(Prot /Lip)f (µg/µmol)	I.E. (%)	Mean size (nm) (P.I.)	Zeta Pot (mV)
Liposome Ps3418-Lys	DMPC:DOPE: CHEMS (4:4:2)	10 ± 4	3 ± 1	27 ± 2	138 ± 2 (<0.1)	–22 ± 2
Liposome Kaer26608-Lys	DMPC:DOPE: CHEMS (4:4:2)	18 ± 6	6 ± 1	26 ± 4	148 ± 5 (<0.1)	–20 ± 1
Liposome Kp2948-Lys	DMPC:DOPE: CHEMS (4:4:2)	11 ± 2	3 ± 1	24 ± 2	136 ± 2 (<0.1)	–21 ± 1
Empty Liposome	DMPC:DOPE: CHEMS (4:4:2)	–	–	–	152 ± 3 (<0.1)	–20 ± 1

Endolysin liposomal formulations prepared with PBS, in the presence of trehalose. Incorporation Efficiency (I.E.) (%) was determined according to equation: (Prot /Lip)f/ (Prot /Lip)i × 100. DMPC – dimyristoyl phosphatidyl choline; DOPE – dioleoyl phosphatidyl ethanolamine; CHEMS - Cholesteryl hemisuccinate. Data presented correspond to mean and SD of at least two independent experiments.

showing activity against different strains of this species, despite not having been tested on *P. aeruginosa*. Endolysins Ps3418-Lys and Kaer26608-Lys from *P. stuartii* and *K. aerogenes* prophages, respectively, showed a broader antibacterial spectrum. Notably no endolysin showed activity against *Streptococcus* spp., *Enterococcus* spp. and *Proteus mirabilis*. Interestingly *P. mirabilis* and *P. stuartii* share the same family (*Morganellaceae*), but still Ps3418-Lys isolated from a *P. stuartii* prophage had no activity against *P. mirabilis*.

4. Discussion

Endolysins are phage enzymes responsible for the degradation of the peptidoglycan wall of the target bacteria and they appear to be one of the most promising antibacterial agents against antibiotic resistance. Here, we have identified three functional endolysins: Kp2948-Lys from *K. pneumoniae* prophage; Ps3418-Lys from *P. stuartii* prophage; and Kaer26608-Lys from *K. aerogenes* prophage. To deliver the endolysins overcoming the outer membrane of the Gram-negative *K. pneumoniae*, we have successfully used liposomes. Liposomes have the ability to fuse with the bacterial outer membrane, enabling the release of the transported drug (Wang et al., 2020). The improved therapeutic effect of different compounds following their incorporation in liposomes is widely reported (Nisini et al., 2018; Gaspar et al., 2008; Lee and Thompson, 2017).

In this study, we showed that endolysins Kp2948-Lys, Ps3418-Lys and Kaer26608-Lys preserved the lytic activity after encapsulation in the lipid composition DMPC:DOPE: CHEMS (at a molar ratio of 4:4:2). These encapsulated endolysins were able to show activity against *K. pneumoniae* 4855 in a concentration of 3.13 µg/mL, being the MIC time point achieved at 48 h after application. Several different formulations of this type of delivery system have already been developed to improve the performance of drugs and other different components. In fact, Ferreira *et al.* (2021) demonstrated that the lipid composition DMPC:DOPE: CHEMS (at a molar ratio of 4:4:2), equal to that used in our work, resulted in a higher loading capacity and incorporation efficiency of the antibiotic rifabutin than DMPC:DMPG, (at a molar ratio of 8:2) and DMPC:SA, (at a molar ratio of 9:1) (Ferreira et al., 2021). These results were also observed for other antibiotics namely levofloxacin and vancomycin (Ferreira et al., 2021). Morais *et al.* (2022) also used the same lipid formulation (DMPC:DOPE: CHEMS, at a ratio of 4:4:2) demonstrating good encapsulation efficiencies (approximately 30 %) for *P. aeruginosa* endolysins, similar to the encapsulation efficiency obtained for our three endolysins (Morais et al., 2022).

The time taken to observe antibacterial activity (48 h), suggests that the delivery of endolysins to the peptidoglycan site and/or fusion to the liposomes is slow and gradual, perhaps due to membrane destabilization. Depending on their composition, as in the present work due to the combination of DOPE and CHEMS, liposomes were prepared aiming to disintegrate and subsequently release incorporated endolysin in a

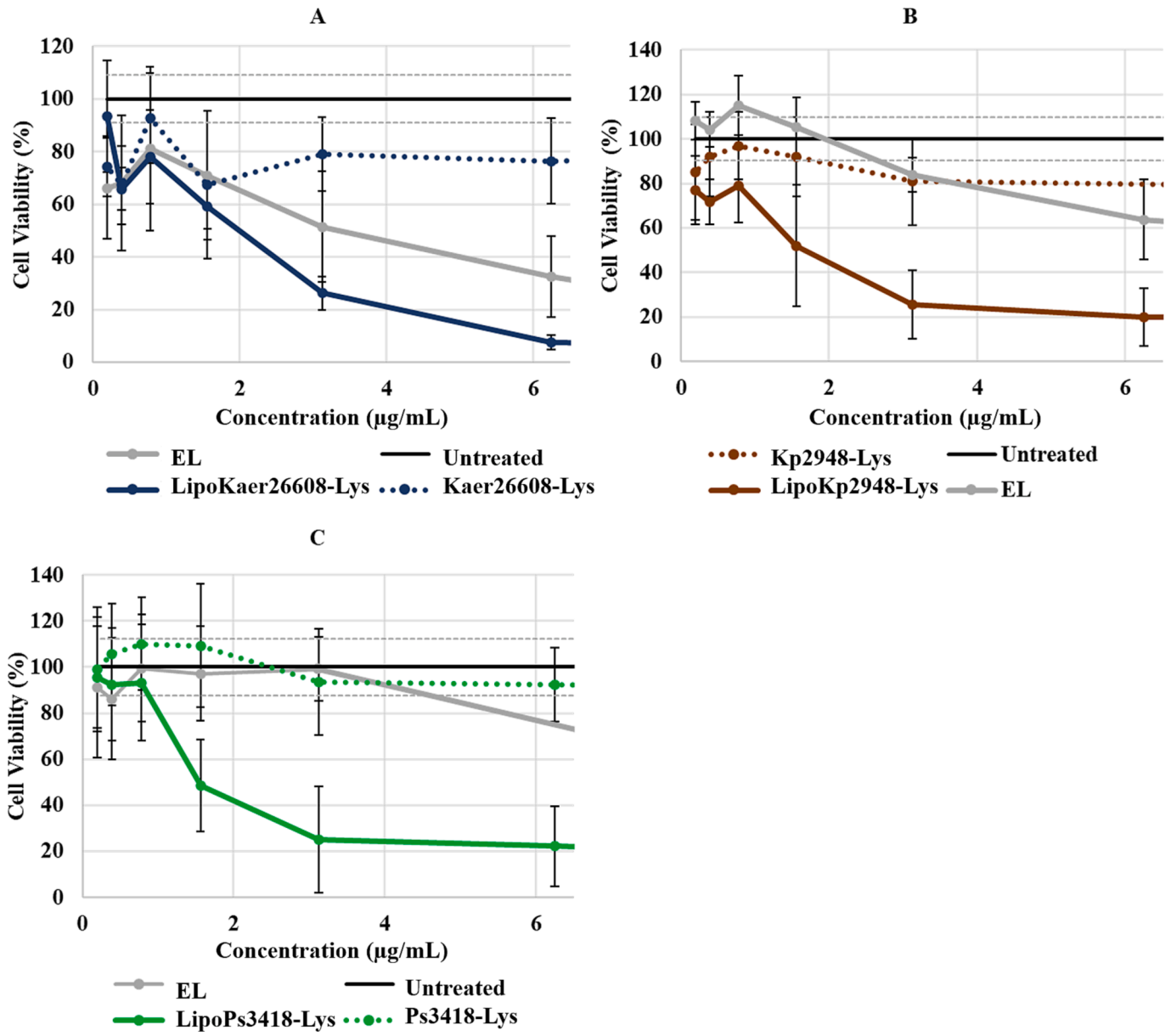

Fig. 5. Antibacterial activity of (A), Kaer26608-Lys, (B) Kp2948-Lys and (C) Ps3418-Lys at their free and encapsulated forms against *K. pneumoniae* 4855. The viability curve for the most effective point (48 h post treatment) is shown. Untreated - Untreated control (bacterial suspension without endolysin) is represented as a solid black line, with the respective 95 % confidence interval represented as dashed black lines; Kp2948-Lys - endolysin not encapsulated; LipoKp2948-Lys - encapsulated endolysin; Ps418 - endolysin not encapsulated; LipoPs3418-Lys - encapsulated endolysin; Kaer26608-Lys - endolysin not encapsulated; LipoKaer26608-Lys - encapsulated endolysin; EL - empty liposomes. Assay performed in triplicate.

Table 6
Antibacterial effect of purified and encapsulated endolysins against *K. pneumoniae*, *P. mirabilis*, *P. aeruginosa*, *Streptococcus* spp. and *Enterococcus* spp. strains.

	LipoKaer26608-Lys	Kaer26608-Lys	LipoPs3418-Lys	Ps3418-Lys	LipoKp2948-Lys	Kp2948-Lys
Streptococcus spp.	No	No	No	No	No	No
Enterococcus spp.	No	No	No	No	No	No
Proteus mirabilis	No	No	No	No	No	No
Klebsiella pneumoniae 4885	Yes (72 h; 1.56 µg/mL)	No	Yes (72 h; 6.25 µg/mL)	No	Yes (72 h; 6.25 µg/mL)	No
Klebsiella pneumoniae 4867	Yes (72 h; 6.25 µg/mL)	No	Yes (72 h; 3.13 µg/mL)	No	Yes (72 h; 6.25 µg/mL)	No
Klebsiella pneumoniae 4859	Yes (72 h; 6.25 µg/mL)	No	Yes (72 h; 6.25 µg/mL)	No	Yes (48 h; 3.13 µg/mL)	No
Pseudomonas aeruginosa	Yes (72 h; 3.13 µg/mL)	No	Yes (72 h; 3.13 µg/mL)	No	n.d	n.d

Yes = With activity; No = Without activity; n.d = not done. For the endolysins, purified or encapsulated, with activity is said the point of time and concentration most effective.

controlled manner (Ferreira et al., 2021). Liposomal formulations have already been successfully used against *K. pneumoniae* for delivery of the antibiotic ciprofloxacin, both *in vitro* and *in vivo*. The administration of liposomes encapsulating ciprofloxacin to rats infected with *K. pneumoniae*, also showed a slow release and a prolonged concentration of antibiotic in the blood and infected tissues (Bakker-Woudenberg et al., 2001).

Recently, LysG24 and LysCA endolysins, from *K. pneumoniae* phage vB_KpnS_MK54, showed a strong antibacterial effect against *K. pneumoniae*, using EDTA as an outer membrane permeabilizer (Lu et al., 2022). They succeeded in showing a reduction of 2.1 log values and 2.5 log values of the *K. pneumoniae* concentration using 100 μg/mL of the recombinant endolysins. In the present work we have observed a similar reduction grade for all three encapsulated endolysins (Kp2948-Lys, Ps3418-Lys and Kaer26608-Lys) but using a considerable smaller concentration of endolysin (6.25 μg/mL against the *K. pneumoniae* strain 4855). This suggests the efficacy of delivery of the liposomal formulation used and the potential of these endolysins that are being studied.

To evaluate the specificity of the encapsulated endolysins, different *K. pneumoniae* strains and different bacterial genera/species were used to test the effective concentration observed (3.13 μg/mL) in the liposome incorporated endolysins. The endolysin Kp2948-Lys led to a reduction in the cell viability in *K. pneumoniae* strains, but not in the other tested genera/species, suggesting that endolysin Kp2948-Lys is species specific. Whether *K. pneumoniae* strain were ESBL-producer alone (strains Kp4855, Kp4859, Kp4867), or co-producing a carbapenemase (strain Kp4885), did not appear to change concentration of encapsulated endolysin to produce an antibacterial effect, except for the encapsulated endolysin Kaer26608-Lys. For this encapsulated endolysin the amount needed to kill was a quarter or half of the amount in the case of presence of carbapenemase enzymes. This suggests that the resistance mechanism involving carbapenemase production increases the required amount of endolysin. Endolysin specificity has been corroborated by others. For instance, purified BSP16Lys endolysin, from *Salmonella* bacteriophage BSP16, encapsulated in liposomes with a lipid composition DPPC, Chol and hexadecylamine (at a ratio of 8:2:1) showed antimicrobial activity against *Salmonella* Typhimurium (Bai et al., 2019). Also, the recombinant endolysin EG-LYS, from the *Enterococcus faecalis* bacteriophage phiM1EF2, had growth inhibitory efficacy on *E. faecalis* strains significantly higher than that observed in the other bacterial species tested (Matsui et al., 2021).

Endolysins Ps3418-Lys and Kaer26608-Lys (isolated from *P. stuartii* and *K. aerogenes* prophages) appear to have a broader antibacterial spectrum, as demonstrated by their antibacterial activity against the *K. pneumoniae* strains and *P. aeruginosa*. Even that with a broader spectrum of action, the spectrum is not total, since these endolysins did not show activity against the Gram-positive *Streptococcus* spp. and *Enterococcus* spp., as well as to the Gram-negative *P. mirabilis*. The specificity overall is not yet entirely conclusive, as further testing will still need to be done to certify this conclusion.

The specificity of endolysins is often determined by the cell wall-binding domains that recognize the connection points in the substrate of the bacteria's cell wall (Murray et al., 2021). This high specificity for certain bacteria limits their antimicrobial action. Thus, also greatly reduces the risk of resistant strain development, often associated with the use of antibiotics with a larger range. The specific killing of the target pathogens ensures that the microflora is not affected (Abdelrahman et al., 2021). Gram-negative bacteria do not require a specific link for endolysins to bind due to the lack of the C-terminal cell-wall-binding domain. Accordingly, we could not find any recognition domain within the primary structure of the tested endolysins (determined by using BLASTp). They do not require this host protective measure since they are naturally protected by the outer membrane. Thus, often Gram-negative endolysins are composed of a single catalytic domain. Therefore, Gram-negative endolysins tend to have a broader range of targets (Murray et al., 2021), as observed here for Ps3418-Lys and Kaer26608-Lys. Although only a single catalytic domain was found in the Gram-negative *K. pneumoniae* specific endolysin Kp2948-Lys it still appears to be specific, with an undetermined mechanism of specificity. Both the outer membrane composition and peptidoglycan structure may contribute to specificity, either by liposome inability to cross the outer membrane, or by peptidoglycan structure not recognized by the endolysin. Our results points to the former as Kp2948-Lys was able to digest other species peptidoglycan in a zymogram assay.

Prophages constitute a huge and still poorly underexplored source of bioproducts, namely endolysins, despite the large amount of available sequences. The results reported here clearly demonstrate that liposomes are useful to overcome the outer bacterial membranes. Moreover, their use may be important not only for endolysin delivery but also for other compounds delivery, namely antibiotics in Gram-negative bacteria. The structure of liposomes similar to biological membranes, associated to fusogenic properties allows the delivery of loaded endolysins by increasing the interaction with peptidoglycan (Lee and Thompson, 2017). Liposomes can also allow controlled, gradual, and sustained released of the component during circulation just as seen in the cellular metabolic activity assay, this optimization is considered an important factor for therapeutic efficacy of liposomal systems (Lee and Thompson, 2017). The applicability of encapsulated endolysins span the multiples fields of the One Health approach, targeting an integrative and unified health for humans, animals, and environment.

In conclusion, this strategy of endolysin incorporated in liposomes is highly promising and may contribute to the fight against *K. pneumoniae* infection as well as other resistant *Enterobacteriaceae*. Future work should include *in vitro* studies to access the toxicity of the liposomal formulations against human cells, and tests *in vivo* using these endolysins encapsulated in liposomes.

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CRediT authorship contribution statement

Tiago Gonçalves: Formal analysis, Investigation, Methodology. **Andreia T. Marques:** Formal analysis, Investigation, Methodology, Writing – original draft. **Vera Manageiro:** . **Luis Tanoeiro:** . **Joana S. Vital:** Investigation, Writing – review & editing. **Aida Duarte:** Data curation, Validation, Writing – review & editing. **Jorge M.B. Vítor:** Validation, Writing – review & editing. **Manuela Caniça:** Data curation, Validation, Writing – review & editing. **Maria Manuela Gaspar:** Conceptualization, Investigation, Writing – review & editing. **Filipa F. Vale:** Conceptualization, Investigation, Funding acquisition, Supervision, Validation, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.

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