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(54) **PLANTARICIN NC8ALPHABETA VARIANTS**

PLANTARICIN NC8ALPHA-BETA VARIANTEN

VAIANTS DE PLANTARICINE NC8ALPHABÊTA

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Description

Field of the Invention

[0001] This invention pertains in general to the field of treatment of infections. More particularly the invention relates to a use of a pharmaceutical composition comprising bacteriocin PLNC8 αβ for the prevention and/or treatment of infections wherein the pharmaceutical composition further comprises at least one antibiotic.

Background of the Invention

[0002] Hospital-acquired infection (HAI), also known as a nosocomial infection, is an infection that is acquired in a hospital or other health care facility. Such an infection can be acquired in hospital, nursing home, rehabilitation facility, outpatient clinic, or other clinical settings. Infection is spread to the susceptible patient in the clinical setting by various means. Health care staff can spread infection, in addition to contaminated equipment, bed linens, or air droplets. It is estimated that 6 million patients in the EU and USA contract a HAI per year, resulting in up to 150 000 deaths annually. Prevention of HAI often includes hospital sanitation protocols regarding uniforms, equipment sterilization, washing, and other preventive measures. Thorough hand washing and/or use of alcohol rubs by all medical personnel before and after each patient contact is one of the most effective ways to combat nosocomial infections. More careful use of antimicrobial agents, such as antibiotics, is also considered vital.

[0003] Among the categories of bacteria most known to infect patients are the ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter*), including MRSA (Methicillin-resistant *Staphylococcus aureus*) and VRE (Vancomycin-resistant *Enterococcus*), *Streptococcus spp* and *Escherichia coli.* Development of new effective antimicrobial strategies in the treatment of infections caused by antibiotic-resistant bacteria presents one of the major challenges in medicine today. Since most infections are caused by pathogens that live protected in complex biofilms, antibacterial substances need a good ability to penetrate or dissolve biofilm. Such ability is usually limited/lacking in traditional antibiotics, which must therefore be compensated with very high concentrations, often 100-1000 times higher than the doses required for bactericidal effects on planktonic bacteria. This overdose contributes to accelerated development of antibiotic resistance and severe cytotoxic effects. Furthermore, infections are often associated with high proteolytic activity caused by both bacteria and the body's immune system, which means that antimicrobial agents may quickly become inactivated.

[0004] Hazem *et al.,* 2016 (https://doi.org/10.1186/s12866-016-0810-8*)* describes antibacterial effects of Lactobacillus and bacteriocin PLNC8 alpha beta on the periodontal pathogen *Porphyromonas gingivalis.* Torbjörn et al., 2017 (https://doi.org/10.1093/femspd/ftx064) describes a dual action of bacteriocin PLNC8 αβ through inhibition of *Porphyromonas gingivalis* infection and promotion of cell proliferation.

[0005] It is known that bacteriocins constitute a promising potential alternative or complement to traditional antibiotics and have several advantages such as low risk of resistance development, limited effects on normal flora and beneficial effects on human tissue. Bacteriocins are a group of bacterially produced peptides used to fight other bacteria. Bacteriocins may have a net positive charge and express amphipathic structures that interact with negatively charged microbial membranes and kill microbes usually through pore-forming mechanisms. These mechanisms are more difficult to evade by developing resistance, compared to metabolic enzymes, which usually are targets for conventional antibiotics.

[0006] Thus, there is a need for alternative method of antibiotic therapy in the prevention or treatment of bacteria and bacterial infections, especially spread of antibiotic resistance in health care (e.g. methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-resistant *Staphylococcus epidermidis* (MRSE), and vancomycin-resistant *Enterococcus* (VRE)).

Summary of the Invention

[0007] Accordingly, the present invention preferably seeks to mitigate, alleviate or eliminate one or more of the above-identified deficiencies in the art and disadvantages singly or in any combination and solves at least the above mentioned problems by providing a pharmaceutical composition comprising a first and a second peptide, wherein the first peptide is a peptide of the bacteriocin PLNC8 αβ, wherein the peptide of the bacteriocin PLNC8 αβ is SVPTSVYTLGIKIL WSA YKHRKTIEKSFNKGFYH SEQ ID NO 2, and wherein the second peptide is DLTTKLWSSWGYYLGKKARWNL SEQ ID NO 31, and wherein the pharmaceutical composition further comprises at least one antibiotic.

[0008] The peptide(s) and the antibiotic act synergistically and enhance the effect of each other.

[0009] Provided is a pharmaceutical composition wherein at least 90% of the amino acids in the first peptide and/or second peptide are D-amino acid residues.

[0010] Such peptides are more stable and less sensitive to proteolytic cleavage compared to their corresponding L-variants.

[0011] Provided is a pharmaceutical composition wherein the antibiotic is selected from the group consisting of antibiotics that inhibit bacterial cell wall synthesis, antibiotics that inhibit nucleic acid synthesis and antibiotics that inhibit protein synthesis.

[0012] Provided is a pharmaceutical composition for use in the treatment or prophylaxis of a bacterial infection.

[0013] Also provided is the use of a pharmaceutical composition in coating at least part of a device to limit colonization of bacteria on the surface of the device.

Brief Description of the Drawings

[0014] These and other aspects, features and advantages of which the invention is capable of will be apparent and elucidated from the following description of embodiments of the present invention, reference being made to the accompanying drawings, in which

Figure 1 shows PLNC8 αβ markedly inhibits the growth and survival of different strains of *S. aureus* and *S. epidermidis.* Different *Staphylococcus* species were cultured for 20 h in the presence of increasing concentrations of PLNC8 αβ (1:1). *S. epidermidis* was generally more susceptible to PLNC8 αβ than *S. aureus.* Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for *Staphylococcus* species in response to PLNC8 αβ.

Figure 2. The molar ratio of PLNC8 α and PLNC8 β is critical for optimal antimicrobial activity. *S. epidermidis* ATCC 12228 was exposed to different molar ratios of PLNC8 α and β for 20 h. A molar ratio of 1:1 between PLNC8 α and PLNC8 β is most efficient at inhibiting and killing *S. epidermidis.* Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for different molar ratios of PLNC8 α and β. *The highest total concentration of the peptides was kept constant at 50 μM, while the concentrations of PLNC8 α and β individually were altered to obtain different molar ratios.

Figure 3. PLNC8 αβ is effective at disrupting *S. epidermidis* biofilms. The biofilm positive strain *S. epidermidis* RP62A was allowed to form biofilms followed by removal of suspended bacteria and then incubation with PLNC8 αβ, PLNC8 α or PLNC8 β for 1 h. A-Absorbance measurement of detached biofilms. B- Crystal violet staining of the remaining attached biofilms. PLNC8 αβ is most efficient and rapid at disrupting biofilms of *S. epidermidis.*

Figure 4. Membrane disrupting and a antimicrobial activity of PLNC8 α and β with L- or D-amino acids. A- CF release was recorded after exposure of liposomes with increasing concentrations of L- or D-variants of PLNC8 α, β or αβ (1:1). B- *S. epidermidis* ATCC 12228 was incubated with increasing concentrations of PLNC8 α and β, alone or in combination (1:1), for 20 h. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for PLNC8 α, β and αβ are indicated.

Figure 5. The L- and D-variant of PLNC8 αβ rapidly permeabilize the plasma membrane of *S. epidermidis.* The uptake of Sytox Green by *S. epidermidis* ATCC 12228 after treatment with 5μM of L-PLNC8 αβ, D-PLNC8 αβ or scrambled-PLNC8 αβ for 2 min, compared to untreated bacteria (C).

Figure 6. The D-form of PLNC8 αβ is more stable and less sensitive to proteolytic cleavage. The peptides (100μM) a) L-PLNC8α b) D-PLNC8α c) L-PLNC8β d) D-PLNC8β were treated with Trypsin (5μM) in Ammonium Bicarbonate buffer (50mM) for 16 h at 37°C before being acidified (2.5% TFA), dried, suspended in H2O +0.1% TFA, desalted (ZipTip) and analyzed by MALDI-ToF MS. Number above the peaks indicate molecular weights (Da) and number in brackets sequences of amino acids. Full-length α- and β-peptide, 1-29 and 1-34, respectively.

Figure 7. (A) Both the L- and D-form of PLNC8 αβ display a low hemolytic activity. Human erythrocytes were incubated with different concentrations (0.5-50 μM) of L- or D-variant of PLNC8 α, β or αβ (1:1) for 1 h. In (B) this is shown for truncated forms α1-15, α1-22, β7-20, β1-20, β7-34.

Figure 8. Amino acid sequences of truncated peptides of PLNC8 α and PLNC8 β.

Figure 9. Antimicrobial activities of truncated forms of PLNC8 β. In A. disruption of the membrane and release of (6)-carboxyfluorescein (CF) from liposomes was obtained with the β-peptides 1-34 (full-length), 7-34, 1-20 and 7-20. In B. when combined with a full length PLNC8 α peptide, effects were also obtained with the other truncated peptides, although at higher concentrations. In C, amino acid sequences of truncated peptides of *L*-PLNC8 β. In D, quantification of 50% CF release with truncated *L*-PLNC8 β, with and without *L*-PLNC8 α. In E, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of truncated PLNC8 β, in the absence or presence of full-length α-peptide, against *S. epidermidis* ATCC 12228. Growth of *S. epidermidis* was inhibited by the full-length β1-34, β7-34, β1-20 and β7-20.

Figure 10. Antimicrobial activities of truncated forms of PLNC8 α. (A) Release of (6)-carboxyfluorescein (CF) from liposomes was obtained with α1-22 and full-length α1-29. When combined with a full length PLNC8 β peptide, effects were also obtained with the other truncated peptides, although at higher concentrations. (C) Amino acid sequences of truncated peptides of *L*-PLNC8 α. (D) Quantification of 50% CF release by truncated *L*-PLNC8 α peptides, with and without *L*-PLNC8 β. (E) Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of truncated PLNC8 α against *S. epidermidis* ATCC 12228. Growth and survival of *S. epidermidis* was inhibited by

α1-29 and α1-22 in combination with the β-peptide.

Figure 11. Antimicrobial activity of a combination of truncated PLNC8 α and PLNC8 β. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of a combination of truncated PLNC8 α and PLNC8 β against *S. epidermidis* ATCC 12228. The inhibition of growth and survival of *S. epidermidis* by β1-20 and β7-20, respectively, was not further enhanced by a co-incubation with α1-22 or α1-15.

Figure 12. Morphological effects of PLNC8 αβ on *S. epidermidis* using TEM and SEM. PLNC8 α caused massive bleb formation and PLNC8 β induced bacterial lysis shown by an extracellular release of intracellular content. PLNC8 αβ was most efficient causing complete bacterial lysis. The truncated forms of PLNC8 β, β1-20 and β7-20, induced fragmentation of the bacterial cell wall and in combination with PLNC8 α *S. epidermidis* went through lysis.

Figure 13. PLNC8 αβ in a formula is effective against *S. epidermidis* and retains its activity after long-term storage. Bacterial lysis was visualized by studying the uptake of Sytox Green by *S. epidermidis* ATCC 12228 exposed to a gel containing different concentrations (5-100 μM) of PLNC8 αβ. The activity of the formula with 100 μM PLNC8 αβ was also tested on blood-agar plates with *S. epidermidis,* at time zero and after long-term storage at 4°C. A gradient of PLNC8 αβ was created by spreading the gel over the agar surface with a plastic loop. Inhibition of bacterial growth is demonstrated by the translucent areas.

Figure 14. PLNC8 αβ is effective against heterogeneous strains of *S. epidermidis. S. epidermidis* isolated from prosthetic joint infections, including heterogeneous glycopeptide intermediate *S. epidermidis* (hGISE), was exposed to L-PLNC8 αβ for 20 h and MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) were determined.

Figure 15. PLNC8 αβ acts synergistically with antibiotics. Synergistic antimicrobial effects between antibiotics and L- or D-PLNC8 αβ. *S. epidermidis* (strain 154) was exposed to L-PLNC8 αβ or D-PLNC8 αβ (3.1 μM), a serial dilution of teicoplanin, vancomycin, rifampicin and gentamicin, alone or in their combination with 3.1 μM L-PLNC8 αβ or D-PLNC8 αβ.

Figure 16. Synergistic antimicrobial effects between teicoplanin and PLNC8 αβ. PLNC8 β and truncated PLNC8 α markedly amplify the inhibitory effects of teicoplanin against *S. epidermidis. S. epidermidis* (strain 154) was exposed to a serial dilution of teicoplanin or full-length/truncated PLNC8 αβ alone or in their combination (serial dilution of teicoplanin and 6.25 μM full-length/truncated PLNC8 αβ),

Figure 17. PLNC8 αβ markedly permeabilizes and kills different species of *Streptococcus. Streptococcus spp* (*S. mutans* (Sm), *S. constellatus* (Sc) and *S. anginosus* (Sa)) were treated with 5μM PLNC8 αβ for 2 min, followed by analysis of uptake of Sytox Green. *S. constellatus* and *S. anginosus* were more susceptible to PLNC8 αβ than *S. mutans,*

Figure 18. PLNC8 αβ causes rapid lysis of *S. aureus,* independent of their resistance to antibiotics. (A) Dose-dependent increase in bacterial lysis, as indicated by Sytox green staining, following exposure to PLNC8 αβ for 5 min. (B) MIC and MBC values for MSSA and MRSA are indicated. (C) Time-kill assay indicated that PLNC8 αβ rapidly kills the bacteria in a dose-dependent manner,

Figure 19. PLNC8 αβ promotes wound healing of human keratinocytes. HaCaT cells were exposed to different concentrations of PLNC8 αβ or MSSA for 24h. (A) PLNC8 αβ promoted wound healing, which was determined *in vitro* using scratch assay. While (B) IL-6 and (C) CXCL8 were increased by *S. aureus,* these inflammatory mediators were not altered by PLNC8 αβ,

Figure 20. PLNC8 αβ antagonizes *S.* aureus-mediated cytotoxicity and inflammatory responses of human keratinocytes. HaCaT cells were infected with MSSA for 1h followed by addition of PLNC8 αβ for 6h. (A) PLNC8 αβ antagonized *S. aureus*-mediated cytotoxicity, which was determined by LDH activity, and promoted cell viability. Secretion of (B) IL-6 and (C) CXCL8 were significantly reduced by the peptides. (D) Gene expression analysis of *il-6* and *cxcl8* confirmed the effects of PLNC8 αβ by preventing infection of keratinocytes by *S. aureus.* Furthermore, intracellular signaling events involves *c-jun* and *c-fos,* suggesting a role for the transcription factor AP-1 via MAPK,

Figure 21. PLNC8 αβ promotes wound healing and reduces inflammatory responses of human keratinocytes. HaCaT cells were infected with MSSA, in the presence or absence of PLNC8 αβ for 24h. (A) PLNC8 promotes wound healing following an infection with *S. aureus.* The increased secretion of (B) IL-6 and (C) CXCL8 by *S. aureus* was significantly reduced by the peptides,

Figure 22. PLNC8αβ inhibits infection and promotes wound healing *in vivo.* Wound healing was evaluated *in vivo* using a porcine wound healing model. Wounds were either left unexposed or infected with *S.aureus* (10^8CFU/ml) for 3 days. Gentamicin (100μg/ml) and/or PLNC8αβ (50μM) were added once every other day and the wounds were monitored for 7 days (a total of 4 doses). The peptide, alone or in combination with gentamicin, antagonized the infection and promoted wound healing,

Figure 23. Aggregation (dotted line) and ATP release (solid line) were recorded to determine bacterial lysis by L-PLNC8 αβ,

Figure 24. PLNC8 αβ causes rapid membrane permeabilization on liposomes. PLNC8 β and PLNC8 αβ (1:1), but not PLNC8 α, of both the (A) L-form and (B) D-form, caused complete lysis of liposomes after 2 min,

Figure 25. CD-spectroscopy of PLNC8 αβ. CD measurements of (A) *L*-PLNC8 αβ and (B) *D*-PLNC8 αβ (100μM each) without (dashed) and with (solid) liposomes (0.5mg/ml, ~660μM) in PBS. Three repeats with PBS as background. Liposome containing samples were incubated for at least 30 min prior to measurements,

Figure 26. IncuCyte live-cell analysis of infected keratinocytes, in the presence or absence of PLNC8 αβ. *S. aureus* MOI:1 caused cell death after 8 h. A single dose of PLNC8 αβ prevented bacterial growth and protected the cells for up to 32 h. The combination PLNC8 αβ/gentamicin (5μg/ml) efficiently eliminated *S. aureus* and prevented an infection, and subsequent cell death, over the entire experimental period (72 h), and

Figure 27. IncuCyte live-cell analysis of infected keratinocytes, in the presence or absence of PLNC8 αβ. A- *S. aureus* MOI:0.1 caused cell death after 10 h. A single dose of PLNC8 αβ prevented bacterial growth and protected the cells for up to 42 h. The combination PLNC8 αβ/gentamicin (5μg/ml) efficiently eliminated *S. aureus* and prevented an infection, and subsequent cell death, throughout the entire experimental period (72 h). B- Bacterial growth, measured by quantifying GFP fluorescence of the bacteria, reached maximum levels after 8-9 h. PLNC8 αβ prevented and delayed bacterial growth up to 38 h, and the combination PLNC8 αβ/gentamicin efficiently eliminated all the bacteria.

Description of embodiments

[0015] The following description focuses on an embodiment of the present disclosure applicable to combating infection, and especially Hospital-acquired infection (HAI) (but also other types of infections) using peptides derived from a *L. plantarum* NC8 bacteriocin used together with antibiotics. However, it will be appreciated that the disclosure is not limited to this application but these peptides may be applied to many other uses, including for example disinfection and coating of surfaces.

[0016] There are several problems associated with combating infections, such as Hospital-acquired infection (HAI). These include: Inadequate treatment strategies for many severe and serious bacterial infections; Development and spread of antibiotic resistance in health care (e.g. methicillin-resistant *Staphylococcus aureus* (MRSA) and *Staphylococcus epidermidis* (MRSE)); Large costs for society for prevention and treatment of infectious diseases (e.g. annual hospital costs of treating healthcare-associated infections (HAIs) in US is estimated to 40 billion dollar and in Sweden to 6.5 billion SEK): Human suffering from infectious diseases (annually approx. 6 million patients with HAIs in US and EU and 150 000 die).

[0017] These problems are not trivial to approach, since they include aspects such as intractable infections in the form of biofilms, high proteolytic activity in infections antagonizing the action of antibacterial agents, limited stability and activity of antibacterial agents, chronic infection and inflammation, and slow and complicated wound healing.

[0018] It was envisaged by the present inventors that specific *Lactobacillus* species indeed may be able to contribute to solving these problems, from its ability to suppress pathogens primarily through expression and secretion of certain bacteriocins.

[0019] *Lactobacillus plantarum* is a highly versatile lactic acid bacterium found in saliva and gastrointestinal tract as well as fermented vegetables, meat and dairy products. *L. plantarum* NC8 has been used as a model strain in many laboratories worldwide, and is a naturally plasmid-free *L. plantarum* strain. *L. plantarum* NC8 has previously been shown to produce a two-peptide bacteriocin, PLNC8 αβ, classified as a class IIb bacteriocin. The inventors have previously shown that PLNC8 αβ is efficient against the periodontal pathogen *Porphyromonas gingivalis* and stimulates cell proliferation (1,2).

[0020] The idea of the disclosure is to exploit the antibacterial effects of bacteriocin PLNC8αβ, unmodified or truncated, in soluble or immobilized form, together with antibiotics, for the prevention and treatment of acute and chronic infections, such as periodontitis, wound infections, implant-associated infections and urinary tract infections. Products based on bacteriocins in conjunction with traditional antibiotics can be of enormous importance in health care, with improved public health and a positive impact on the social economy.

[0021] Since development and spread of antibiotic resistance in health care primarily concerns methicillin-resistant *Staphylococcus aureus* (MRSA) and *Staphylococcus epidermidis* (MRSE), the effect of PLNC8αβ on different strains of *S. aureus* and *S. epidermidis* were studied. As can be seen in Fig. 1 and Table 1, PLNC8αβ markedly inhibited the growth and the survival of all bacterial strains (Fig. 1).

Table 1

Bacteria	Characteristics
***S. aureus* ATCC 29213 (MSSA)**	Methicillin sensitive
***S. aureus* CCUG 35601 (MRSA)**	Methicillin resistant
***S. epidermidis* ATCC 12228**	Biofilm negative

(continued)

***S. epidermidis* RP62A**	Biofilm positive
***S. epidermidis* N15**	Isolated from nose of a healthy individual
***S. epidermidis* 117**	Isolated from an infected hip joint prosthesis

L-PLNC8 αβ	MIC	MBC
***S. aureus* ATCC 29213 (MSSA)**	12.5	25
***S. aureus* CCUG 35601 (MRSA)**	12.5	25
***S. epidermidis* ATCC 12228**	6.25	12.5
***S. epidermidis* RP62A**	6.25	6.25
***S. epidermidis* N15**	6.25	6.25
***S. epidermidis* 117**	12.5	12.5

[0022] Further, it was probed if the antimicrobial effect could be enhanced using combination therapy. In combination therapy, combinations of antimicrobial agents are utilized for the prevention of the development of resistance and to shorten the length of treatment time. It was investigated whether combinations of PLNC8 αβ together with different traditional antibiotics would be effective in the treatment of *S. epidermidis.* In Fig. 15, results are summarized for PLNC8 αβ together with rifampicin, vancomycin, gentamicin or teicoplanin. Here it was surprisingly found that PLNC8 αβ decreased MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) of teicoplanin more than 15-fold against *S. epidermidis* (Fig. 15). A combination of PLNC8 αβ and rifampicin was found to be even more effective.

[0023] MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) of rifampicin was lowered more than 100-fold when treating *S. epidermidis* in the presence of L-PLNC8 αβ or D-PLNC8 αβ. Furthermore, L-PLNC8 αβ decreased MIC and MBC of gentamicin 15-30-fold against *S. epidermidis.* L-PLNC8 αβ or D-PLNC8 αβ lowered MIC and MBC of vancomycin 2-fold. (Fig. 15 and Table 2).

Table 2. Antimicrobial effect is enhanced using PLNC8 αβ combination therapy.

	***S. epidermidis* ATCC 12228**	
Antimicrobial agent	**MIC**	**MBC**
L-PLNCβ αβ (μM)	6.25	12.5
D-PLNC8 αβ (μM)	12.5	12.5
Vancomycin (μg/ml)	1.5	3.1
Vancomycin/*L*-PLNC8 αβ	0.78	1.5
Vancomycin/*D*-PLNC8 αβ	0.78	1.5
Teicoplanin (μg/ml)	1.5	1.5
Teicoplanin/*L*-PLNC8 αβ	<0.097	<0.097
Teicoplanin/*D*-PLNC8 αβ	<0.097	<0.097
Rifampicin (μg/ml)	0.25	0.5
Rifampicin/*L*-PLNC8 αβ	<0.0019	<0.0019
Rifampicin/*D*-PLNC8 αβ	0.0019	0.0019
Gentamicin (μg/ml)	0.31	0.31
Gentamicin/*L*-PLNC8 αβ	<0.0097	<0.0097
Gentamicin/*D*-PLNC8 αβ	<0.0097	<0.0097

[0024] This showed a surprisingly strong synergistic effect, with up to hundred-fold decrease of MIC and MBC of the antibiotic against specific bacteria. Without being bound to theory, this may be due to the membrane permeabilizing effect of PLNC8 αβ, which may damage bacterial membranes and thus facilitate passage for the antibiotics, which thus

more easily reach their intracellular targets (e.g., ribosomes, RNA polymerase). The consequence is that the concentration of antibiotics can be significantly lowered with reduced problems of both antibiotic resistance and cytotoxic side effects.

[0025] The membrane permeabilizing effect is shown in figure 23, where L-PLNC8 αβ effectively lyses *S. epidermidis,* which is demonstrated by a dose-dependent release of ATP. It is also demonstrated that bacteria aggregates when exposed to low concentrations of L-PLNC8 αβ. Also, in figure 24, it is shown that that PLNC8 αβ causes rapid membrane permeabilization of liposomes. PLNC8 β and PLNC8 αβ (1:1), but not PLNC8 α, of both the *L*-form and *D*-form, caused complete lysis of liposomes after 2 min.

[0026] The synergistic antimicrobial effect between PLNC8 αβ and traditional antibiotics against resistant strains of *Staphylococcus* is shown in table 3 below. Here the effects of vancomycin or teicoplanin combined with *L*-PLNC8 αβ against methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant *Staphylococcus epidermidis* (MRSE) are shown.

[0027] In figure 25, CD-spectroscopy shows that both *L*- and D-PLNC8 αβ has an ordered secondary structure in liposomes, which indicates that the α-helices are arranged in a definite order for the peptide to be active.

Table 3. Synergistic antimicrobial effect between PLNC8 αβ and traditional antibiotics against resistant strains of *Staphylococcus.*

			S. epidermidis					
	MRSA		**154**		**126***		**157***	
Antimicrobial agent	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
L-PLNC8 αβ (μM)	12.5	25	6.25	12.5	12.5	50	12.5	>50
Vancomycin (μg/ml)	1.5	3.1	1.5	3.1	3.1	3.1	6.25	6.25
Vancomycin/ *L*-PLNC8 αβ (10μM)	<0.097	0.39	<0.097	<0.097	<0.097	0.78	6.25	6.25
Vancomycin/ *L*-PLNC8 αβ (5μM)	<0.097	0.78	<0.097	<0.097	<0.097	0.78	6.25	6.25
Teicoplanin (μg/ml)	0.78	3.1	1.5	1.5	3.1	6.25	12.5	25
Teicoplanin/ *L*-PLNC8 αβ (10μM)	<0.097	0.39	<0.097	<0.097	<0.097	0.39	12.5	25
Teicoplanin/ *L*-PLNC8 αβ (5μM)	<0.097	0.78	<0.097	<0.097	<0.097	0.39	12.5	25

*hGISE strains

[0028] This shows that combination therapy with PLNC8 αβ and antibiotics is an efficient treatment strategy. This was further shown during trials using ESKAPE pathogens and *Escherichia.coli,* one of the leading causes of nosocomial infections throughout the world. The acronym ESKAPE includes six pathogenic bacterial species (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacte rbaumannii, Pseudomonas aeruginosa* and *Enterobacter*). These bacteria have become resistant to multiple antibiotics and are associated with higher rates of morbidity and mortality, indicating the need for new strategies to prevent and treat these types of infections. ESKAPE pathogens are prioritized by WHO to promote research and development of new antimicrobials, since multidrugresistance is a serious threat to global public health. Infections caused by these pathogens are often hospital-acquired, and pose a particular threat to patients requiring medical devices, such as catheters, ventilators and implants.

[0029] As can be seen in table 4, PLNC8αβ alone does not affect the growth of *E. coli,* however a sub-MIC concentration of the peptides significantly enhanced the effects of different antibiotics.

[0030] Similarly, PLNC8αβ alone is both inhibitory and bactericidal against *Enterococcus faecium,* and addition of sub-MIC concentrations significantly enhanced the effects of different antibiotics. This is shown in table 5 below.

[0031] Also, in table 6, it is shown that although PLNC8αβ alone does not affect the growth of *Pseudomonas aeruginosa,*

addition of sub-MIC concentration of the peptides enhanced the effects of different antibiotics.

Table 4. PLNC8αβ markedly enhances the inhibitory and bactericidal effects of antibiotics against *Escherichia coli*

	Non-ESBL *E. coli*		**Non-ESBL *E. coli***		**ESBL-producing *E. coli***		**ESBL-producing *E. coli***	
Antimicrobialagent	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
PLNC8αβ (μM)	>50	>50	>50	>50	>50	>50	>50	>50
Gentamicin	1.56	1.56	1.56	3.125	>50	>50	3.125	6.25
Gentamicin/PLNC8α β*	0.78	0.78	0.195	0.195	>50	>50	<0.09 7	<0.09 7
Rifampicin	6.25	12.5	6.25	25	12.5	12.5	12.5	12.5
Rifampicin/PLNC8α β*	0.78	1.56	1.56	1.56	<0.097	0.195	<0.09 7	0.195
Ciprofloxacin	0.012 5	0.012 5	0.006 25	0.006 25	>0.1	>0.1	>0.1	>0.1
Ciprofloxacin/PLNC 8αβ*	0.012 5	0.012 5	0.000 78	0.001 5	<0.000 19	<0.000 19	0.000 78	0.000 78
Teicoplanin	>50	>50	>50	>50	>50	>50	>50	>50
Teicoplanin/PLNC8α β*	>50	>50	>50	>50	<0.097	<0.097	<0.09 7	<0.09 7
*Peptide concentration in combination with antibiotics is 10 μM								

Table 5. PLNC8αβ markedly enhances the inhibitory and bactericidal effects of antibiotics against *Enterococcus faecium.*

	E. faecium		***E.* faecium**		**Vancomycin resistant *E.* faecium (VRE)**	
Antimicrobialagent	MIC	MBC	MIC	MBC	MIC	MBC
PLNC8αβ (μM)	6.25	6.25	3.1	6.25	3.1	25
Gentamicin	50	>50	50	50	>50	>50
Gentamicin/PLNC8αβ*	3.1	3.1	1.5	1.5	>50	>50
Rifampicin	>50	>50	25	>50	<0.097	3,1
Rifampicin/PLNC8αβ*	50	>50	12,5	25	<0.097	<0.097
Ciprofloxacin	>50	>50	>50	>50	>50	>50
Ciprofloxacin/PLNC8αβ*	<0.097	<0.097	<0.097	<0.097	<0.097	25
Teicoplanin	**>**50	>50	0,39	25	0.39	50
Teicoplanin/PLNC8αβ*	>50	>50	<0.097	<0.097	<0.097	0.39
*Peptide concentration in combination with antibiotics is 1.5 μM						

Table 6. PLNC8αβ markedly enhances the inhibitory and bactericidal effects of antibiotics against *Pseudomonas aeruginosa.*

	P. aeruginosa		***P.* aeruginosa**	
Antimicrobialagent	MIC	MBC	MIC	MBC
PLNC8αβ (μM)	>50	>50	>50	>50
Levofloxacin	1	2	4	4

(continued)

	P. aeruginosa		*P.* aeruginosa	
Antimicrobialagent	MIC	MBC	MIC	MBC
Levofloxacin/PLNC8αβ*	0.25	0.25	0.013	0.12
Meropenem	25	50	25	25
Meropenem/PLNC8αβ*	0.19	0.39	0.39	0.78
Ciprofloxacin	0.25	0.25	0.016	0.016
Ciprofloxacin/PLNC8αβ*	0.5	1	0.016	0.063
*Peptide concentration in combination with antibiotics is 15 μM				

[0032] Many hospital-acquired bacterial infections are found in superficial infections and severe infections associated with chronic wounds and insertion of medical devices, including catheters and prosthetic joint implants. This may subsequently increase the risk for development of life-threatening conditions, such as sepsis.

[0033] Keratinocytes constitute the predominant cell type in the epidermis. Although the primary function of keratinocytes is forming a physical barrier against microorganisms, these cells also participate in the initiation of an inflammatory response against invading microorganisms.

[0034] In figure 18, it is shown that PLNC8αβ is inhibitory and bactericidal against *S.aureus,* independent of their irresistance patterns against antibiotics.

[0035] In figure 19, It is shown that PLNC8 αβ promotes wound healing of human keratinocytes, which was determined *in vitro* using scratch assay. While *S. aureus* increased IL-6 and CXCL8, these inflammatory mediators were not altered by PLNC8 αβ.

[0036] In figure 20, it is shown that the peptides efficiently counteract the cytotoxic and inflammatory effects of *S.aureus* on human keratinocytes.

[0037] The infected cells are damaged which leads to increased secretion of IL-6 and CXCL8. Here, a significant reduction of these secretions can be attributed to the peptides. In figure 21, it is shown that PLNC8 promotes wound healing following an infection with *S. aureus.*

[0038] Using a porcine wound healing model, it was also shown that PLNC8αβ inhibits infection and promotes wound healing *in vivo.* In figure 22, the peptide, alone or in combination with gentamicin, antagonized the infection by *S. aureus* and promoted wound healing.

[0039] In figure 26, IncuCyte live-cell analysis of keratinocytes infected by *S. aureus,* (MOI: 1) in the presence or absence of PLNC8 αβ. A single dose of PLNC8 αβ prevented bacterial growth and protected the cells for up to 32 h. Bacterial growth without peptides reached maximum levels after 8-9 h. The combination PLNC8 αβ/gentamicin (5μg/ml) efficiently eliminated *S. aureus* and prevented an infection, and subsequent cell death, over the entire experimental period (72 h).

[0040] Figure 27. IncuCyte live-cell analysis of keratinocytes infected by *S. aureus,* (MOI:0.1) in the presence or absence of PLNC8 αβ. A single dose of PLNC8 αβ prevented bacterial growth and protected the cells for up to 42 h. Bacterial growth without peptides reached maximum levels after 10 h. The combination PLNC8 αβ/gentamicin (5μg/ml) efficiently eliminated *S. aureus* and prevented an infection, and subsequent cell death, throughout the entire experimental period (72 h).

[0041] Another aspect of bacterial defense against antibiotics is the formation of bacterial biofilms. The bacterial biofilms seem to create resistance to antibiotics, disinfectant chemicals and to phagocytosis and other components of the innate and adaptive inflammatory defense system. As such, it is vital that a treatment can combat the formation of bacterial biofilms but also disrupt an already existing biofilm.

[0042] Therefore, the bacteriocins were not only tested using bacteria in a planktonic state, but also using biofilms consisting of *S. epidermidis.* It was found that PLNC8 αβ efficiently disrupted the biofilms and killed the bacteria (shown in figure 3). Also, the α and β peptide of PLNC8 exerted by themselves, although at higher concentrations, disruptive effects on the biofilms.

[0043] A biofilm is a structured consortium of bacteria embedded in a self-produced polymer matrix consisting of polysaccharides, protein and extracellular DNA. Gradients of nutrients and oxygen exist from the top to the bottom of biofilms and the bacterial cells located in nutrient poor areas have decreased metabolic activity and increased doubling times. These more or less dormant cells are therefore responsible for some of the tolerance to antibiotics. Thus, it is of importance that the antimicrobial agent can penetrate the biofilm to expose the biofilm bacteria to the antibiotic or antimicrobial agent and there exert antibacterial effect.

[0044] However, indications are that e.g. *Staphylococcus* biofilms are not totally impervious to antibiotics, and certain fluorescently tagged antimicrobials (such as daptomycin) have been shown to penetrate the biofilms of *S. aureus* and *S. epidermidis* by diffusion.

[0045] In the disclosure, it was hypothesized that the biofilm penetration of PLNC8 αβ could be increased through modifying the bacteriocins. Thus, truncated forms of PLNC8 αβ were developed. These shortened forms of the bacteriocins diffuse more rapidly into the biofilm due to their limited size. It was then investigated whether these truncated forms express antibacterial activities similar to the native bacteriocin or if they are even more effective.

[0046] Truncated peptides of PLNC8 α and PLNC8 β, respectively, were constructed in sequences of 6-7 amino acids, to correspond to the number of amino acids required for formation of an alpha helix (shown in Fig. 8). The effects of truncated PLNC8 α and PLNC8 β were tested on both a liposome system (resembling bacteria) and on *S. epidermidis.* Disruption of the liposome membranes, revealed by release of (6)-carboxyfluorescein (CF), was obtained with the β-peptides 1-34 (full-length), 7-34, 1-20 and 7-20 (Fig. 9). When combined with a full length PLNC8 α peptide, effects were also obtained with the other truncated peptides, although at higher concentrations. As such, it was surprisingly found that growth of *S. epidermidis* was most efficiently inhibited by sequence β1-20 and β7-20, respectively, and these truncated peptides were as effective, or even more effective, than the full-length native PLNC8 β (1-34) (Fig. 9).

[0047] It was further found that the peptide β-sequences β7-13 and β14-20 are crucial for the effects of PLNC8 β and are more efficient when combined with β1-6. Thus, the peptide β1-20 is most effective in inhibiting *S. epidermidis.* Furthermore, it was found that the effects of β1-20 and β7-20 were not further enhanced in combination with the full-length α-peptide.

[0048] The antimicrobial activities of truncated forms of PLNC8 α were further probed, as shown in Fig. 10 and Table 2. The truncated form 1-22 of the α-peptide and the full-length α-peptide (1-29) disrupted the membrane of the liposomes, revealed by a release of carboxyfluorescein. In combination with the β-peptide, α1-22 exerted inhibitory and bactericidal effects on *S. epidermidis* (Fig. 10).

[0049] A combination of truncated α1-22 or α1-15 with β1-20 or β7-20 and the effect on MIC and MBC against *S. epidermidis* is shown in figure 11. α1-22 and α1-15 did not further enhance the inhibitory effects of β1-20 and β7-20.

[0050] As such, the innovation pertains to a combination of PLNC8 αβ and antibiotics for synergistic effects, with several fold decrease of MIC and MBC of the antibiotic against specific bacteria. Furthermore, by truncating PLNC8 αβ) to shorter α and β peptides (e.g. α1-22, β1-20 and β7-20), synergistic antibacterial properties are retained, while higher diffusion rates in bacterial biofilms are obtained. This since the diffusion coefficients increase strongly as the system size increases. In a gel, such as a bacterial biofilm, diffusion is even more affected by particle size, since larger particles will also have a higher risk of becoming entrapped in pores of the gel.

[0051] Thus, in one embodiment, a pharmaceutical composition comprising a first and a second peptide, wherein the first peptide is a peptide of PLNC8 αβ, wherein the peptide of PLNC8 αβ is SVPTSVYTLGIKII,WSAYKHRKTIEKSFNKGFYH (SEQ ID NO 2), and wherein when the second peptide is DLTTKLWSSWGYYLGKKARWNL (SEQ ID NO 31),, and wherein the pharmaceutical composition further comprises at least one antibiotic.

[0052] Thus, according to the disclosure which is not part of the invention, a composition comprising a combination of full length α and truncated or full length β together with antibiotics or full length β and truncated or full length α together with antibiotics or full length α and full length β provide a surprisingly high antimicrobial effect as can be seen in Table 2 (high synergistic effects, with several fold decrease of MIC and MBC). Furthermore, the combinations comprising truncated α and/or β also has the advantage of higher diffusion rates resulting in better activity effect against bacterial biofilms.

[0053] According to one embodiment which is not part of the invention peptide B' has at least 90%, 95%, 96%, 97%, 98% or 99% sequence identity (%SI) with an amino acid sequence selected from the group consisting of SEQ ID NO 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, and 2.

[0054] In one embodiment which is not part of the invention, peptide B' is a sequence selected from the group consisting of SEQ ID NO 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, and 2.

[0055] In one embodiment which is not part of the invention, the peptide A' has at least 90%, 95%, 96%, 97%, 98% or 99% sequence identity (%SI) with an amino acid sequence selected from the group consisting of SEQ ID NO 1, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37 and 4.

[0056] In one embodiment which is not part of the invention, the peptide A' has a sequence selected from the group consisting of SEQ ID NO 1, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37 and 4.

[0057] In one embodiment which is not part of the invention, the peptide A' has at least 90%, 95%, 96%, 97%, 98% or 99% sequence identity (%SI) with an amino acid sequence selected from the group consisting of SEQ ID NO 1, 25, 26, 27, 28, 29, 30, and 31.

[0058] In one embodiment which is not part of the invention, the peptide A' has a sequence selected from the group consisting of SEQ ID NO 1, 25, 26, 27, 28, 29, 30, and 31.

[0059] In one embodiment which is not part of the invention, the first peptide is DLTTKLWSSWGYYLGKKARWNLKHPYVQF (SEQ ID NO 1), and the second peptide is chosen from SVPTSVYTLGIKILWSAYKHRKTIEKSFNKGFYH

(SEQ ID NO 2), SVPTSVYTLGIKILWSAYKH (SEQ ID NO 5), and YTLGIKILWSAYKH (SEQ ID NO 3).

[0060] In one embodiment which is not part of the invention, the first peptide is DLTTKLWSSWGYYLGKKARWN-LKHPYVQF (SEQ ID NO 1), and the second peptide is SVPTSVYTLGIKII,WSAYKHRKTIEKSFNKGFYH (SEQ ID NO 2).

[0061] In one embodiment which is not part of the invention, the first peptide is DLTTKLWSSWGYYLGKKARWN-LKHPYVQF (SEQ ID NO 1), and the second peptide is SVPTSVYTLGIKILWSAYKH (SEQ ID NO 5).

[0062] In one embodiment which is not part of the invention, the first peptide is DLTTKLWSSWGYYLGKKARWN-LKHPYVQF (SEQ ID NO 1), and the second peptide is YTLGIKILWSAYKH (SEQ ID NO 3).

[0063] In one embodiment which is not part of the invention, the first peptide is SVPTSVYTLGIKII,WSAYKHRKTIEKS-FNKGFYH (SEQ ID NO 2), and the second peptide is chosen from DLTTKLWSSWGYYLGKKARWNL (SEQ ID NO 31).

[0064] In one embodiment which is not part of the invention, the first peptide is SVPTSVYTLGIKII,WSAYKHRKTIEKS-FNKGFYH (SEQ ID NO 2), and the second peptide is chosen from DLTTKLWSSWGYYLG (SEQ ID NO 4).

[0065] Sequence identity (%SI) as described herein may be assessed by any convenient method. Programs that compare and align pairs of sequences, like ALIGN (Myers and Miller, CABIOS, 4:11-17, 1988), FASTA (Pearson, Methods in Enzymology, 183:63-98, 1990) and gapped BLAST (Altschul et al., Nucleic Acids Res., 25:3389-3402, 1997), or BLASTP (Devereux et al., Nucleic Acids Res., 12:387, 1984) can be used for this purpose. If no such resources are at hand, according to one embodiment, sequence identity (%SI) can be calculated as (%SI) = 100% * (Nr of identical residues in pairwise alignment) / (Length of the shortest sequence).

[0066] A list of peptide sequences is supplied in table 7.

Table 7: SEQUENCE LIST

PEPTIDE SEQUENCE ID	PEPTIDE SEQUENCE (AA)	Peptide decription
1	DLTTKLWSSWGYYLGKKARWNLKHPYVQF	α1-29
25	DLTTKLWSSWGYYLGKKARWNLKHPYVQ	α1-28
26	DLTTKLWSSWGYYLGKKARWNLKHPYV	α1-27
27	DLTTKLWSSWGYYLGKK ARWNLKHPY	α1-26
28	DLTTKLWSSWGYYLGKKARWNLKHP	α1-25
29	DLTTKLWSSWGYYLGKKARWNLKH	α1-24
30	DLTTKLWSSWGYYLGKKARWNLK	α1-23
31	DLTTKLWSSWGYYLGKKARWNL	α1-22
32	DLTTKLWSSWGYYLGKKARWN	α1-21
33	DLTTKLWSSWGYYLGKKARW	α1-20
34	DLTTKLWSSWGYYLGKKAR	α1-19
35	DLTTKLWSSWGYYLGKKA	α1-18
36	DLTTKLWSSWGYYLGKK	α1-17
37	DLTTKLWSSWGYYLGK	α1-16
4	DLTTKLWSSWGYYLG	α1-15
5	SVPTSVYTLGIKILWSAYKH	β1-20
6	VPTSVYTLGIKILWSAYKH	β2-20
7	PTSVYTLGIKILWSAYKH	β3-20
8	TSVYTLGIKILWSAYKH	β4-20
9	SVYTLGIKILWSAYKH	β5-20
10	VYTLGIKILWSAYKH	β6-20
3	YTLGIKILWSAYKH	β7-20
11	YTLGIKILWSAYKHR	β7-21
12	YTLGIKILWSAYKHRK	β7-22
13	YTLGIKILWSAYKHRKT	β7-23

(continued)

PEPTIDE SEQUENCE ID	PEPTIDE SEQUENCE (AA)	Peptide decription
14	YTLGIKILWSAYKHRKTI	β7-24
15	YTLGIKILWSAYKHRKTIE	β7-25
16	YTLGIKILWSAYKHRKTIEK	β7-26
17	YTLGIKILWSAYKHRKTIEKS	β7-27
18	YTLGIKILWSAYKHRKTIEKSF	β7-28
19	YTLGIKILWSAYKHRKTIEKSFN	β7-29
20	YTLGIKILWSAYKHRKTIEKSFNK	β7-30
21	YTLGIKILWSAYKHRKTIEKSFNKG	β7-31
22	YTLGIKILWSAYKHRKTIEKSFNKGF	β7-32
23	YTLGIKILWSAYKHRKTIEKSFNKGFY	β7-33
24	YTLGIKILWSAYKHRKTIEKSFNKGFYH	β7-34
2	SVPTSVYTLGIKILWSAYKHRKTIEKSFNKGFYH	β1-34

[0067] To verify the synergy effect of peptides of PLNC8 αβ together with antibiotics, antibiotics from the three largest groups of antibiotics were selected and tested; antibiotics that inhibit bacterial cell wall synthesis, antibiotics that inhibit nucleic acid synthesis and antibiotics that inhibit protein synthesis. The combination of antibiotics and PLNC8 αβ provides a powerful synergistic effect, and reduces (up to 100 times) MIC and MBC for antibiotics from the three classes. As can be seen in Fig. 15 teicoplanin, vancomycin, rifampicin, and gentamicin were evaluated. Of these, synergistic effects were (from high to low) rifampicin [100-fold], gentamicin [15-30-fold] teicoplanin [10-fold] and vancomycin [2-fold]. The highest effect was thus shown for a combination of PLNC8 αβ and rifampicin.

[0068] In tables 4, 5 and 6, the synergy effects of PLNC8 αβ together with antibiotics selected from a group consisting of gentamicin, rifampicin, ciprofloxacin, teicoplanin, levofloxacin, and meropenem, are shown.

[0069] Thus, in one embodiment, the antibiotic is selected from the group consisting of antibiotics that inhibit bacterial cell wall synthesis, antibiotics that inhibit nucleic acid synthesis and antibiotics that inhibit protein synthesis.

[0070] In one further embodiment, the antibiotic is selected from a group consisting of gentamicin, rifampicin, ciprofloxacin, teicoplanin, levofloxacin, meropenem and vancomycin.

[0071] In one further embodiment, the antibiotic is selected from a group consisting of gentamicin, rifampicin, ciprofloxacin, teicoplanin, levofloxacin, and meropenem.

[0072] In one further embodiment, the antibiotic is selected from a group consisting of rifampicin, gentamicin, teicoplanin and vancomycin.

[0073] In one further embodiment, the antibiotic is selected from a group consisting of rifampicin, gentamicin, and teicoplanin.

[0074] Bacterial biofilms may also seek to combat antibiotics by a reaction with the antimicrobial agent. Similarly, infections are often associated with high proteolytic activity caused by both bacteria and the body's immune system, which means that antimicrobial peptides or proteins may be inactivated.

[0075] In the disclosure, it was hypothesized that the inactivation through proteolytic activity often targets specific sequence motifs. Hypothetically bacteriocin modifications altering the susceptibility to these target attacks could increase the lifetime, and thereby the effect, of the bacteriocin. However, such modifications risk altering the molecular structure of the peptide, which may affect the peptide function.

[0076] Under the hypothesis that a structurally stable structure might be provided if the whole peptide was modified, all L-amino acids of the peptides were replaced by D-amino acids (all alpha amino acids but glycine can exist in either of two enantiomers). It was also hypothesized that this could affect proteolytic cleavage of the peptides and thus increase efficacy. The effects of the L- and D-variants of PLNC8 αβ were tested on both a liposome system (resembling bacteria) and on *S. epidermidis.* The D-variant of PLNC8 αβ was almost as effective in destroying liposomes and inhibiting/killing *S. epidermidis* as the L-variant (Fig. 4). The perturbation of the plasma membrane of *S. epidermidis* was equally rapid (2 min) for the L- and D-variant, respectively, of PLNC8 αβ (Fig. 5). Figure 15 shown that the synergistic effect is maintained when treating *S. epidermidis* with rifampicin or tecioplanin in the presence of L-PLNC8 αβ or D-PLNC8 αβ (Fig. 15).

[0077] To analyze whether PLNC8 αβ with D-amino acids is more stable and less sensitive to proteolytic cleavage

compared to the L-variant of PLNC8αβ; D-PLNC8α, D-PLNC8β, L-PLNC8α and L-PLNC8β were exposed to trypsin and the presence of proteolytic fragments was analyzed with MALDI-TOF mass spectrometry (Fig. 6). While trypsin generated several fragments of both the α- and β-peptide of L-PLNC8, no obvious fragmentation was observed of the α- and β-peptide of D-PLNC8. Thus, the D-variants are more resistant to trypsin-mediated degradation than the L-variants.

[0078] Furthermore, to clarify whether PLNC8 αβ (the L- and D-variant) exerts cytotoxic effects, lysis of erythrocytes isolated from human whole blood was investigated. However, no hemolytic activity was observed (Fig. 7). Thus, in one embodiment, least 90% of the amino acids in the first peptide and/or second peptide are D-amino acid residues.

[0079] In figure 25, CD measurements of (A) *L*-PLNC8 αβ and (B) *D*-PLNC8 αβ with or without liposomes are shown. Bacteriocins are often unstructured in solution but typically adopt a more ordered secondary structure when bound to the bacterial cell membrane as a result of membrane partitioning. However, results indicate that both *L*- and *D*-PLNC8 αβ has an ordered secondary structure in liposomes.

[0080] The advantages of the D-variants are increased stability and less sensitivity to proteolytic cleavage. This results in a longer lifetime of the D-variant peptides and thus prolonged antibacterial effect.

[0081] Non-natural or modified amino acids can be introduced that enable convenient coupling chemistries, including click-chemistry approaches. The bacteriocins can also be modified with either N- or C-terminal azide groups to enable copper-free click reaction with e.g. cyclooctyne conjugated polymers. Using biodegradable polymers such as hyaluronic acid (HA), the release rate will be dependent on the hydrolysis rate of the biopolymer backbone and can be tuned to a certain extent by using different polymers. Interestingly, hyaluronidase is expressed by *S. aureus,* as a virulence factor, degrading polysaccharides between cells and thereby enabling spreading of the infection. Thus, if the biodegradable polymer is HA, the release rate of the peptides will increase in the presence of *S. aureus.*

[0082] PLNC8αβ is a two peptide bacteriocin, so in order to investigate the role of the PLNC8 α chain and PLNC8 β chain, respectively, in the inhibitory and bactericidal action of the bacteriocin, the effects of different molar ratios between the peptides on *S. epidermidis* were studied. It was found that a molar ratio of 1:1 is most efficient at inhibiting and killing *S. epidermidis* (Fig. 2). However, a ratio of between PLNC8 α chain and PLNC8 β chain of 1:1 to 1:7 also showed a good effect.

[0083] Thus, in one embodiment, the first and second peptides are present in a in a molar ratio of from between 5:1 to 1:20, preferably 1:1 to 1:7, most preferably 1:1.

[0084] The composition may comprise between 10 nM to 50 μM of the first peptide and/or of the second peptide. As shown in Fig. 15, concentrations within the micromolar range effectively reduce *S. epidermidis* in the presence of an antibiotic.

[0085] Thus, in one embodiment, the pharmaceutical composition comprises between 10 nM to 50 μM of the first peptide and/or of the second peptide.

[0086] As can be seen in Fig. 15, MIC and MBC of rifampicin was lowered more then 100-fold when treating *S. epidermidis* in the presence of L-PLNC8 αβ or D-PLNC8 αβ, resulting in an effective amount already at 0.0019 μg/ml.

[0087] Thus, in one embodiment, the pharmaceutical composition comprises the antibiotic in an amount of at between 0.002 μg/ml to 50 μg/ml, such as at least 0.01 μg/ml to 5 μg/ml, such as at least 0.1 μg/ml to 1 μg/ml, such as at least 0.8 μg/ml.

[0088] Thus, in one embodiment, the pharmaceutical composition comprises the antibiotic in an amount of at least 0.78 μg/ml of vancomycin, at least 0.097 μg/ml for teicoplanin, at least 0.0019 for rifampicin and at least 0.0097 for gentamicin.

[0089] Traditionally, one may think of antibiotics treatment as administered orally. Such treatment may lead to unwanted side effects, such as affecting or even destroying the protective flora or stimulates the development of antibiotics resistance. Such treatment may also lead to changes in the intestinal bacterial composition, which may result in superinfection by fungi and other infective organisms.

[0090] PLNC8 αβ together with an antibiotic may beneficially be administered locally, in the form of a solution, cream, a gel or in immobilized form (as described further under coating below).

[0091] The formulations may further include a solvent and/or a variety of excipients, for instance to stabilize the peptides and suppress aggregation, such as solubilizers, surfactants, bulking agents (such as carbohydrates), thickeners (such as polymers) to increase solution viscosity, preservatives, vehicles, salts or sugars to stabilize proteins and to obtain physiological tonicity and osmolality and/or buffering agents to control pH.

[0092] Thus, in one embodiment, the composition is formulated as a solution, a cream, a gel, or an ointment or formulated in immobilized form as a coating on a device.

[0093] In another embodiment, the pharmaceutical composition is for use in the treatment or prophylaxis of a bacterial infection.

[0094] In one embodiment, the composition is administered locally on the site of infection, such as topically.

[0095] To be able to treat local infections, e.g. chronic wounds, PLNC8 αβ may be linked or associated with a supporting material. To test this, PLNC8 αβ was loaded in a formula (gel) consisting of gelatin and glycerol. PLNC8 αβ in the gel rapidly *lysed S. epidermidis* and the PLNC8 αβ-containing gel totally inhibited the growth of the bacteria on agar plates

(Fig. 13). The activity of PLNC8 αβ in the gel was stable after long-term storage at 4°C for at least 180 days.

[0096] Thus, in one embodiment, the composition is formulated as a gel, wherein the gel further comprises gelatine and glycerol.

[0097] The effect of formulating the composition as a gel is to provide a localized, long-term antibacterial effect.

[0098] The results indicate that PLNC8 αβ is effective against many pathogens that are responsible for causing severe hospital- and community acquired infections usually hard to treat (Fig. 1-3, 11, 14, 17-18, 26-27, and Table 4-6). Furthermore, PLNC8 αβ has synergistic effects with a wide range of antibiotics and can enhance their effects by 2-130-fold (Fig. 15-16, 22, 26-27 and Table 2-7). These results suggest that severe infections caused by antibiotic resistant bacteria may be efficiently treated by applying combination therapy of PLNC8 αβ with low concentrations of antibiotics.

[0099] In one embodiment, the bacterial infection is caused by *Staphylococcus spp* (including MRSA, MRSE), *Streptococcus* spp (e.g. *S. mutans, S. constellatus, S. anginosus*), *Enterococcus faecium* (including VRE), *Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter* spp and/or *Escherichia coli.*

[0100] In one embodiment, the bacterial infection is caused by *Staphylococcus spp, Streptococcus spp,* such as *S. mutans, S. constellatus, S. anginosus.*

[0101] In on embodiment, the bacterial infection is caused by *Staphylococcus spp* and/or *Streptococcus spp.*

[0102] The bacterial infection may be caused by gram-negative bacteria.

[0103] The bacterial infection may be caused by gram-positive bacteria.

[0104] The bacterial infection may be caused by *Escherichia coli.*

[0105] The bacterial infection may be caused by *Enterococcus* ssp.

[0106] The bacterial infection may be caused by *Pseudomonas aeruginosa.*

[0107] The bacterial infection may be caused by *Porphyromonas gingivalis.*

[0108] Such bacteria are a common cause of hospital-acquired infection (HAI). Among the categories of bacteria most known to infect patients are the ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter*), including MRSA (Methicillin-resistant *Staphylococcus aureus*) and VRE (Vancomycin-resistant *Enterococcus*), *Streptococcus spp* and *Escherichia coli.* Thus, one advantage of the present disclosure is that infections caused by bacteria which are resistant to conventional antibiotics may be treated.

[0109] Bacterial infection and inflammation are sometimes linked to implants, caused by the bacterial adherence and colonization in the implant area. Treatment may include removing dead tissue, antibiotics, and improved hygiene. Preventive measures include polish the implant surface, to minimize bacterial adherence, which is a time consuming and costly procedure. Thus, implant coating or treatment with antibacterial material would minimize these incidences and avoid the high cost of producing a highly polished surface on implant.

[0110] Thus, a coating comprising the first and second peptide of the disclosure (i.e. PLNC8 αβ) together with an antibiotic may be used to impart bacterial resistance to a coating for an implant.

[0111] Similarly, such a coating may be used for any medical device, or part of a medical device, where bacterial colonization on the surface should be prevented.

[0112] The medical device may also be a band-aid comprising the first and second peptide (i.e. PLNC8 αβ) and antibiotic of the disclosure. This would help facilitate local administration on a wound or infection site. The bacteriocin and antibiotic may either be tethered to a polymeric scaffold via a flexible linker or physically entrapped in a biopolymeric matrix, its bactericidal property will be retained, or even improved because of its high local concentration

[0113] In one embodiment, the composition is formulated in immobilized form as a coating on a device, wherein the device is chosen from the group consisting of a wound dressing, an orthopedic implant, a dental implant, a urinary catheter and an urinary stent.

[0114] In one embodiment, a pharmaceutical composition is used in coating at least part of a device to limit colonization of bacteria on the surface of the device.

[0115] In one further embodiment, the device is a medical device, such as a prosthesis or a wound dressing.

[0116] In one further embodiment, the bacteria are *Staphylococcus spp* (including MRSA, MRSE), *Streptococcus spp* (e.g. *S. mutans, S. constellatus, S. anginosus*), *Enterococcus faecium* (including VRE), *Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter spp* and/or *Escherichia coli.*

[0117] In one further embodiment, the bacteria are *Staphylococcus spp* and/or *Streptococcus spp,* such as *S. mutans, S. constellatus, S. anginosus.*

Conclusions

[0118] Thus, it has been shown that a combination of full length α and truncated or full length β together with antibiotics or full length β and truncated or full length α together with antibiotics or truncated α and truncated length β, have a rapid and direct effect on different pathogens without expressing any toxic effects on surrounding human cells. In addition, it was found that this combination enhances, 2-130-fold, the effect and sensitivity of antibiotics. Substitution of L-amino

acids of PLNC8α/β by D-amino acids does not change the anti-bacterial effects of the bacteriocin. However, the D-form of PLNC8α/β is much more stable against proteolytic cleavage and is thus adapted for a therapeutical use *in vivo.*

[0119] The data indicates that the use of a combination of full length α and truncated or full length β together with antibiotics or full length β and truncated or full length α together with antibiotics or truncated α and truncated length β is very well suited for the treatment of infections. This combination can be administered locally in soluble form in gels (ointments, creams) and in immobilized form, e.g. on wound dressings, orthopedic implants, dental implants, urinary catheters and stents, and act antibacterially with no cytotoxic side effects.

[0120] Such a combinations provide the following advantages: They act very fast (sec-min); are effective and very potent (nano-micromolar doses); have a wide anti-bacterial spectrum - both against gram-negative and gram-positive bacteria; facilitate and/or enhance the absorption, activity and efficacy of different antibiotics; enable the use of lower doses of antibiotics, which reduce resistance development; enable treatment of complex infections caused by multiple pathogens including multiresistant bacteria, such as MRSA, in suspension or biofilm; low or no effects on normal flora; low or no cytotoxic effects; simple and stable; cheap production.

[0121] This means that PLNC8 αβ and antibiotic combination according to the disclosure is in many respects superior to the various products currently on the market, such as traditional antibiotics, antiseptics and other more unspecific antibacterial substances.

[0122] Today there is no method of counteracting and treating chronic infections, for example caused by bacterial biofilms. Treatment of biofilms with antibiotics is very ineffective and costly, and there is also a risk that the protective normal flora is eradicated and that antibiotic resistance develops. Here it has been shown that PLNC8αβ acts synergistically with antibiotics and can effectively attack different pathogens, in suspension or biofilm. Thus it constitutes a more specific, potent and direct anti-bacterial treatment of troublesome infections and associated diseases, and thus lead to less human suffering and greater health-economic effects compared to current forms of treatment.

[0123] The disclosure can be implemented in any suitable form or any combination of forms. Although the present disclosure has been described above with reference to (a) specific embodiment(s), it is not intended to be limited to the specific form set forth herein. Rather, the disclosure is limited only by the accompanying claims and, other embodiments than the specific above are equally possible within the scope of these appended claims, e.g. different than those described above.

[0124] In the claims, the term "comprises/comprising" does not exclude the presence of other elements or steps. Furthermore, although individually listed, a plurality of means, elements or method steps may be implemented. Additionally, although individual features may be included in different claims, these may advantageously be combined, and the inclusion in different claims does not imply that a combination of features is not feasible and/or advantageous. In addition, singular references do not exclude a plurality. The terms "a", "an", "first", "second" etc do not preclude a plurality.

Experimental Section

Bacterial culture conditions

[0125] *Staphylococcus aureus* CCUG 35601 (MRSA, Culture Collection, University of Gothenburg) and *Staphylococcus aureus* ATCC 29213 (MSSA, ATCC, Manassas, VA). *Staphylococcus epidermidis* ATCC 12228 (ATCC, Manassas, VA), RP62A, N15 and 10 clinical isolates of *Staphylococcus epidermidis* that have previously been characterized. Isolated *Escherichia coli, Enterococcus faecium* (including VRE), *Pseudomonas aeruginosa, Klebsiella pneumoniae, Enterobacter spp* and *Acinetobacter baumannii* were obtained from Örebro University hospital. Isolated *Streptococcus mutans, Streptococcus constellatus* and *Streptococcus anginosus* were obtained from Malmö University. The bacteria were grown on Luria-Bertani (LB) agar plates, supplemented with 5% defibrinated horse blood, and incubated at 37°C overnight. Single colonies were inoculated into 5 ml of LB broth and incubated on a shaker (300 rpm) at 37°C overnight. The bacterial concentration was determined by viable count and adjusted to correlate with approximately 10^9 CFU/ml.

Peptide synthesis

[0126] All chemicals were bought from Sigma Aldrich unless otherwise noted and used without further purification. PLNC8α (H2N-DLTTKLWSSWGYYLGKKARWNLKHPYVQF-COOH), PLNC8β (H2N SVPTSVYTLGIKIL WSA YKHRKTIEKSFNKGFYH-COOH), scrambled-PLNC8α (H2N-TWLKYGHGDAKLWSWSKPLNLTFRYQYVK-COOH), scrambled-PLNC8β (H2N-LKLWNTYGTFSRFYTSKSEVKIAHGIKSIHVPYK-COOH), and truncated forms of PLNC8α and PLNC8β were synthesized using conventional Fmoc chemistry on a Quartet automated peptide synthesizer (Protein Technologies, Inc) in a 100 μmol scale. Peptide elongations were performed using a four-fold excesses of amino acid (Iris biotech gmbh) and activator (TBTU, Iris biothech gmbh) and using an eight-fold excesses of base (DIPEA). Fmoc removal was accomplished by treatment with Piperidine (20% in DMF, v/v). All peptides were cleaved from their solid support using a mixture of TFA, triisoproylsilane and water (95:2.5:2.5, v/v/v) for 2 h before being, filtered, concentrated

and precipitated twice in cold diethylether. Crude peptides were purified on a C-18 reversed phase column (Kromatek HiQ-Sil C18HS) attached to a semi preparative HPLC system (Dionex) using an aqueous gradient of acetonitrile (10-46%) containing 0.1% TFA. Mass identity of all peptides was confirmed by MALDI-ToF MS (Applied biosystems) using α-cyano-4-hydroxycinnamic acid as matrix.

[0127] To study the effects and stability of D-forms of PLNC8α and PLNC8β, the L-form of amino acids was substituted with the D-form of amino acids during peptide synthesis. The sensitivity to proteolytic cleavage of D-PLNC8α, D-PLNC8β, L-PLNC8α and L-PLNC8β was analyzed by exposing the peptides to Trypsin for 16h, whereafter the presence of proteolytic fragments was determined with MALDI-TOF mass spectrometry.

Liposome preparation

[0128] Liposomes were prepared by dry film formation, hydration and finally extrusion through a polycarbonate membrane to form monodisperse large unilamellar vesicles. The lipids 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine (POPC) (Avanti Polar Lipids, Alabaster, USA) were mixed at molar ratios 1:99, 5:95 and 10:90 while dissolved in chloroform. A dry lipid film was formed by evaporation of the chloroform by nitrogen flow and overnight lyophilization. The film was hydrated with either 10 mM phosphate buffer (PB) pH 7 or 10 mM phosphate buffer saline (PBS) pH 7, and the solution was vortexed for 1 min and put on a shaker for 1 h before extruded 21 times through a 100 nm pore-sized polycarbonate membrane. For fluorescence leakage assay the lipid film was hydrated with buffer (PBS) containing self-quenching concentration (50 mM) of 5(6)-carboxyfluorescein (CF) (Sigma Aldrich) and liposomes were prepared as described above. Removal of unencapsulated CF was done by gel filtration using a PD-25 column (GE Healthcare, Uppsala, Sweden) and liposomes with encapsulated CF were eluted with PBS.

Fluorescence leakage assay

[0129] Leakage of the liposome encapsulated fluorophore CF due to additions of the bacteriocins was recorded using a fluorescence plate reader (Infinite 200, Tecan, Austria) where λ_{ex} = 485 nm and λ_{em} = 520 nm. CF was encapsulated at self-quenching concentration, and CF release results in an increased fluorescence signal. Liposomes were diluted to 25 μM (total lipid concentration) in PBS, followed by additions of 0, 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1 and 2 μM of the L-form or D-form of PLNC8 α and β, separately and combined, and truncated forms of PLNC8 α, separately and combined with PLNC8 β, and truncated forms of PLNC8 β, separately and combined with PLNC8 α. In order to estimate the maximum release from each sample a final addition of 0.5 % Triton X-100 was made at the end of all measurements and the total amount of CF (100% release) was estimated after 15 min incubation. The CF release is presented as percentage release for each time interval (measurements taken every minute). The percentage CF release is calculated as $100 \times (F - F_0)/(F_T - F_0)$ where F_0 is the initial fluorescence intensity of CF before peptide addition, F is the fluorescence intensity of CF at time point t and F_T is the maximum fluorescence after the addition of Triton X-100. Results are shown in Fig. 4.

Antimicrobial activity of PLNC8 αβ

[0130] The broth microdilution method was used to determine minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC). Two-fold serial dilutions of the peptides were used and the final concentrations ranged from 0.097-50 μM. The final concentrations of the antibiotics vancomycin and teicoplanin ranged from 0.097-50 μg/ml, while rifampicin ranged from 0.0019-1 μg/ml and gentamicin 0.0097-5 μg/ml. The effect of bacteriocin-antibiotic combinations was accomplished by using the same concentration series of antibiotics with a constant concentration of PLNC8 αβ (3.1 μM) in all the wells. The MIC was determined visually and spectroscopically (620 nm) as the first concentration that completely inhibited bacterial growth. All concentrations that resulted in complete inhibition of bacterial growth were cultured (10 μl) on blood-agar plates, and the lowest concentration where no growth was observed on agar represented the MBC. All experiments were repeated at least three times.

Microscopy

[0131] The fluorescent dye Sytox® Green, which can only cross damaged membranes and fluoresce upon binding to nucleic acids, was used to study the antimicrobial activity of PLNC8 αβ on *S. epidermidis, S. aureus* (MSSA, MRSA) and *Streptococcus spp.* The bacteria were washed and resuspended in Krebs-Ringer Glucose buffer (KRG) (120 mM NaCl, 4.9 mM KCl, 1.2 mM $MgSO_4$, 1.7 mM KH_2PO_4, 8.3 mM Na_2HPO_4, and 10 mM glucose, pH 7.3) and incubated in the presence or absence of different combinations of PLNC8 αβ in 96-well microtiter plates for 2 min. Images were captured with Olympus BX41 at 40x magnification.

[0132] Electron microscopy was used to visualize the damage of bacteria caused by PLNC8 αβ. Briefly, bacteria were pelleted and washed with Krebs-Ringer Glucose buffer (KRG) (120 mM NaCl, 4.9 mM KCl, 1.2 mM $MgSO_4$, 1.7 mM KH_2PO_4, 8.3 mM Na_2HPO_4, and 10 mM glucose, pH 7.3). The bacteria were then treated with different concentrations of PLNC8 αβ in a ratio of 1:1 for 5 min, followed by fixation in 2.5% glutaraldehyde in 0.1M phosphate buffer, pH 7.3. Critical point drying was applied for specimens for SEM coated with Gold using a Sputter coater. Specimens for TEM were washed in 0.1M phosphate buffer, postfixed in 2% osmium tetroxide in 0.1M phosphate buffer for 2 hours and embedded into LX-112 (Ladd, Burlington, Vermont, USA). Ultrathin sections (approximately 50-60 nm) were cut by a Leica ultracut UCT/ Leica EM UC 6 (Leica, Wien, Austria). Sections were contrasted with uranyl acetate followed by lead citrate and examined in a Hitachi HT 7700 (Tokyo, Japan). Digital images were taken by using a Veleta camera (Olympus Soft Imaging Solutions, GmbH, Münster, Germany). Representative images of three independent experiments can be seen in Fig. 12.

Circular Dichroism (CD) spectroscopy

[0133] Bacteriocins are often unstructured in solution but typically adopt a more ordered secondary structure when bound to the bacterial cell membrane as a result of membrane partitioning. Circular dichroism spectroscopy measurements were performed on a Chirascan (Applied Photophysics, United Kingdom) using a 1 mm cuvette at room temperature. A wavelength scan of 195-280 nm was recorded 3 times for each sample, averaged and baseline corrected using PB buffer (pH 7.4, 10 mM). In all samples, the concentration of each peptide was 30 μM, prepared in PB buffer. In experiments with liposomes the final lipid concentration was 660 μM (0.5 mg/ml). To compensate for the different total peptide concentrations used, the averaged data were converted to mean residue ellipticity (MRE).

Proteolytic degradation

[0134] Full length PLNC8 αβ (100 μM) in both L-and D-form was subjected to Trypsin (0.125 mg/ml, ~5 μM) in ammonium bicarbonate buffer (50 mM, pH 8.5) for 16 hours at 37 °C. Sample solutions were acidified by adding 2.5 % TFA and dried in an exicator at room temperature. Samples were resuspended in MQ-water containing 0.1% TFA, desalted using ZipTip-C18 columns (Millipore) and analyzed using MALDI-ToF MS (UltraflexXtreme, Bruker Daltonics) with α-cyano-4-hydroxycinnamic acid as matrix.

Hemolysis

[0135] The hemolytic activity of the peptides was investigated by collecting blood from healthy volunteers in heparinized vacutainers. The blood was centrifuged at 600 ×g for 5 min and the erythrocyte pellet was washed three times in PBS. The cells were then suspended in PBS and added to 96-well plates (15% erythrocyte suspension/well), containing the peptides with two-fold serial dilution. The plates were incubated for 1 h at 37°C followed by centrifugation for 5 min at 900 ×g and measurement of the supernatants at 540 nm. Haemolytic activity (%) was calculated by subtracting the negative control from all values and normalization against the positive control (0.5% Triton X-100), that was set to 100%. All experiments, each in duplicate, were repeated three times.

Aggregation and ATP release

[0136] Aggregation and extracellular release of ATP were used to study the effects of PLNC8 αβ on the bacteria. ATP was registered using a luciferin/luciferase bioluminescence assay (Sigma, St. Louis, Mo, USA) in bacterial suspensions (2.5×10^8 CFU/ml). The bacteria were exposed to different concentrations of PLNC8 αβ, and real-time changes in light transmission and bioluminescence were recorded in a Chronolog lumi-aggregometer (Chrono-Log, Haverton, PA, USA) for 30 min. The levels of ATP were calculated based on the bioluminescence signals recorded in response to known concentrations of ATP.

Bacterial biofilms

[0137] *S. epidermidis* RP62A was inoculated into 5 ml of LB broth and incubated on a shaker at 37°C overnight. The bacterial culture was diluted 1:100 into fresh media and 100 μl of bacterial suspension per well was added in a 96-well microtiter plate and incubated statically at 37°C for 20 h. The wells were washed three times by submerging the plate into a container with distilled water to remove unattached cells. Fresh LB media was added to each well (100 μl) followed by addition of the peptides in different concentrations. The plate was incubated statically for 1 h. Detached material in the wells were transferred to a new microtiter plate for absorbance measurements at 620 nm. The remaining attached biofilms were stained with 0.1% crystal violet for 15 min before the plate was washed four times in distilled water as

mentioned above and allowed to dry at room temperature for 2 h. The crystal violet was solubilized in 30% acetic acid for 15 min and the absorbance quantified at 550 nm. Each experiment, with three replicates, was repeated three times.

Cell culture conditions

[0138] Human keratinocytes (HaCaT) were cultured in Dulbecco's modified Eagle medium (DMEM, Fisher Scientific, New York, USA) supplemented with 10% FBS (FBS, Invitrogen Ltd, Paisley, UK) incubated in a stable environment at 95% air, 5% CO_2 and 37°C. The cells were used at passages 1-20.

[0139] HaCaT cells were cultured overnight in a 24-well plate at a seed-count of 2×10^5 cells per well. The cells were either treated with PLNC8 $\alpha\beta$ (6.25, 12.5 and 25 μM) or MSSA (MOI: 0.1, 1 and 10) alone for 24 h. Co-stimulation was performed by infection of human keratinocytes, in the presence or absence of PLNC8 $\alpha\beta$, for 24 h, or infection of the cells for 1 h followed by addition of different concentrations of PLNC8 $\alpha\beta$ for 6 h. Furthermore, infection of keratinocytes, with or without PLNC8 $\alpha\beta$, were monitored in real-time for 72 hours using IncuCyte Live-cell Analysis System. Bacterial load was quantified by measuring the fluorescence of green fluorescent protein (GFP) and cell viability was determined by measuring the fluorescent dye Draq7 that stains nuclei of dead cells.

Enzyme-linked immunosorbent assay (ELISA)

[0140] ELISA was performed on supernatants retrieved from human keratinocytes that were exposed to MSSA and PLNC8 $\alpha\beta$. The levels of CXCL8 (Human IL-8 ELISA MAX Deluxe, Nordic Biosite, Sweden) and IL-6 (Human IL-6 ELISA MAX Deluxe, Nordic Biosite, Sweden) were quantified according to the manufacturer's instructions.

Reverse transcription quantitative PCR (RT-qPCR)

[0141] RT-qPCR was used to determine gene expression levels of a selected number of genes. Briefly, RNA was extracted using GeneJET™ RNA Purification Kit (Fermentas, Sweden) according to the manufacturer's recommendations. Reverse transcription (100 ng RNA/sample) was performed using iScript cDNA Synthesis Kit (Biorad, Sweden). Thermal cycling conditions for SYBR Green (Maxima® SYBR Green/ROX qPCR Master Mix, Fermentas, Sweden) consisted of a denaturation step at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Gene expression was analyzed using a 7900 HT real-time PCR instrument (Applied Biosystems). The obtained Ct values were normalized against *gapdh.* Relative quantification of gene-expression was determined by using the $\Delta\Delta$Ct method. Fold change was generated by using the formula $2^{\Delta\Delta Ct}$.

Porcine wound model

[0142] Full-thickness wounds measuring 1.5×1.5 cm were created on the dorsum of the pig and covered with sterile wound chambers (S2Medical, Linköping). Three wounds were created per condition with the following conditions: control (PBS), PLNC8 $\alpha\beta$, MSSA, MSSA/PLNC8 $\alpha\beta$, MSSA/gentamicin and MSSA/gentamicin/ PLNC8 $\alpha\beta$. The wounds were either left untreated (sterile PBS), treated with PLNC8 $\alpha\beta$ (50 μM) or infected with MSSA (10^8 CFU/ml). The pig was returned to the pen and monitored during recovery from anesthesia. After three days, the wounds were washed with sterile PBS and treatment was started (gentamicin 100 μg/ml, PLNC8 $\alpha\beta$ 50μM or a combination of both gentamicin and PLNC8 $\alpha\beta$, 10μg/ml and 50μM, respectively). This procedure was repeated every other day for seven days (a total of four doses of treatment of the infected wounds).

LDH cytotoxicity assay

[0143] Cytotoxicity of HaCaT cells was determined by measuring extracellular lactate dehydrogenase (LDH) activity using LDH cytotoxicity assay. The procedure was performed using Thermo Scientific ™ Pierce ™ LDH Cytotoxicity Assay Kit according to the manufacturer's instructions. The method relies on the fact that the cytosolic enzyme LDH is released into the surrounding cell culture media if the cell membrane is damaged. Extracellular LDH undergoes an enzymatic reaction, which combined with the assay chemicals culminates in the formation of a red formazan compound which then can be measured in a photo spectrometer at 490nm. Cytotoxic effects were calculated relative to the untreated cells that were set to 0.

Statistical analysis

[0144] All data were analyzed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). One-way ANOVA with Bonferroni's post hoc test was used for the comparisons between the different treatments. P-values are referred to

as *p<0.05; **p<0.01; ***p<0.001.

Ethics statement

[0145] This work deals with clinical bacterial isolates from human infections. No tissue material or other biological material was stored from the patients, only subcultured bacterial isolates. Swedish law does not require ethical approval for work with bacterial isolates from humans. All information regarding these isolates was anonymized. Animal experiments were performed under the strict regulation of the Ethics Committee for Animal Experimentation, with all the appropriate ethical permissions.

Results

[0146] The effects of PLNC8αβ on different strains of *S. aureus* and *S. epidermidis* were studied (Table 1). PLNC8αβ markedly inhibited the growth and the survival of all bacterial strains (Fig. 1).

Table 1

Bacteria	Characteristics
***S. aureus* ATCC 29213 (MSSA)**	Methicillin sensitive
***S. aureus* CCUG 35601 (MRSA)**	Methicillin resistant
***S. epidermidis* ATCC 12228**	Biofilm negative
***S. epidermidis* RP62A**	Biofilm positive
***S. epidermidis* N15**	Isolated from nose of a healthy individual
***S. epidermidis* 117**	Isolated from an infected hip joint prosthesis

L-PLNC8 αβ	MIC	MBC
***S. aureus* ATCC 29213 (MSSA)**	12.5	25
***S. aureus* CCUG 35601 (MRSA)**	12.5	25
***S. epidermidis* ATCC 12228**	6.25	12.5
***S. epidermidis* RP62A**	6.25	6.25
***S. epidermidis* N15**	6.25	6.25
***S. epidermidis* 117**	12.5	12.5

[0147] In addition, PLNC8 αβ permeabilized and killed *Streptococcus spp* (Fig. 17), *Pseudomonas aeruginosa. Escherichia coli and Enterococcus faecium (Table 4-6).*

[0148] In order to investigate the role of PLNC8 α and PLNC8 β, respectively, in the inhibitory and bactericidal action of the bacteriocin, the effects of different molar ratios between the peptides on *S. epidermidis* were studied. It was found that a PLNC8 α to PLNC8 β molar ratio of 1:1 is most efficient at inhibiting and killing *S. epidermidis* (Fig. 2). However, ratios between 1: 1 and 1:7 were also found to be effective.

[0149] Since most bacteria grow and are a part of complex biofilms, where they often are more resistant against antibiotic treatment compared to when they exist in a planktonic state, the effects of PLNC8 αβ on biofilms consisting of *S. epidermidis* were tested. It was found that PLNC8 αβ efficiently disrupted the biofilms and killed the bacteria (Fig. 3). Also the α and β peptide of PLNC8 exerted by themselves, although at higher concentrations, disruptive effects on the biofilms.

[0150] The antibacterial activity of PLNC8 αβ may *in vivo* be restricted by proteolytic activity exerted by proteases from both bacteria and human cells. In order to circumvent the problem with a proteolytic cleavage of PLNC8 αβ, the L-form of amino acids, that normally occurs in peptides such as PLNC8 αβ, was substituted with the D-form of amino acids. The effects of the L- and D-variants of PLNC8 αβ were tested on both a liposome system (resembling bacteria) and on *S. epidermidis.* It was found that the D-variant of PLNC8 αβ was as effective in destroying liposomes and inhibiting and/or killing *S. epidermidis* as the L-variant (Fig. 4). Furthermore, the perturbation of the plasma membrane of *S. epidermidis* was equally rapid (2 min) for the L- and D-variant, respectively, of PLNC8 αβ (Fig. 5).

[0151] To analyze whether PLNC8 αβ with D-amino acids is more stable and less sensitive to proteolytic cleavage compared to the L-variant of PLNC8αβ; D-PLNC8α, D-PLNC8β, L-PLNC8α and L-PLNC8β were exposed to trypsin and the presence of proteolytic fragments was analyzed with MALDI-TOF mass spectrometry (Fig. 6). While trypsin generated several fragments of both the α- and β-peptide of L-PLNC8, no obvious fragmentation was observed of the α- and β-peptide of D-PLNC8.

[0152] To clarify whether PLNC8 αβ (the L- and D-variant) exerts cytotoxic effects, lysis of erythrocytes isolated from human whole blood was investigated. However, no hemolytic activity was observed (figure 7).

[0153] Truncated forms of PLNC8 αβ express antibacterial activities similar to the native bacteriocin or are even more effective. Truncated peptides of PLNC8 α and PLNC8 β, respectively, were constructed in sequences of 6-7 amino acids corresponding to the number of amino acids needed for formation of an alpha helix (figure 8). The effects of truncated PLNC8 α and PLNC8 β were tested on both a liposome system (resembling bacteria) and on *S. epidermidis.* Disruption of the liposome membranes, revealed by release of (6)-carboxyfluorescein (CF), was obtained with the β-peptides 1-34 (full-length), 7-34, 1-20 and 7-20 (figure 9). When combined with PLNC8 α, effects were also obtained with the other truncated peptides, although at higher concentrations.

[0154] Interestingly, growth *of S. epidermidis* was most efficiently inhibited by sequence β1-20 and β7-20, respectively, and these truncated peptides were more effective than the full-length native PLNC8 β (1-34) (Fig. 9).

[0155] The peptide β-sequences β7-13 and β14-20 are crucial for the effects of PLNC8 β and are more efficient when combined with β1-6. Thus, the peptide β1-20 is most effective in inhibiting *S. epidermidis.*

[0156] The truncated form1-22 of the α-peptide and the full-length α-peptide (1-29) disrupted the membrane of the liposomes, revealed by a release of carboxyfluorescein (figure 10). However, the different truncated forms of the α-peptide had no significant effects on *S. epidermidis.* In combination with the β-peptide, α1-22 exerted inhibitory and bactericidal effects (Fig. 10).

[0157] To be able to treat local infections, e.g. chronic wounds, PLNC8 αβ was used with a supporting material. PLNC8 αβ was loaded in a formula (gel) consisting of gelatin and glycerol. PLNC8 αβ in the gel rapidly lysed *S. epidermidis* and the PLNC8 αβ-containing gel totally inhibited the growth of the bacteria on agar plates (Fig. 13). The activity of PLNC8 αβ in the gel was stable after long-term storage at 4°C for at least 180 days.

[0158] Heterogeneous glycopeptide intermediate *S. epidermidis* (hGISE) is common in prosthetic joint infections (PJIs). Glycopeptide treatment, such as treatment with vancomycin and teicoplanin, is not sufficient in many cases of PJIs. We found that PLNC8 αβ effectively inhibits different strains of *S. epidermidis* isolated from PJIs, including *S. epidermidis* (hGISE) (figure 14). The D-form of PLNC8 αβ is almost as effective as the L-form in inhibiting strain *S. epidermidis* 154 (Fig. 15).

[0159] Combination therapy is utilized both to prevent the development of antibiotic resistance and to shorten the length of treatment. The effect of the combination of L-PLNC8 αβ or D-PLNC8 αβ with different antibiotics belonging to different classes was also shown: the cell wall synthesis inhibitors vancomycin and teicoplanin, the nucleic acid synthesis inhibitor rifampicin and the protein synthesis inhibitor gentamicin, in the treatment *of S. epidermidis.*

[0160] Both L-PLNC8 αβ and D-PLNC8 αβ decreased MIC and MBC of teicoplanin more than 10-fold against *S. epidermidis* (Fig. 15). A combination of PLNC8 αβ and rifampicin was even more effective. MIC and MBC of rifampicin were lowered more than 100-fold when treating *S. epidermidis* in the presence of L-PLNC8 αβ or D-PLNC8 αβ (Fig. 15). Furthermore, L-PLNC8 αβ and D-PLNC8 αβ decreased MIC and MBC of gentamicin more than 30-fold against *S. epidermidis.* However, L-PLNC8 αβ or D-PLNC8 αβ lowered MIC and MBC of vancomycin 2-fold (Fig. 15).

[0161] A combination of the truncated α-peptide 1-22 with full-length β-peptide decreased MIC and MBC of teicoplanin more then 10-fold against *S. epidermidis* (Fig. 15), i.e. the same effects as with PLNC8 αβ (Fig. 14). α1-22 and β1-20 lowered MIC and MBC of teicoplanin approximately 4-fold, however, full-length α-peptide and β1-20 had no effects (Fig. 16). As can be seen in table 8 below, the full-length and truncated PLNC8 β and PLNC8 α markedly amplify the inhibitory and bactericidal effects of teicoplanin and rifampicin against *S. epidermidis.*

Table 8. Teicoplanin and rifampicin against *S. epidermidis*

Antimicrobial agent	MIC	MBC
Teicoplanin (μg/ml)	1.5	1.5
Rifampicin (μg/ml)	0.25	0.5

(continued)

Antimicrobial agent	MIC	MBC
α/B1-20(μM)	12.5	>50
Teicoplanin/α/β1-20 (6.25 μM)	0.78	1.5
Rifampicin/α/β1-20 (6.25 μM)	0.25	0.5
α1-22/β(μM)	25	50
Teicoplanin/α1-22/β (6.25 μM)	<0.097	0.39
Rifampicin/α1-22/β(6.25 μM)	0.063	0.25
α1-22/β1-20 (μM)	12.5	>50
Teicoplanin/α1-22/β1-20 (6.25 μM)	0.39	1.5
Rifampicin/α1-22/β1-20 (6.25 μM)	0.25	0.5

[0162] A combination of the truncated α-peptide 1-22 with full-length β-peptide decreased MIC of rifampicin approximately 4-fold against *S. epidermidis*. α1-22 and β1-20, respectively full-length α-peptide and β1-20, have 2-fold effect (Fig. 16).

[0163] PLNC8 αβ rapidly and markedly permeabilized and killed different species of *Streptococcus* (*S. mutans, S. constellatus* and *S. anginosus*). *S. constellatus* and *S. anginosus* were more susceptible to PLNC8 αβ than *S. mutans* (Fig. 17).

[0164] PLNC8 αβ dose-dependently and rapidly lysed and killed *S. aureus,* independent of their resistance to antibiotics (MSSA and MRSA) (Fig 18).

[0165] PLNC8 αβ promoted wound healing *in vitro* of human keratinocytes. determined using scratch assay. *S. aureus* increased IL-6 and CXCL8, however, these inflammatory mediators were not altered by PLNC8 αβ (Fig 19).

[0166] PLNC8 αβ antagonized *S*. aureus-mediated cytotoxicity and inflammatory responses, and promoted cell viability, of human keratinocytes. Secretion of IL-6 and CXCL8 were significantly reduced by the peptides, which was confirmed by gene expression analysis of *il-6* and *cxcl8*. Intracellular signaling events involve *c-jun* and *c-fos,* suggesting a role for the transcription factor AP-1 via MAPK (Fig. 20).

[0167] PLNC8 αβ promoted wound healing *in vitro* of human keratinocytes following an infection with *S. aureus* and reduced bacteria-induced secretion of IL-6 and CXCL8 (Fig.21).

[0168] PLNC8αβ inhibited infection and promoted wound healing *in vivo,* shown in a porcine wound healing model. The peptide, alone or in combination with gentamicin, antagonized the infection and promoted wound healing (Fig 22).

[0169] PLNC8 αβ effectively lysed *S. epidermidis* demonstrated by a dose-dependent release of ATP. (Fig 23)

[0170] PLNC8 β and PLNC8 αβ (1:1), but not PLNC8 α, of both the *L*-form and *D*-form, caused complete lysis of liposomes after 2 min (Fig.24)

[0171] CD-spectroscopy indicated that both *L*- and *D*-PLNC8 αβ has an ordered secondary structure in liposomes (Fig. 25).

[0172] IncuCyte live-cell analysis of keratinocytes infected by *S. aureus,* (MOI:1) showed that PLNC8 αβ prevented bacterial growth and protected the cells for up to 32 h. Bacterial growth without peptides reached maximum levels after 8-9 h The combination PLNC8 αβ/gentamicin efficiently eliminated *S. aureus* and prevented an infection, and subsequent cell death, over the entire experimental period (72 h) (Fig. 26).

[0173] IncuCyte live-cell analysis of keratinocytes infected by *S. aureus,* (MOI:0.1) showed that PLNC8 αβ prevented bacterial growth and protected the cells for up to 42 h. Bacterial growth without peptides reached maximum levels after 10 h. The combination PLNC8 αβ/gentamicin efficiently eliminated *S. aureus* and prevented an infection, and subsequent cell death, throughout the entire experimental period (72 h). (Fig. 27).

[0174] PLNC8αβ alone did not affect the growth of *Escherichia coli,* however a sub-MIC concentration of the peptides significantly enhanced the effects of different antibiotics (Table 4).

[0175] PLNC8αβ alone was both inhibitory and bactericidal against *Enterococcus faecium,* and addition of sub-MIC concentrations significantly enhanced the effects of different antibiotics (Table 5).

[0176] PLNC8αβ alone did not affect the growth of *Pseudomonas aeruginosa,* however, sub-MIC concentration of the peptides enhanced the effects of different antibiotics (Table 6).

References

[0177]

1) Khalaf, H., Nakka S., Sandén, C., Svärd, A., Scherbak, N., Hultenby, K., Aili, D., Bengtsson, T. (2016) Antibacterial

effects of Lactobacillus and bacteriocin PLNC8 αβ on the periodontal pathogen Porphyromonas gingivalis, BMC Microbiology, 18:88.
2) Bengtsson, T., Zhang, B., Selegård, R., Wiman, E., Aili, D., Khalaf, H. (2017). Dual action bacteriocin PLNC8 αβ through inhibition of Porphyomonas gingivalis infection and promotion of cell proliferation. Pathogens and Disease, 2017 Jun 12. Doi: 10.1093/femspd/ftx064

Claims

1. A pharmaceutical composition comprising a first and a second peptide, wherein the first peptide is a peptide of the bacteriocin PLNC8 αβ,

   wherein the peptide of the bacteriocin PLNC8 αβ is
   SVPTSVYTLGIKIL WSA YKHRKTIEKSFNKGFYH SEQ ID NO 2,
   wherein the second peptide is
   DLTTKLWSSWGYYLGKKARWNL SEQ ID NO 31,
   and wherein the pharmaceutical composition further comprises at least one antibiotic.

2. The pharmaceutical composition according to claim 1, wherein the antibiotic is selected from the group consisting of antibiotics that inhibit bacterial cell wall synthesis, antibiotics that inhibit nucleic acid synthesis and antibiotics that inhibit protein synthesis.

3. The pharmaceutical composition according to any one of the preceding claims, wherein the antibiotic is selected from the group consisting of gentamicin, rifampicin, ciprofloxacin, teicoplanin, levofloxacin, meropenem and vancomycin.

4. The pharmaceutical composition according to any one of the preceding claims, wherein at least 90% of the amino acids in the first peptide and/or second peptide are D-amino acid residues.

5. The pharmaceutical composition according to any one of the preceding claims, wherein the first and second peptides are present in a molar ratio of from between 5:1 to 1:20, preferably 1:1 to 1:7, most preferably 1:1.

6. The pharmaceutical composition according to any one of the preceding claims, wherein the pharmaceutical composition comprises between 100 nM to 50 μM of the first peptide and/or of the second peptide.

7. The pharmaceutical composition according to any one of the preceding claims, wherein the pharmaceutical composition comprises the antibiotic in an amount of between 0.002 μg/ml to 50 μg/ml, such as at least 0.01 μg/ml to 5 μg/ml, such as at least 0.1 μg/ml to 1 μg/ml, such as at least 0.8 μg/ml

8. The pharmaceutical composition according to any one of the preceding claims, wherein the composition is formulated as a solution, a cream, a gel, or an ointment or formulated in immobilized form as a coating on a device.

9. The pharmaceutical composition according to claim 8, wherein the composition is formulated:

   as a gel, wherein the gel further comprises gelatine and glycerol, or
   in immobilized form as a coating on a device, wherein the device is chosen from the group consisting of a wound dressing, an orthopedic implant, a dental implant, a urinary catheter and a urinary stent.

10. A pharmaceutical composition for use in the treatment or prophylaxis of a bacterial infection, wherein the pharmaceutical composition is a pharmaceutical composition according to anyone of claims 8 or 9.

11. The pharmaceutical composition for use according to claim 10, wherein the bacterial infection is caused by *Staphylococcus spp* (including MRSA, MRSE), *Streptococcus spp* (e.g. *S. mutans, S. constellatus, S. anginosus*), *Enterococcus faecium* (including VRE), *Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter spp* and/or *Escherichia coli.*

12. The pharmaceutical composition for use according to claim 10 or 11, wherein the composition is administered locally on the site of infection, such as topically.

13. Use of a pharmaceutical composition according to any one of claims 1 to 11 in coating at least part of a device to limit colonization of bacteria on the surface of the device.

14. Use of a pharmaceutical composition according to claim 13, wherein the device is a medical device, such as a prosthesis or a wound dressing.

15. Use of a pharmaceutical composition according to claim 13 or 14, wherein the bacteria are *Staphylococcus spp* (including MRSA, MRSE), *Streptococcus spp* (e.g. *S. mutans, S. constellatus, S. anginosus*), *Enterococcus faecium* (including VRE), *Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter spp* and/or *Escherichia coli.*

Patentansprüche

1. Pharmazeutische Zusammensetzung, die ein erstes und ein zweites Peptid umfasst, wobei das erste Peptid ein Peptid des Bakteriocins PLNC8 αβ ist,

   wobei das Peptid des Bakteriocins PLNC8 αβ SVPTSVYTLGIKILWSAYKHRKTIEKSFNKGFYH SEQ ID NO 2 ist,
   wobei das zweite Peptid DLTTKLWSSWGYYLGKKARWNL SEQ ID NO 31 ist,
   und wobei die pharmazeutische Zusammensetzung weiter mindestens ein Antibiotikum umfasst.

2. Pharmazeutische Zusammensetzung nach Anspruch 1, wobei das Antibiotikum aus der Gruppe ausgewählt ist, die aus Antibiotika besteht, die die bakterielle Zellwandsynthese hemmen, Antibiotika, die die Nukleinsäuresynthese hemmen, und Antibiotika, die die Proteinsynthese hemmen.

3. Pharmazeutische Zusammensetzung nach einem der vorstehenden Ansprüche, wobei das Antibiotikum aus der Gruppe ausgewählt ist, die aus Gentamicin, Rifampicin, Ciprofloxacin, Teicoplanin, Levofloxacin, Meropenem und Vancomycin besteht.

4. Pharmazeutische Zusammensetzung nach einem der vorstehenden Ansprüche, wobei mindestens 90 % der Aminosäuren in dem ersten Peptid und/oder den zweiten Peptid D-Aminosäurenreste sind.

5. Pharmazeutische Zusammensetzung nach einem der vorstehenden Ansprüche, wobei das erste und das zweite Peptid in einem molaren Verhältnis von zwischen 5:1 bis 1:20, bevorzugt 1:1 bis 1:7, am besten 1:1 vorhanden sind.

6. Pharmazeutische Zusammensetzung nach einem der vorstehenden Ansprüche, wobei die pharmazeutische Zusammensetzung zwischen 100 nM bis 50 μM des ersten Peptids und/oder des zweiten Peptids umfasst.

7. Pharmazeutische Zusammensetzung nach einem der vorstehenden Ansprüche, wobei die pharmazeutische Zusammensetzung das Antibiotikum in einer Menge von zwischen 0,002 μg/ml bis 50 μg/ml, wie etwa mindestens 0,01 μg/ml bis 5 μg/ml, wie etwa mindestens 0,1 μg/ml bis 1 μg/ml, wie etwa mindestens 0,8 μg/ml umfasst.

8. Pharmazeutische Zusammensetzung nach einem der vorstehenden Ansprüche, wobei die Zusammensetzung als eine Lösung, eine Creme, ein Gel, oder eine Salbe formuliert ist, oder in immobilisierter Form als eine Beschichtung auf einer Vorrichtung formuliert ist.

9. Pharmazeutische Zusammensetzung nach Anspruch 8, wobei die Zusammensetzung wie folgt formuliert ist:

   als ein Gel, wobei das Gel weiter Gelatine und Glyzerin umfasst, oder
   in immobilisierter Form als eine Beschichtung auf einer Vorrichtung, wobei die Vorrichtung aus der Gruppe ausgewählt ist, die aus einem Wundverband, einem orthopädischen Implantat, einem Zahnimplantat, einem Harnkatheter oder einem Harnstent besteht.

10. Pharmazeutische Zusammensetzung zur Verwendung bei der Behandlung oder Prophylaxe einer bakteriellen Infektion, wobei die pharmazeutische Zusammensetzung eine pharmazeutische Zusammensetzung nach einem der Ansprüche 8 oder 9 ist.

11. Pharmazeutische Zusammensetzung zur Verwendung nach Anspruch 10,
wobei die bakterielle Infektion eine Infektion ist, die von *Staphylococcus* spp (einschließlich MRSA, MRSE), *Streptococcus spp* (z. B. *S. mutans, S. constellatus, S. anginosus*), *Enterococcus faecium* (einschließlich VRE), *Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter spp und/oder Escherichia coli verursacht wird.*

12. Pharmazeutische Zusammensetzung zur Verwendung nach Anspruch 10 oder 11,
wobei die Zusammensetzung lokal an der Infektionsstelle, wie etwa topisch, verabreicht wird.

13. Verwendung einer pharmazeutischen Zusammensetzung nach einem der Ansprüche 1 bis 11 zum Beschichten mindestens eines Teils einer Vorrichtung, um die Besiedelung mit Bakterien auf der Oberfläche der Vorrichtung einzuschränken.

14. Verwendung einer pharmazeutischen Zusammensetzung nach Anspruch 13, wobei
die Vorrichtung eine medizinische Vorrichtung, wie eine Prothese oder ein Wundverband, ist.

15. Verwendung einer pharmazeutischen Zusammensetzung nach Anspruch 13 oder 14,
wobei die Bakterien *Staphylococcus spp* (einschließlich MRSA, MRSE), *Streptococcus spp* (z. B. *S. mutans, S. constellatus, S. anginosus*), *Enterococcus faecium* (einschließlich VRE), *Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter spp und/oder Escherichia coli sind.*

Revendications

1. Composition pharmaceutique comprenant un premier et un second peptide, dans laquelle le premier peptide est un peptide de la bactériocine PLNC8 αβ,

dans laquelle le peptide de la bactériocine PLNC8 αβ est SVPTSVYTLGIKILWSAYKHRKTIEKSFNKGFYH SEQ ID N° 2,
dans laquelle le second peptide est DLTTKLWSSWGYYLGKKARWNL SEQ ID N° 31,
et dans laquelle la composition pharmaceutique comprend en outre au moins un antibiotique.

2. Composition pharmaceutique selon la revendication 1, dans laquelle l'antibiotique est sélectionné dans le groupe constitué des antibiotiques qui inhibent la synthèse des parois cellulaires bactériennes, des antibiotiques qui inhibent la synthèse des acides nucléiques et des antibiotiques qui inhibent la synthèse des protéines.

3. Composition pharmaceutique selon l'une quelconque des revendications précédentes, dans laquelle l'antibiotique est sélectionné dans le groupe constitué de la gentamicine, de la rifampicine, de la ciprofloxacine, de la téicoplanine, de la lévofloxacine, du méropénème et de la vancomycine.

4. Composition pharmaceutique selon l'une quelconque des revendications précédentes, dans laquelle au moins 90 % des acides aminés dans le premier peptide et/ou le second peptide sont des résidus d'acides D-aminés.

5. Composition pharmaceutique selon l'une quelconque des revendications précédentes, dans laquelle les premier et second peptides sont présents en un rapport molaire entre 5:1 à 1:20, de préférence 1:1 à 1:7, le plus préférentiellement de 1:1.

6. Composition pharmaceutique selon l'une quelconque des revendications précédentes, dans laquelle la composition pharmaceutique comprend entre 100 nM à 50 μM du premier peptide et/ou du second peptide.

7. Composition pharmaceutique selon l'une quelconque des revendications précédentes, dans laquelle la composition pharmaceutique comprend l'antibiotique en une quantité entre 0,002 μg/ml à 50 μg/ml, telle qu'au moins 0,01 μg/ml à 5 μg/ml, telle qu'au moins 0,1 μg/ml à 1 μg/ml, telle qu'au moins 0,8 μg/ml

8. Composition pharmaceutique selon l'une quelconque des revendications précédentes, dans laquelle la composition est formulée sous la forme d'une solution, d'une crème, d'un gel ou d'un onguent ou formulée sous une forme immobilisée en tant que revêtement sur un dispositif.

9. Composition pharmaceutique selon la revendication 8, dans laquelle la composition est formulée :

sous la forme d'un gel, dans laquelle le gel comprend en outre de la gélatine et du glycérol, ou
sous une forme immobilisée en tant que revêtement sur un dispositif, dans laquelle le dispositif est choisi dans le groupe constitué d'un pansement, d'un implant orthopédique, d'un implant dentaire, d'un cathéter urinaire et d'un stent urinaire.

10. Composition pharmaceutique pour une utilisation dans le traitement ou la prophylaxie d'une infection bactérienne, dans laquelle la composition pharmaceutique est une composition pharmaceutique selon l'une quelconque des revendications 8 ou 9.

11. Composition pharmaceutique pour une utilisation selon la revendication 10,
dans laquelle l'infection bactérienne est provoquée par *les espèces Staphylococcus* (y compris MRSA, MRSE), *les espèces Streptococcus* (par exemple *S. mutans, S. constellatus, S. anginosus*), *Enterococcus faecium* (y compris VRE), *Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, les espèces Enterobacter et/ou Escherichia coli.*

12. Composition pharmaceutique pour une utilisation selon la revendication 10 ou 11,
dans laquelle la composition est administrée localement sur le site d'infection, tel que par voie topique.

13. Utilisation d'une composition pharmaceutique selon l'une quelconque des revendications 1 à 11 dans le revêtement d'au moins une partie d'un dispositif pour limiter la colonisation de bactéries sur la surface du dispositif.

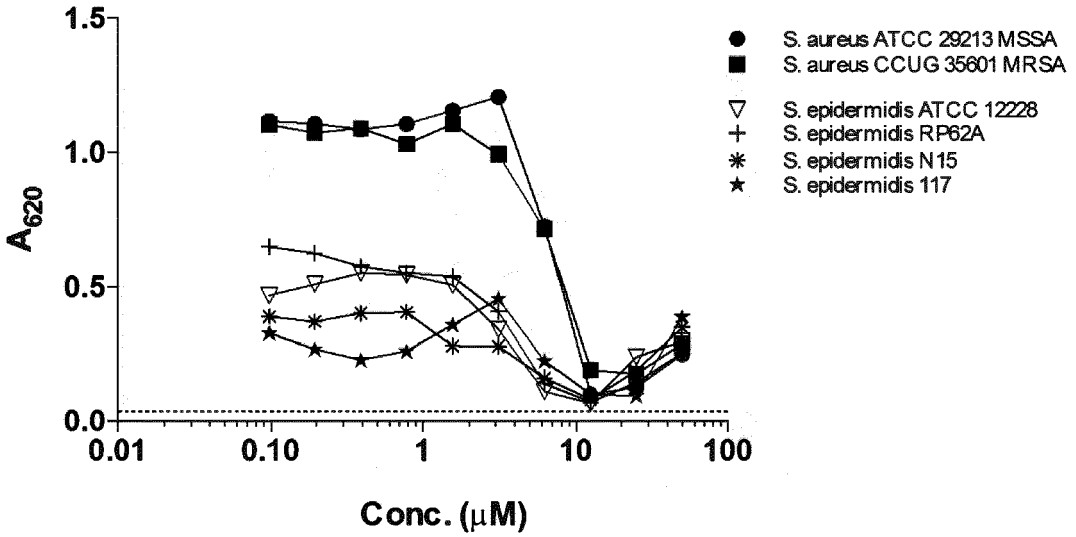
14. Utilisation d'une composition pharmaceutique selon la revendication 13, dans laquelle
le dispositif est un dispositif médical, tel qu'une prosthèse ou un pansement.

15. Utilisation d'une composition pharmaceutique selon la revendication 13 ou 14,
dans laquelle les bactéries sont *les espèces Staphylococcus* (y compris MRSA, MRSE), *les espèces Streptococcus* (par exemple *S. mutans, S. constellatus, S. anginosus*), *Enterococcus faecium* (y compris VRE), *Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, les espèces Enterobacter et/ou Escherichia coli.*

Figure 1

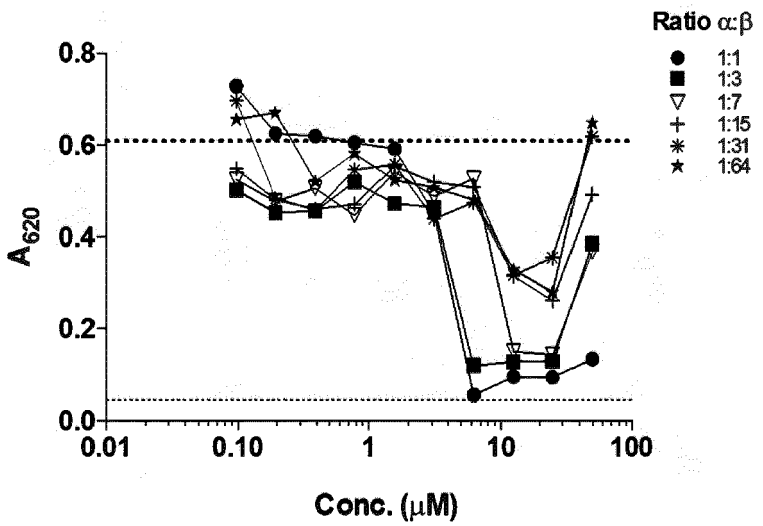
Table 1

Bacteria	Characteristics
S. aureus ATCC 29213 (MSSA)	Methicillin sensitive
S. aureus CCUG 35601 (MRSA)	Methicillin resistant
S. epidermidis ATCC 12228	Biofilm negative
S. epidermidis RP62A	Biofilm positive
S. epidermidis N15	Isolated from nose of a healthy indivual
S. epidermidis 117	Isolated from an infected hip joint prosthesis



L-PLNC8 αβ	MIC	MBC
S. aureus ATCC 29213 (MSSA)	12.5	25
S. aureus CCUG 35601 (MRSA)	12.5	25
S. epidermidis ATCC 12228	6.25	12.5
S. epidermidis RP62A	6.25	6.25
S. epidermidis N15	6.25	6.25
S. epidermidis 117	12.5	12.5

Figure 2



Ratio α:β*	MIC	MBC
1:1	6.25	12.5
1:3	6.25	25
1:7	12.5	50
1:15	50	>50
1:31	50	>50
1:64	50	>50

Figure 3

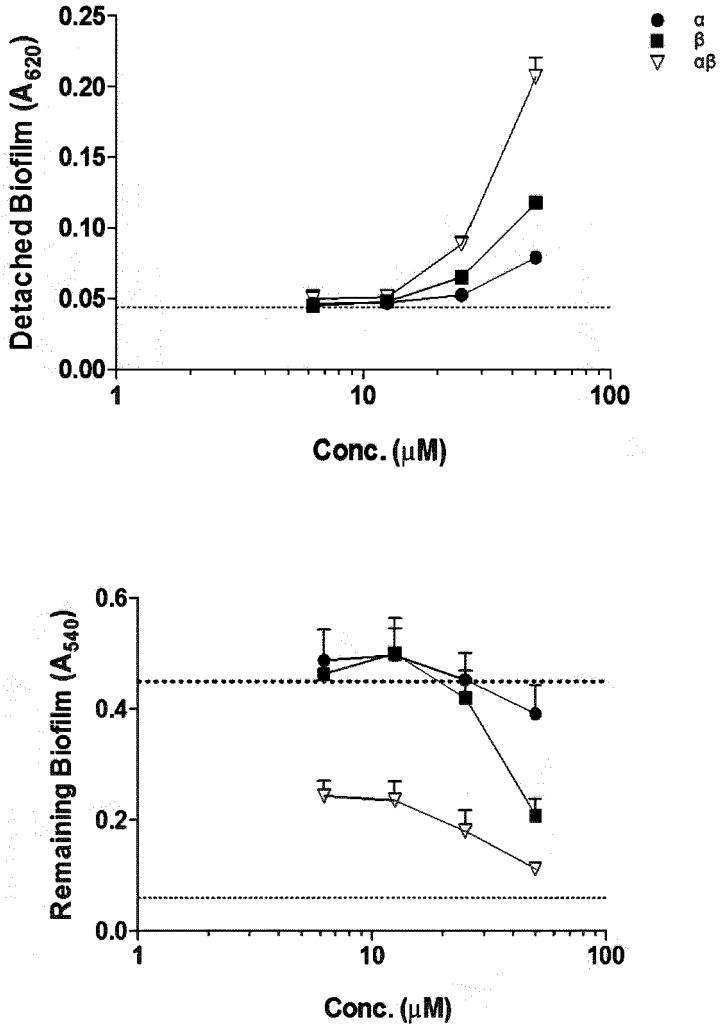
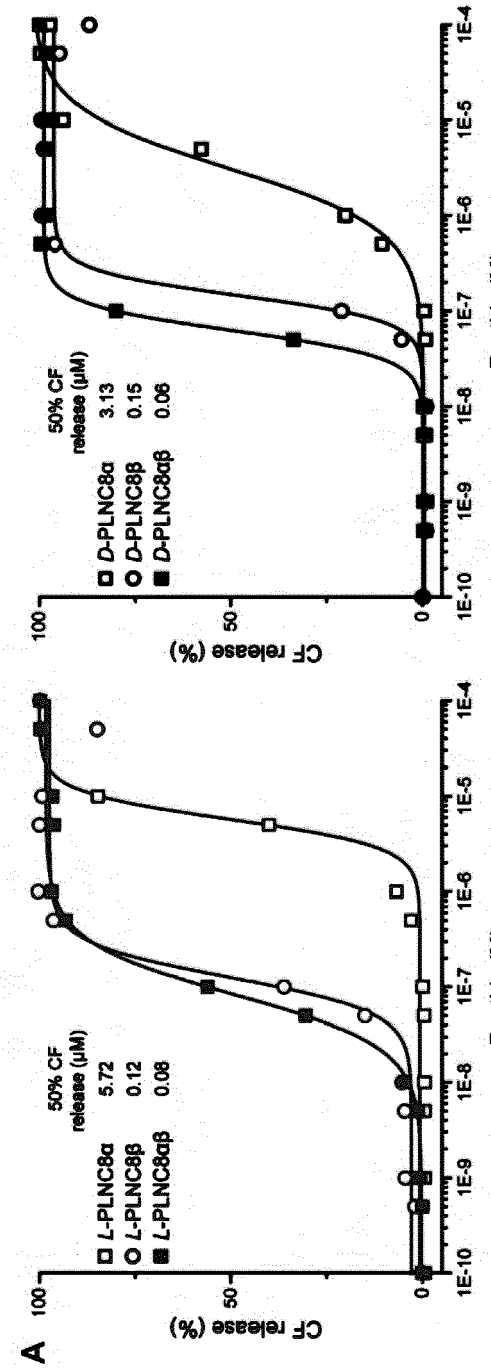

Detached Biofilm (A_{620})
0.25
0.20
0.15
0.10
0.05
0.00
1
10
100
Conc. (μM)
α
β
αβ
Remaining Biofilm (A_{540})
0.6
0.4
0.2
0.0
1
10
100
Conc. (μM)

Figure 4



B

L-PLNC8	MIC (μM)	MBC (μM)
α	50	>50
β	50	>50
αβ	6.25	12.5

D-PLNC8	MIC (μM)	MBC (μM)
α	>50	>50
β	50	>50
αβ	12.5	12.5

Figure 5

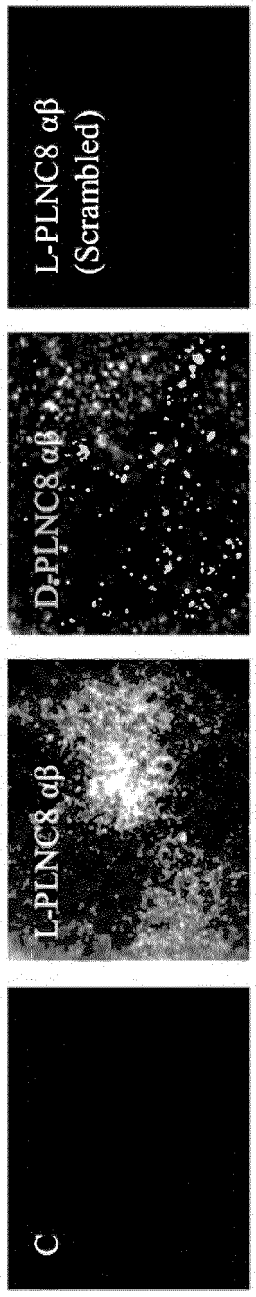


Figure 6

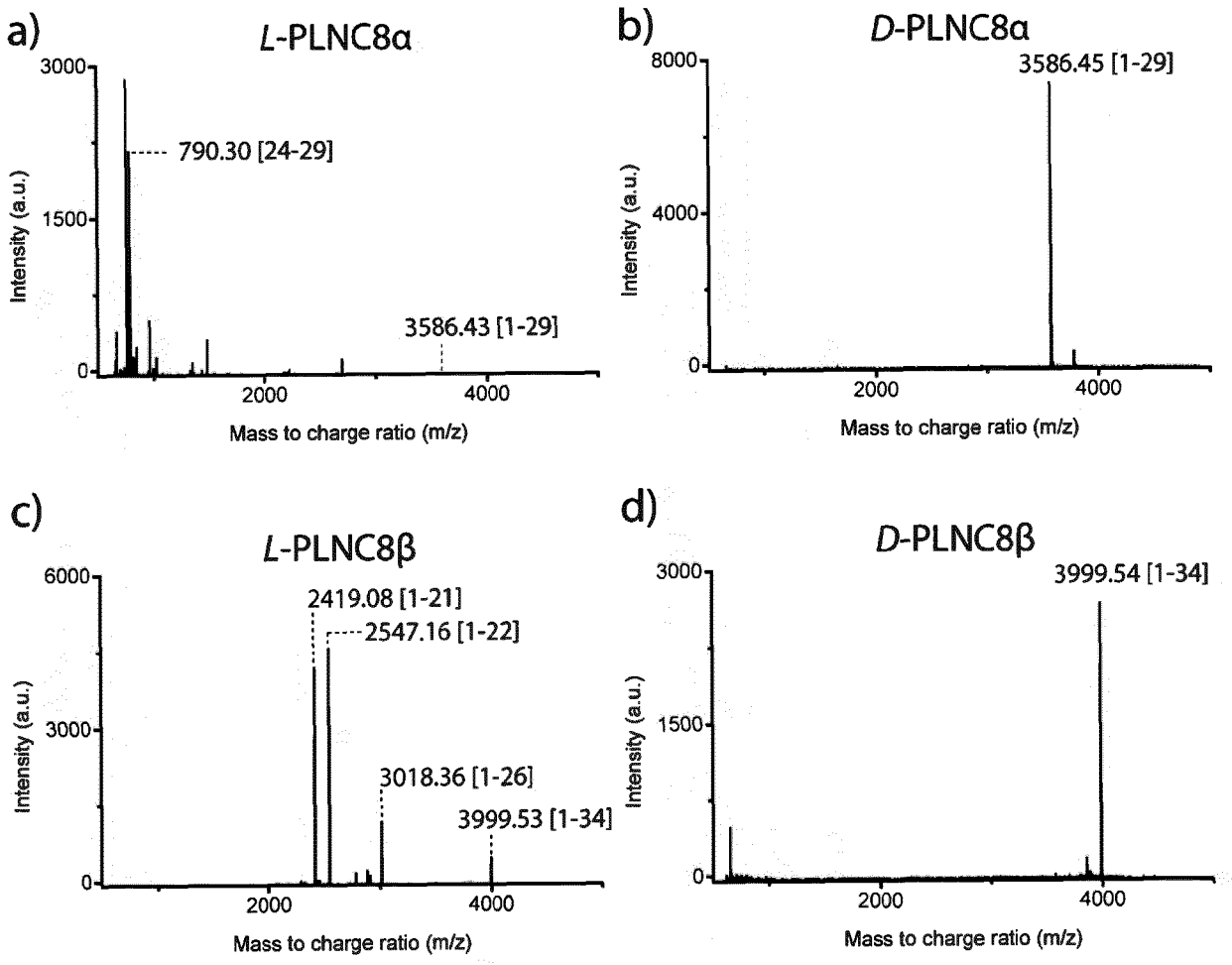

a)
L-PLNC8α
3000
1500
0
Intensity (a.u.)
790.30 [24-29]
3586.43 [1-29]
2000
4000
Mass to charge ratio (m/z)
b)
D-PLNC8α
8000
4000
0
Intensity (a.u.)
3586.45 [1-29]
2000
4000
Mass to charge ratio (m/z)
c)
L-PLNC8β
6000
3000
0
Intensity (a.u.)
2419.08 [1-21]
2547.16 [1-22]
3018.36 [1-26]
3999.53 [1-34]
2000
4000
Mass to charge ratio (m/z)
d)
D-PLNC8β
3000
1500
0
Intensity (a.u.)
3999.54 [1-34]
2000
4000
Mass to charge ratio (m/z)

Figure 7

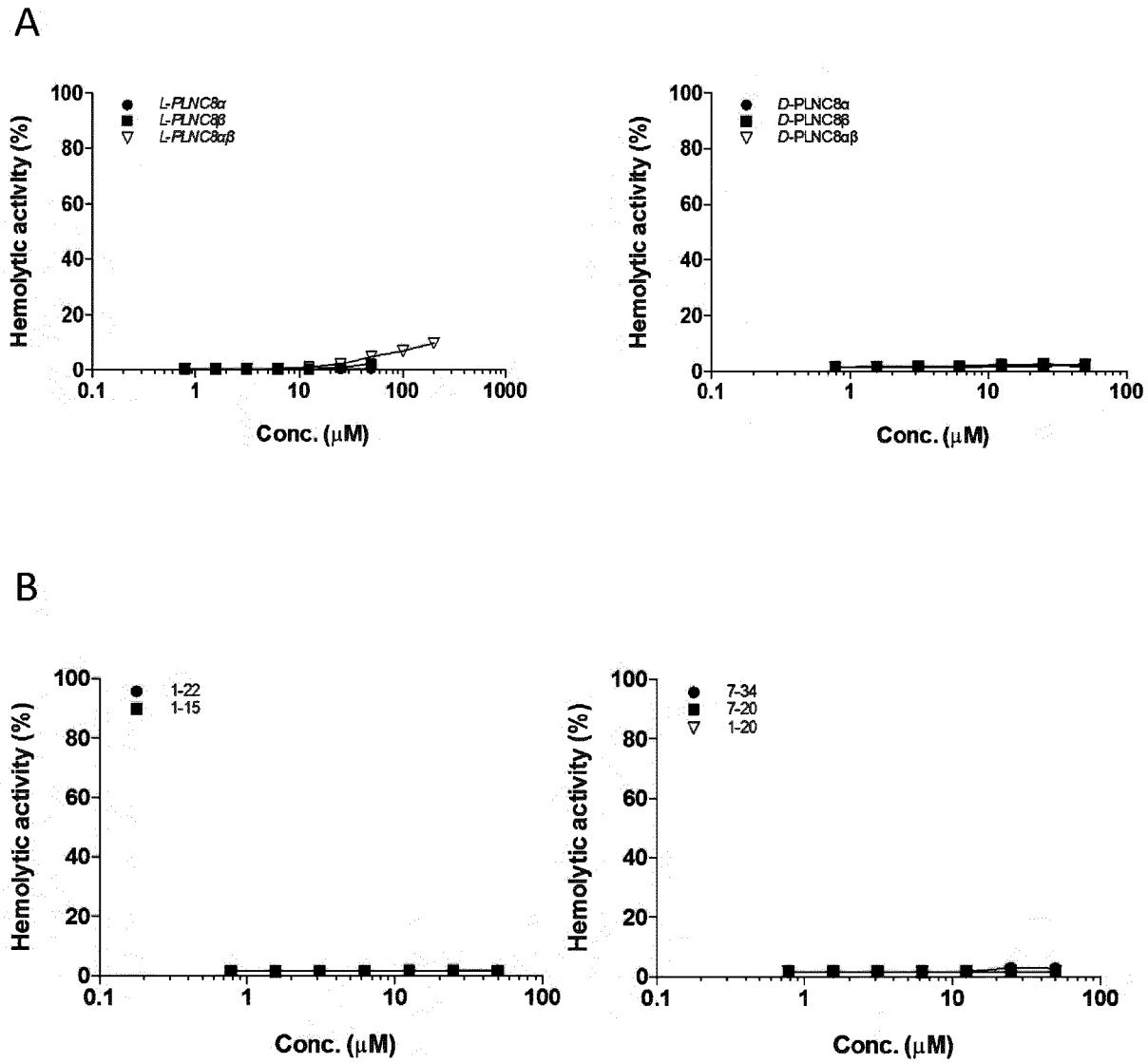

A
L-PLNC8α
L-PLNC8β
L-PLNC8αβ
Hemolytic activity (%)
Conc. (μM)
D-PLNC8α
D-PLNC8β
D-PLNC8αβ
Hemolytic activity (%)
Conc. (μM)
B
1-22
1-15
Hemolytic activity (%)
Conc. (μM)
7-34
7-20
1-20
Hemolytic activity (%)
Conc. (μM)

Figure 8

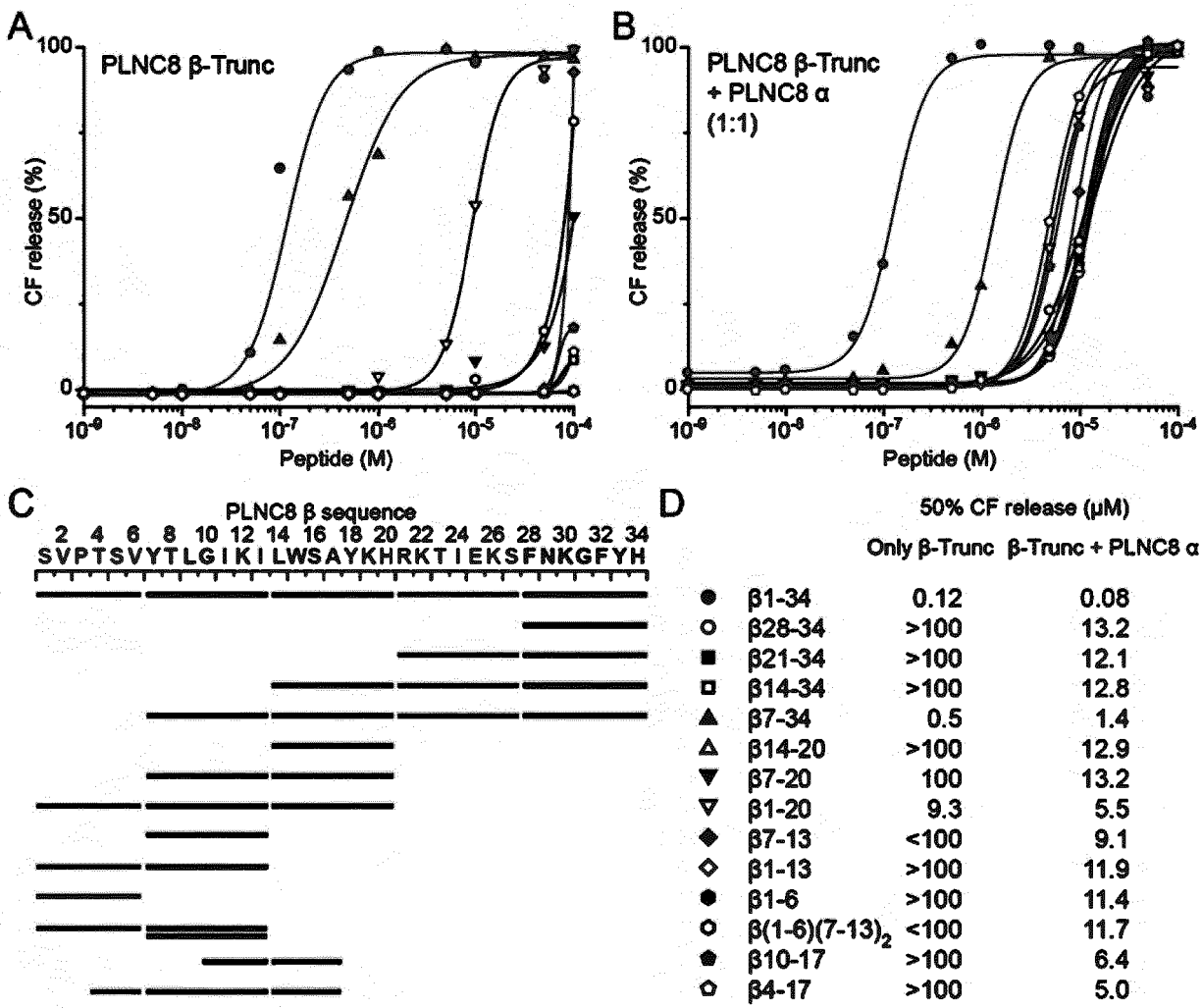
PLNC8α

Peptide ID	MW	H_2N- 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29 -COOH
23-29	918.05																							K	H	P	Y	V	Q	F
16-29	1815.13																K	K	A	R	W	N	L	K	H	P	Y	V	Q	F
9-29	2642.02									S	W	G	Y	Y	L	G	K	K	A	R	W	N	L	K	H	P	Y	V	Q	F
1-29	3587.09	D	L	T	T	K	L	W	S	S	W	G	Y	Y	L	G	K	K	A	R	W	N	L	K	H	P	Y	V	Q	F
16-22	915.09																K	K	A	R	W	N	L							
9-22	1741.99									S	W	G	Y	Y	L	G	K	K	A	R	W	N	L							
1-22	2687.06	D	L	T	T	K	L	W	S	S	W	G	Y	Y	L	G	K	K	A	R	W	N	L							
9-15	844.91									S	W	G	Y	Y	L	G														
1-15	1789.98	D	L	T	T	K	L	W	S	S	W	G	Y	Y	L	G														
1-8	963.08	D	L	T	T	K	L	W	S																					

PLNC8β

Peptide ID	MW	H_2N- 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34 -COOH
28-34	912																												F	N	K	G	F	Y	H
21-34	1754.98																					R	K	T	I	E	K	S	F	N	K	G	F	Y	H
14-34	2640.99														L	W	S	A	Y	K	H	R	K	T	I	E	K	S	F	N	K	G	F	Y	H
7-34	3429.96							Y	T	L	G	I	K	I	L	W	S	A	Y	K	H	R	K	T	I	E	K	S	F	N	K	G	F	Y	H
14-20	904.02														L	W	S	A	Y	K	H														
7-20	1693.00							Y	T	L	G	I	K	I	L	W	S	A	Y	K	H														
1-20	2263.63	S	V	P	T	S	V	Y	T	L	G	I	K	I	L	W	S	A	Y	K	H														
1-34	4000.6	S	V	P	T	S	V	Y	T	L	G	I	K	I	L	W	S	A	Y	K	H	R	K	T	I	E	K	S	F	N	K	G	F	Y	H
7-13	806.99							Y	T	L	G	I	K	I																					
$(1\text{-}6)(7\text{-}13)_2$	2166.6	S	V	P	T	S	V	Y	T	L	G	I	K	I	Y	T	L	G	I	K	I														
1-6	588.65	S	V	P	T	S	V																												
1-13	1377.62	S	V	P	T	S	V	Y	T	L	G	I	K	I																					
10-17	887.08										G	I	K	I	L	W	S	A																
4-17	1551.82				T	S	V	Y	T	L	G	I	K	I	L	W	S	A																	

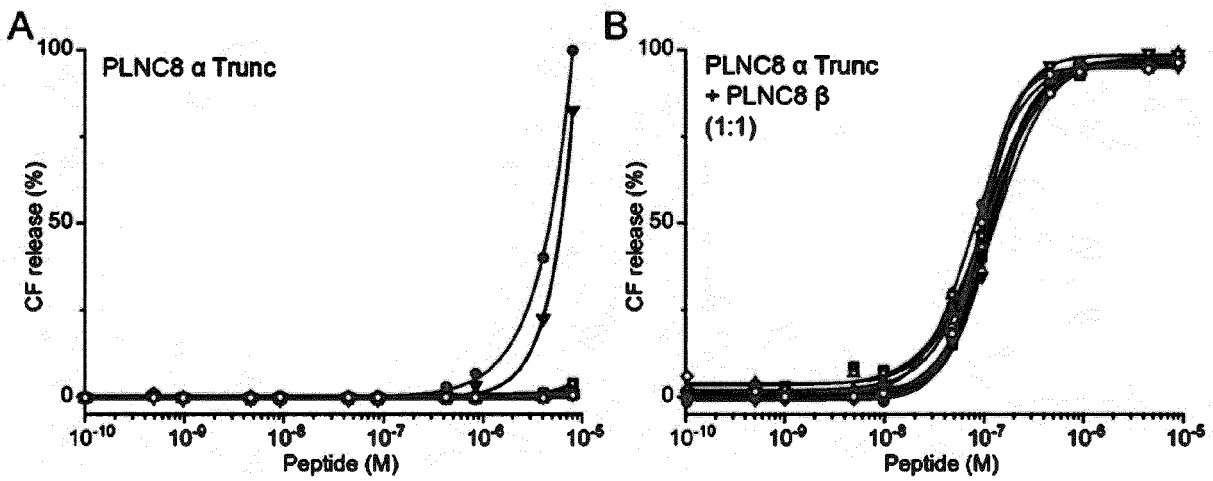
Figure 9



E

Peptide	MIC (μM)	MBC (μM)	MIC/α (μM)	MBC/α (μM)
28-34	>50	>50	>50	>50
21-34	>50	>50	>50	>50
14-34	>50	>50	>50	>50
7-34	50	>50	12.5	25
14-20	>50	>50	>50	>50
7-20	25	>50	25	>50
1-20	12.5	>50	12.5	>50
1-34	50	>50	6.25	12.5
7-13	>50	>50	>50	>50
1-6	>50	>50	>50	>50
1-13	>50	>50	>50	>50
$(1\text{-}6)(7\text{-}13)_2$	>50	>50	>50	>50
10-17	>50	>50	>50	>50
4-17	>50	>50	>50	>50

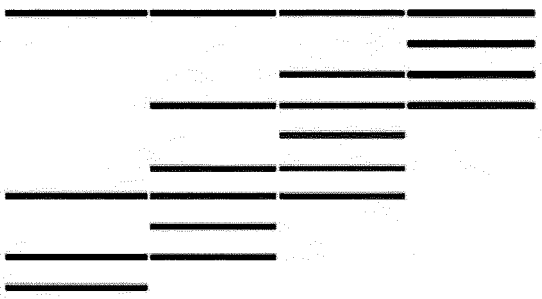
Figure 10



C

PLNC8 α sequence

2	4	6	8	10	12	14	16	18	20	22	24	26	28
D	L	T	T	K	L	W	S	S	W	G	Y	Y	L	G	K	K	A	R	W	N	L	K	H	P	Y	V	Q	F



D

		50% CF release (μM)	
		Only α-Trunc	α-Trunc + PLNC8 β
●	α1-29	5.8	0.08
○	α23-29	>10	0.13
■	α16-29	>10	0.12
□	α9-29	>10	0.11
▲	α16-22	>10	0.11
△	α9-22	>10	0.11
▼	α1-22	7.6	0.13
▽	α9-15	>10	0.11
◆	α1-15	>10	0.09
◇	α1-8	>10	0.10

E

Peptide	MIC (μM)	MBC (μM)	MIC/β (μM)	MBC/β (μM)
23-29	>50	>50	>50	>50
16-29	>50	>50	>50	>50
9-29	>50	>50	50	>50
1-29	>50	>50	6.25	12.5
16-22	>50	>50	>50	>50
9-22	>50	>50	50	>50
1-22	>50	>50	25	25
9-15	>50	>50	50	>50
1-15	>50	>50	>50	>50
1-8	>50	>50	>50	>50

Figure 11

Peptide (1:1)	MIC	MBC
α1-22/β1-20	12.5	>50
α1-22/β7-20	25	>50
α1-15/β1-20	25	>50
α1-15/β7-20	25	>50

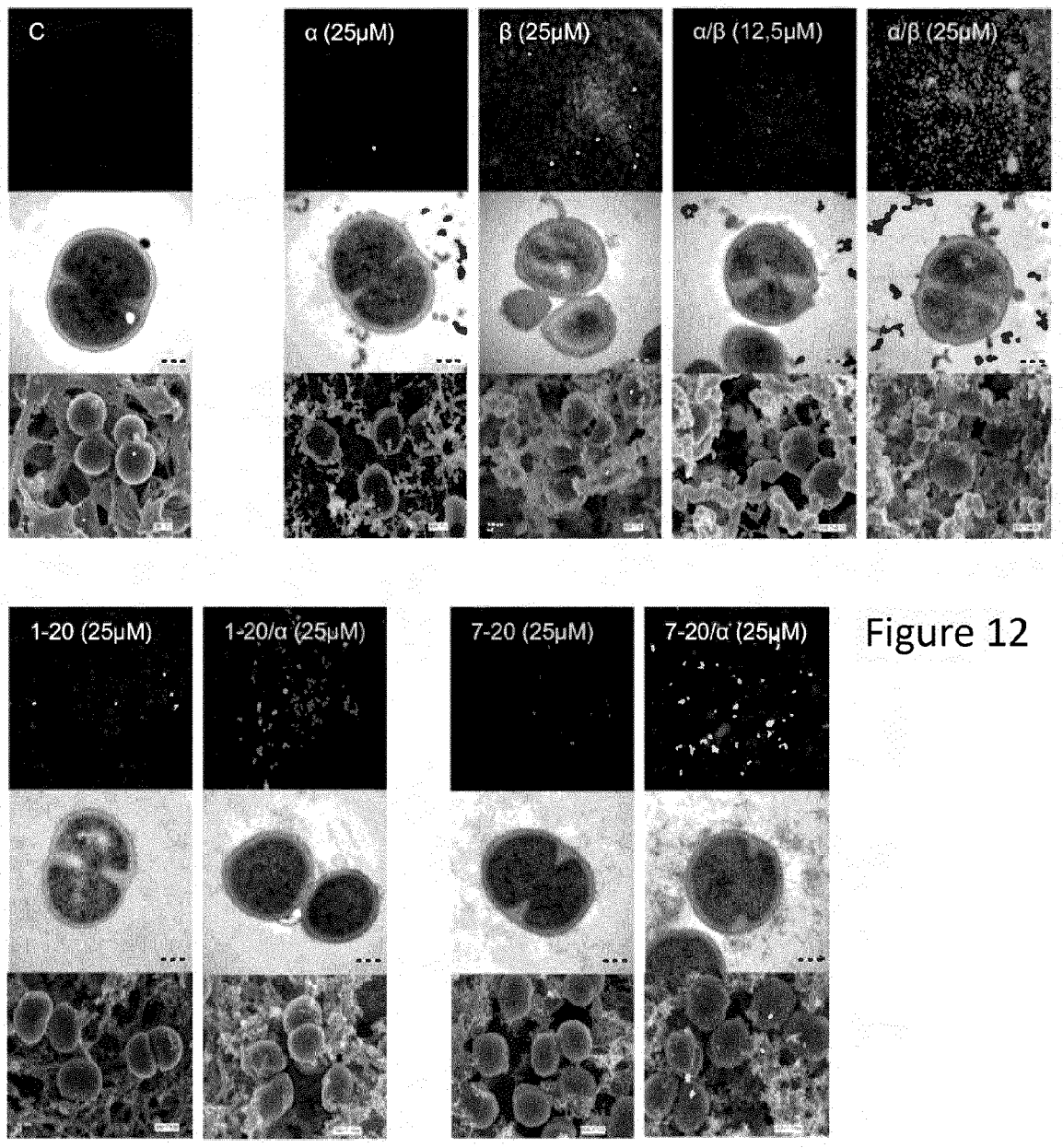

C
α (25μM)
β (25μM)
α/β (12,5μM)
α/β (25μM)
1-20 (25μM)
1-20/α (25μM)
7-20 (25μM)
7-20/α (25μM)
Cell wall (nm)
Fluorescence intensity (AU)
C
α
β
αβ 12.5μM
αβ 25μM
β1-20
β1-20/α
β7-20
β7-20/α

Figure 12

Figure 13

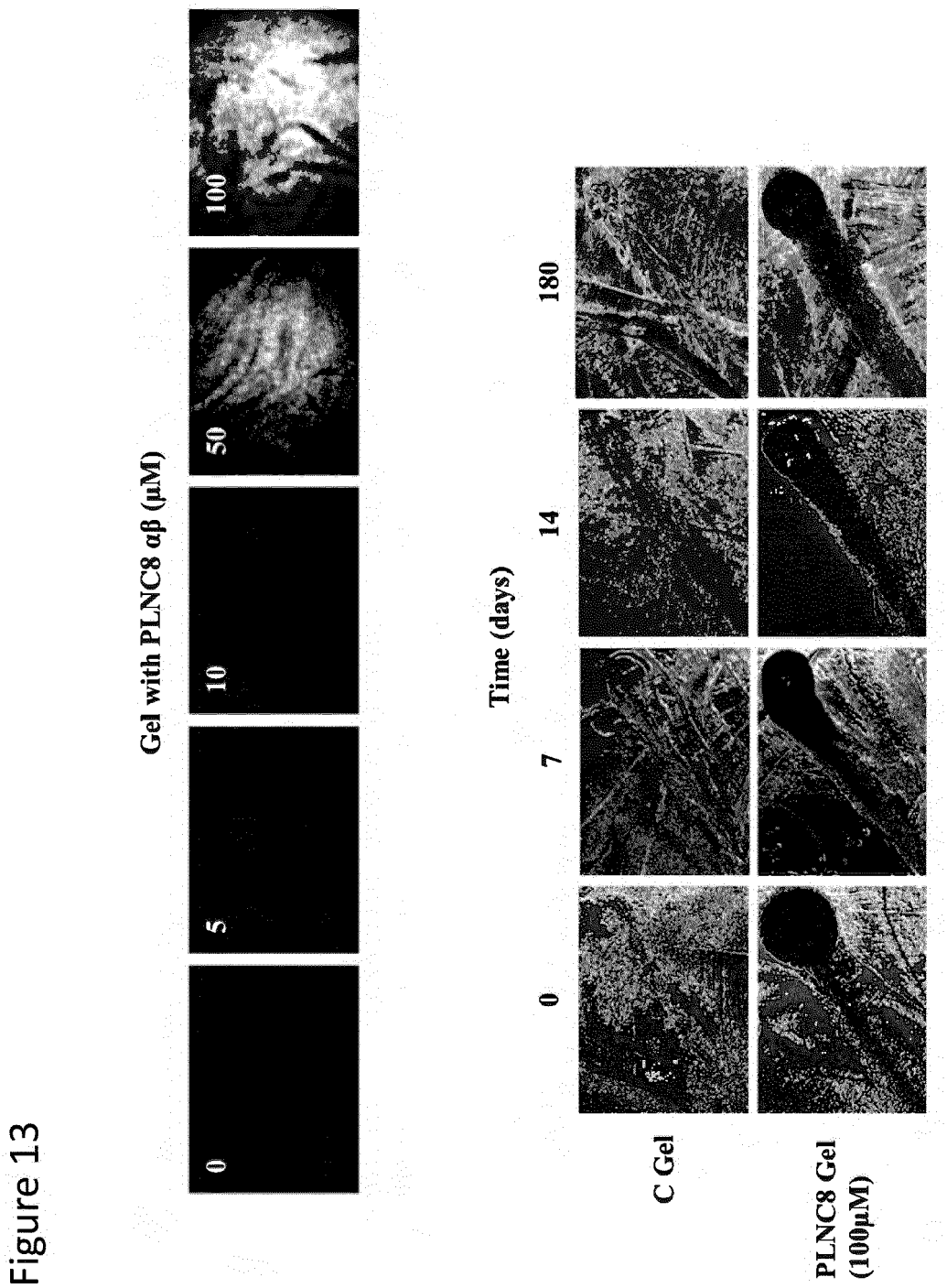

Gel with PLNC8 αβ (μM)
0
5
10
50
100
Time (days)
0
7
14
180
C Gel
PLNC8 Gel (100μM)

Figure 14

	Strain	MIC	MBC
hGISE	157	12.5	>50
	126	12.5	50
	145	6.25	>50
	109	6.25	>50
	127	12.5	50
	117	6.25	25
	154	6.25	12.5
	138	6.25	6.25
	152	6.25	12.5
	124	6.25	6.25

Figure 15

Antimicrobial agent	*S. epidermidis* ATCC 12228 MIC	MBC
L-PLNC8 αβ (μM)	6.25	12.5
D-PLNC8 αβ (μM)	12.5	12.5
Vancomycin (μg/ml)	1.5	3.1
Vancomycin/*L*-PLNC8 αβ	0.78	1.5
Vancomycin/*D*-PLNC8 αβ	0.78	1.5
Teicoplanin (μg/ml)	1.5	1.5
Teicoplanin/*L*-PLNC8 αβ	<0.097	<0.097
Teicoplanin/*D*-PLNC8 αβ	<0.097	<0.097
Rifampicin (μg/ml)	0.25	0.5
Rifampicin/*L*-PLNC8 αβ	<0.0019	<0.0019
Rifampicin/*D*-PLNC8 αβ	0.0019	0.0019
Gentamicin (μg/ml)	0.31	0.31
Gentamicin/*L*-PLNC8 αβ	<0.0097	<0.0097
Gentamicin/*D*-PLNC8 αβ	<0.0097	<0.0097

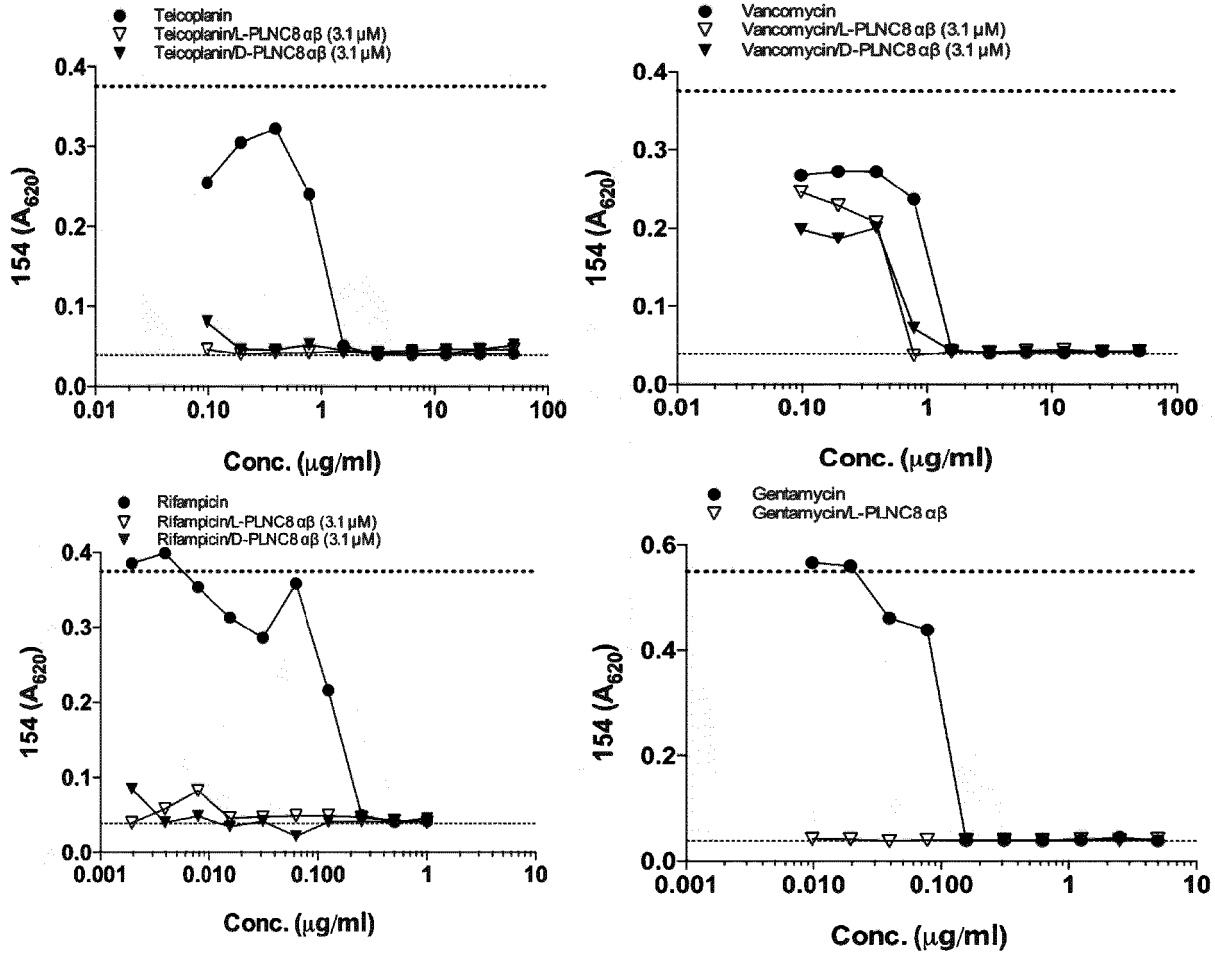


Figure 16

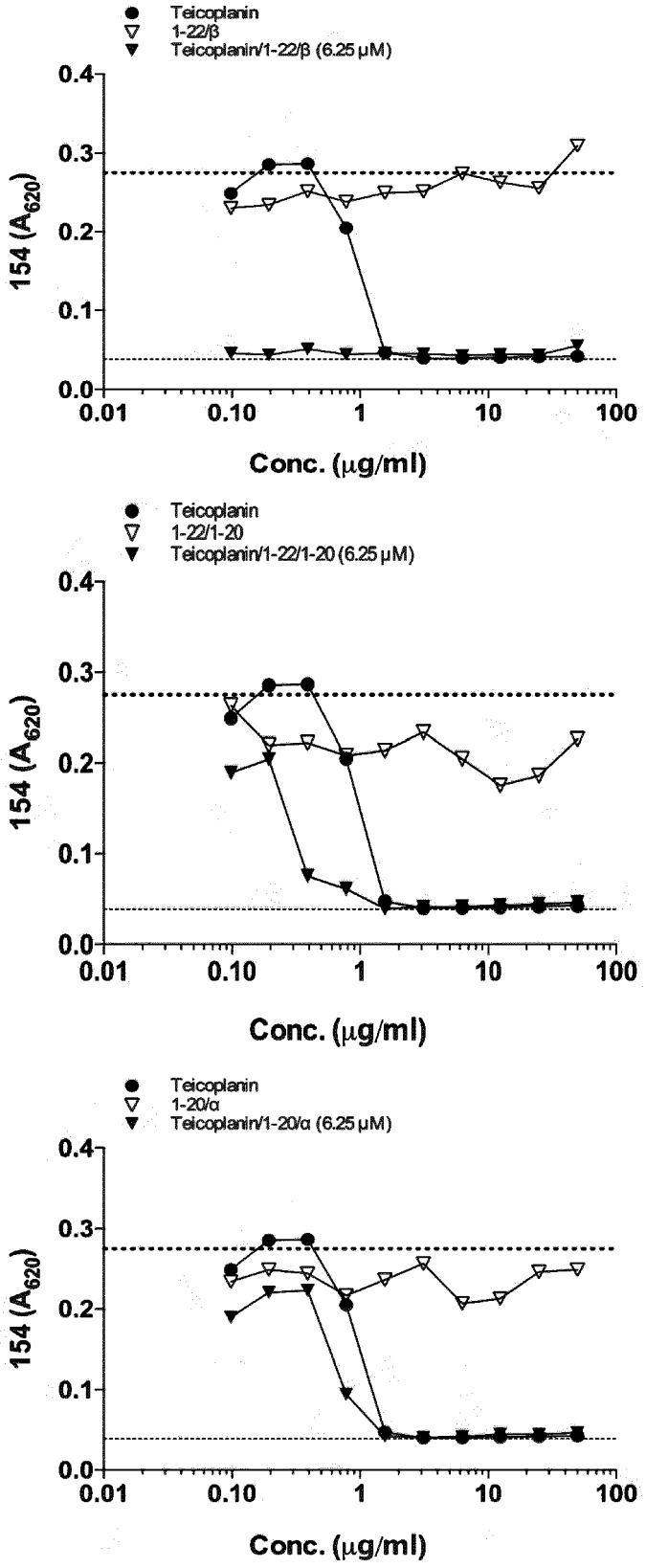

Teicoplanin
1-22/β
Teicoplanin/1-22/β (6.25 μM)
154 (A620)
0.4
0.3
0.2
0.1
0.0
0.01
0.10
1
10
100
Conc. (μg/ml)
Teicoplanin
1-22/1-20
Teicoplanin/1-22/1-20 (6.25 μM)
154 (A620)
Conc. (μg/ml)
Teicoplanin
1-20/α
Teicoplanin/1-20/α (6.25 μM)
154 (A620)
Conc. (μg/ml)

Figure 17

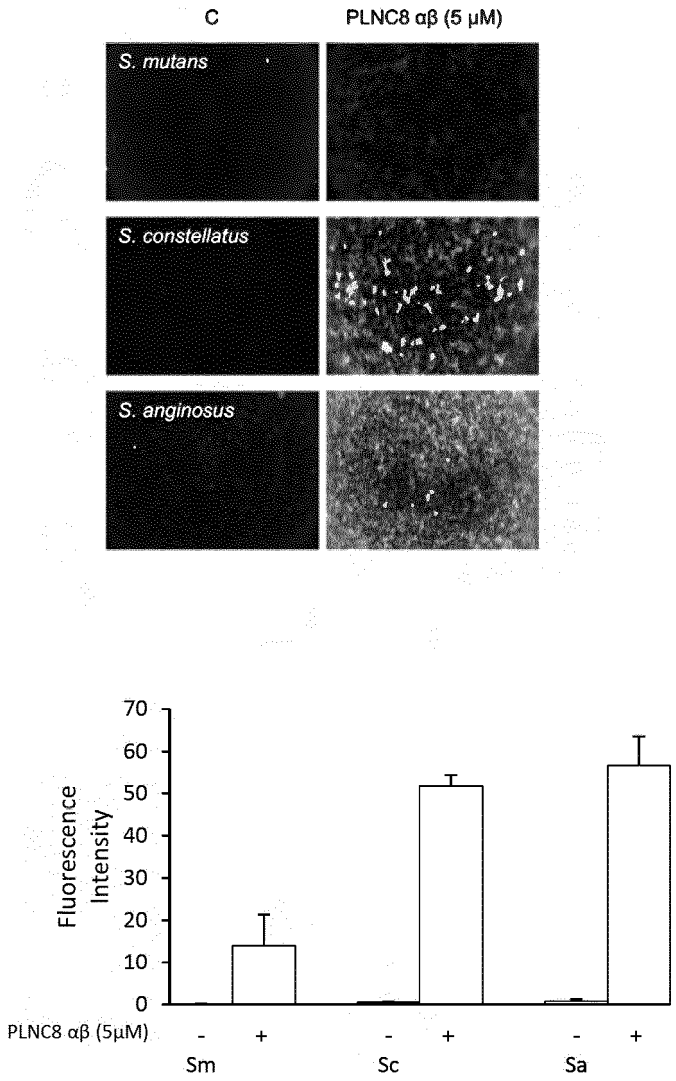

C
PLNC8 αβ (5 μM)
S. mutans
S. constellatus
S. anginosus
Fluorescence Intensity
70
60
50
40
30
20
10
0
PLNC8 αβ (5μM)
-
+
-
+
-
+
Sm
Sc
Sa

Figure 18

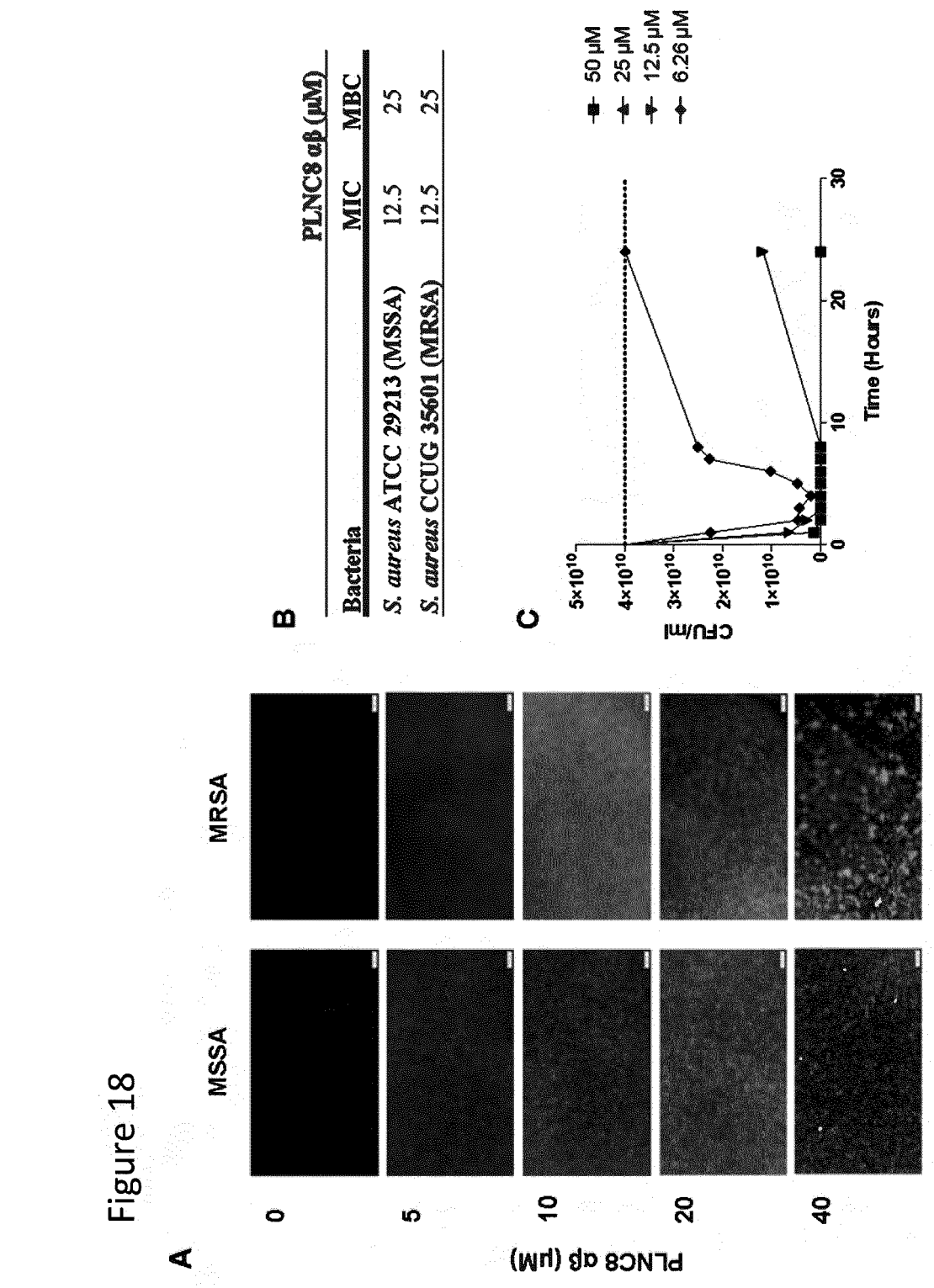

A
MSSA
MRSA
PLNC8 αβ (μM)
0
5
10
20
40
B
PLNC8 αβ (μM)
Bacteria
MIC
MBC
S. aureus ATCC 29213 (MSSA)
12.5
25
S. aureus CCUG 35601 (MRSA)
12.5
25
C
50 μM
25 μM
12.5 μM
6.26 μM
CFU/ml
5×10^10
4×10^10
3×10^10
2×10^10
1×10^10
0
0
10
20
30
Time (Hours)

Figure 19

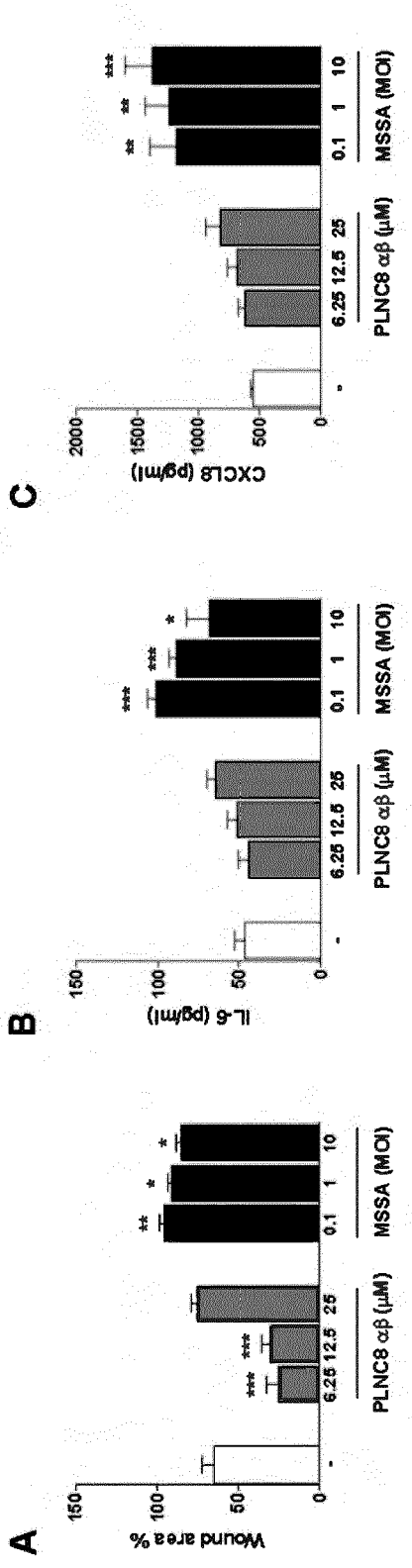
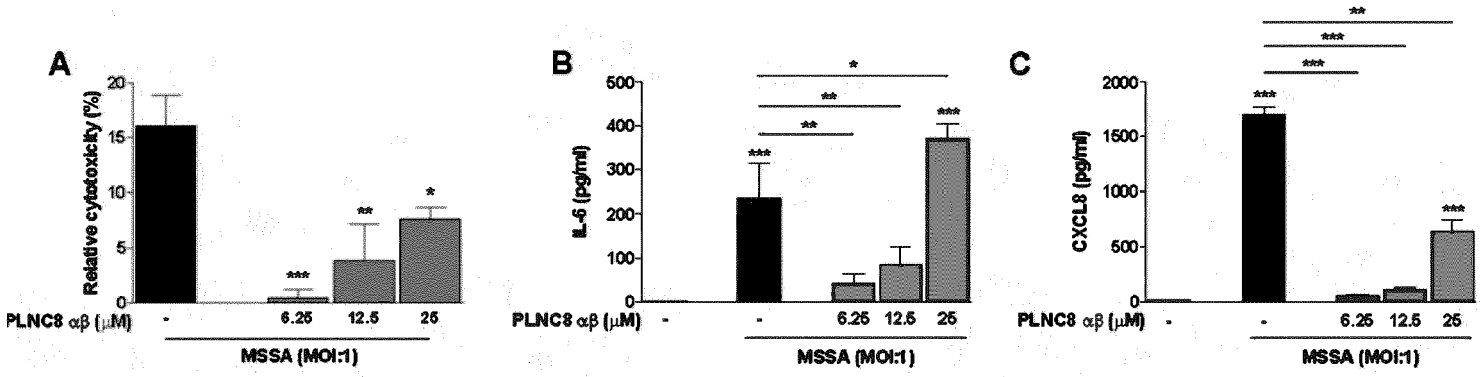

A
Wound area %
150
100
50
0
-
6.25
12.5
25
PLNC8 αβ (μM)
0.1
1
10
MSSA (MOI)
B
IL-6 (pg/ml)
C
CXCL8 (pg/ml)
2000
1500
1000
500

Figure 20



D

	MSSA (MOI:1)				
		PLNC8αβ (μM)			
Gene	-	-	6.25	12.5	25
il-6	1.0±0.15	2.8±0.18	2.9±0.34	4.3±0.31	4.7±0.74
cxcl8	1.0±0.04	3.6±0.19	1.38±0.04	1.8±0.37	3.3±0.04
p50	1.0±0.16	0.9±0.03	1.0±0.09	1.4±0.18	1.5±0.12
p65	1.0±0.02	0.8±0.12	1.1±0.19	1.3±0.29	1.7±0.42
c-jun	1.0±0.04	2.2±0.12	1.8±0.21	1.9±0.27	4.7±0.67
c-fos	1.0±0.59	4.2±0.23	1.9±0.28	2.1±0.18	3.2±1.0

Figure 21

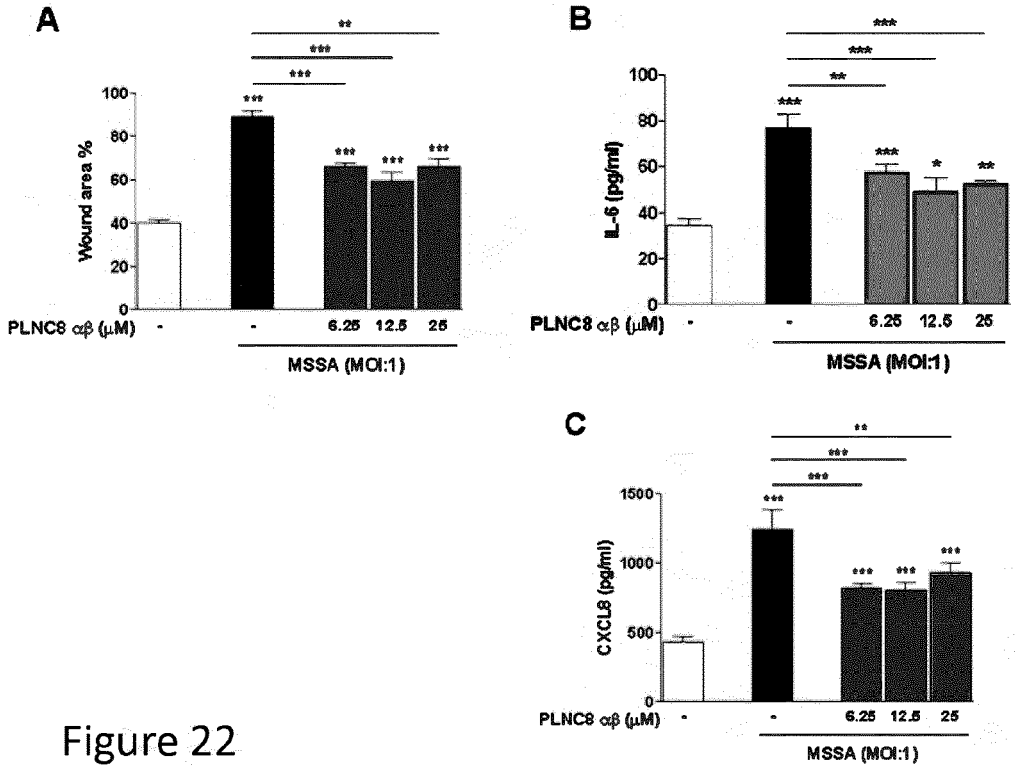


Figure 22

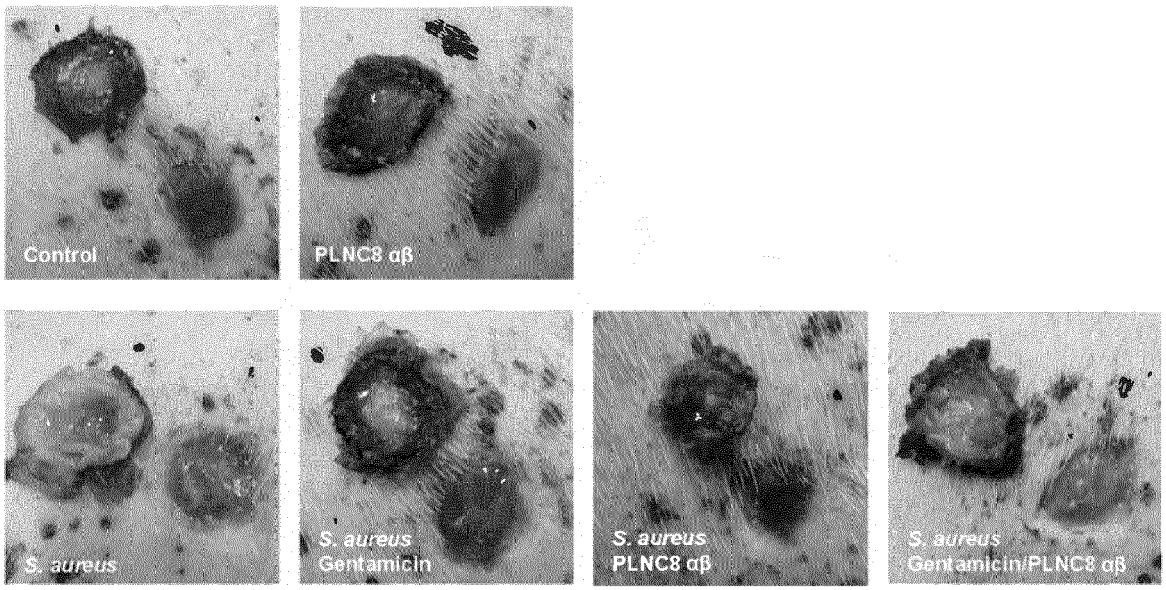


Figure 23

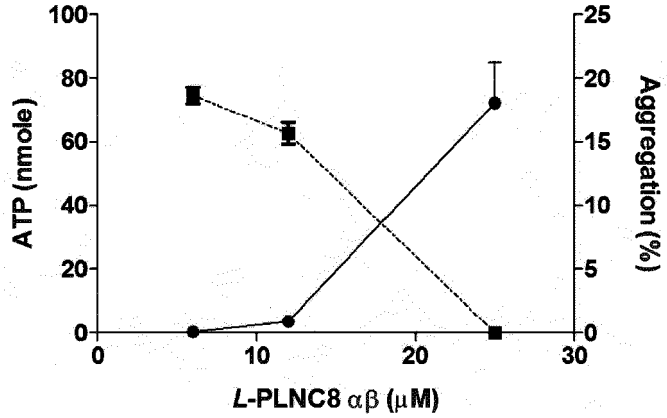


Figure 24

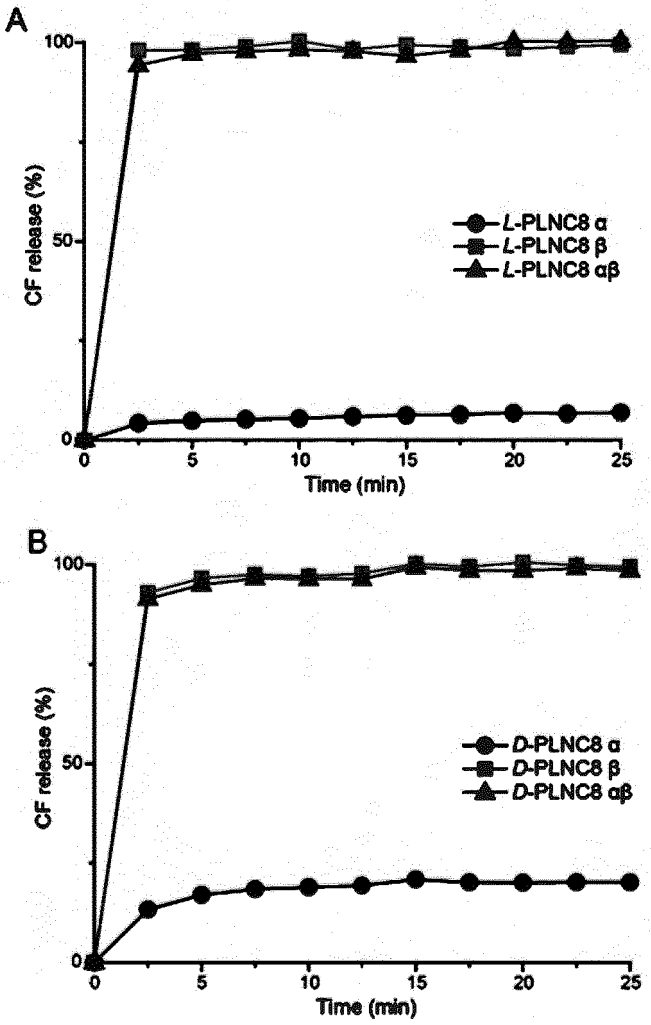


Figure 25

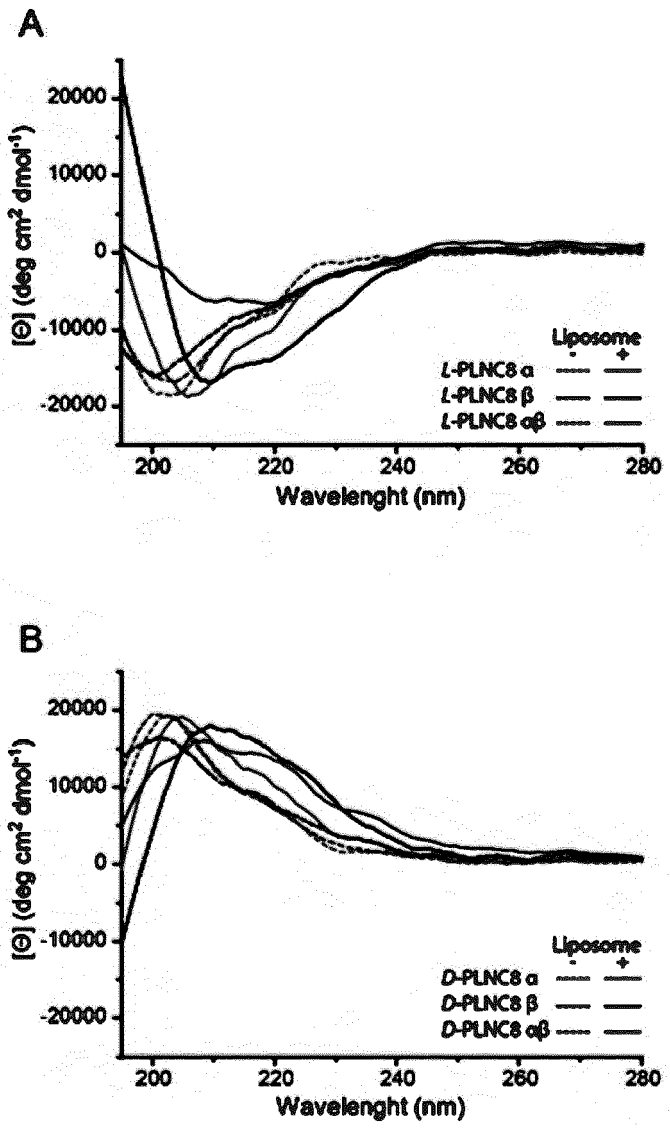

A
20000
10000
0
-10000
-20000
[Θ] (deg cm² dmol⁻¹)
200
220
240
260
280
Wavelenght (nm)
Liposome
-
+
L-PLNC8 α
L-PLNC8 β
L-PLNC8 αβ
B
20000
10000
0
-10000
-20000
[Θ] (deg cm² dmol⁻¹)
200
220
240
260
280
Wavelenght (nm)
Liposome
-
+
D-PLNC8 α
D-PLNC8 β
D-PLNC8 αβ

Figure 26

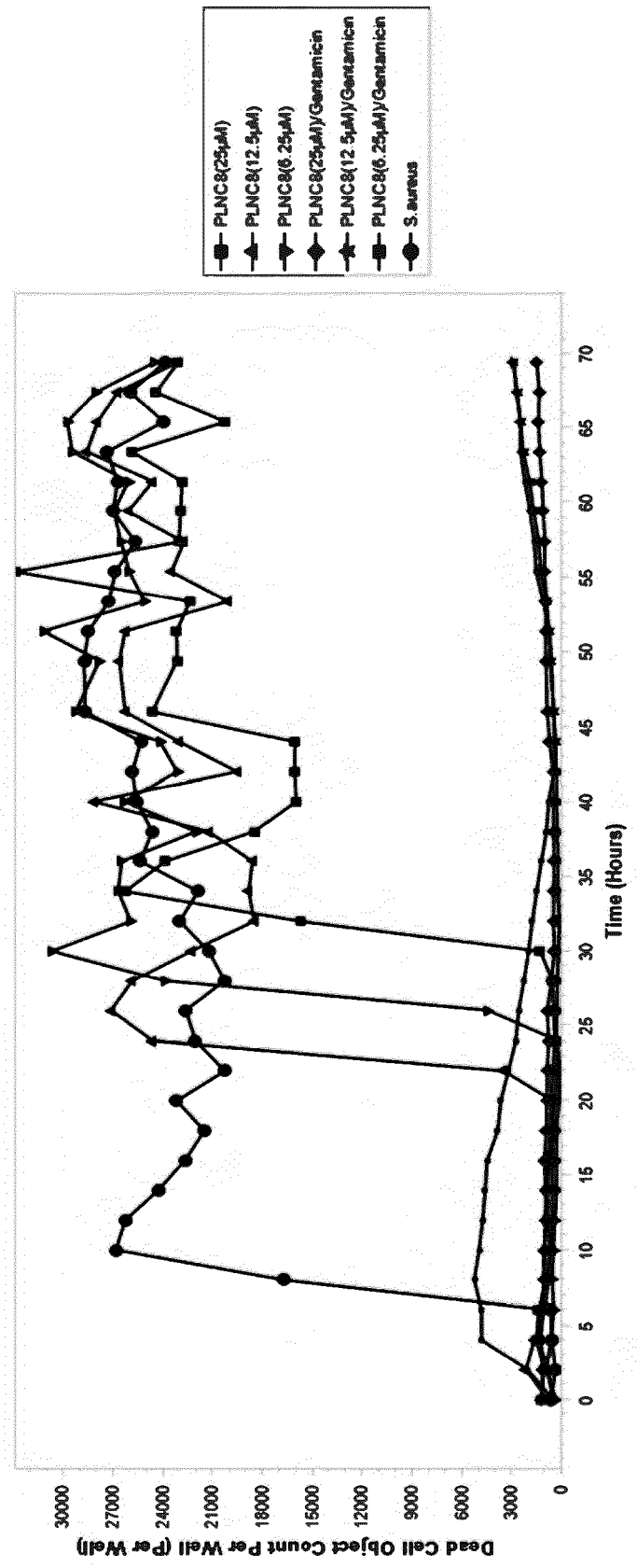
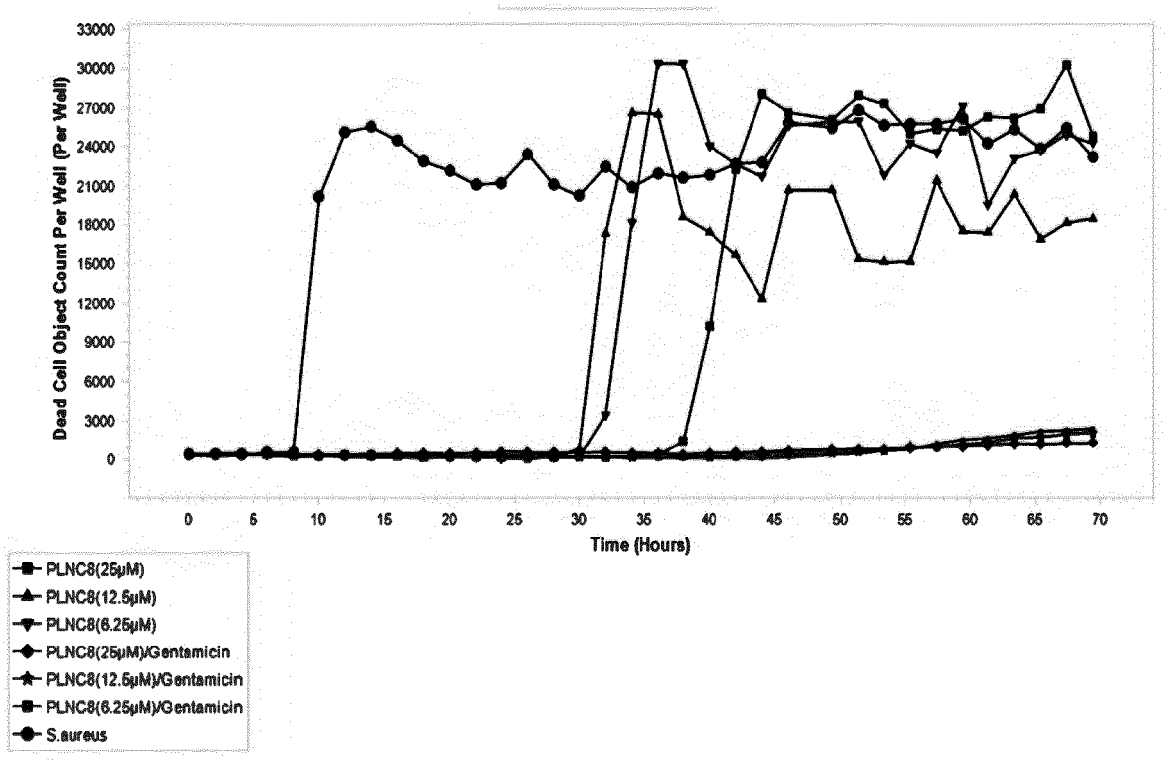
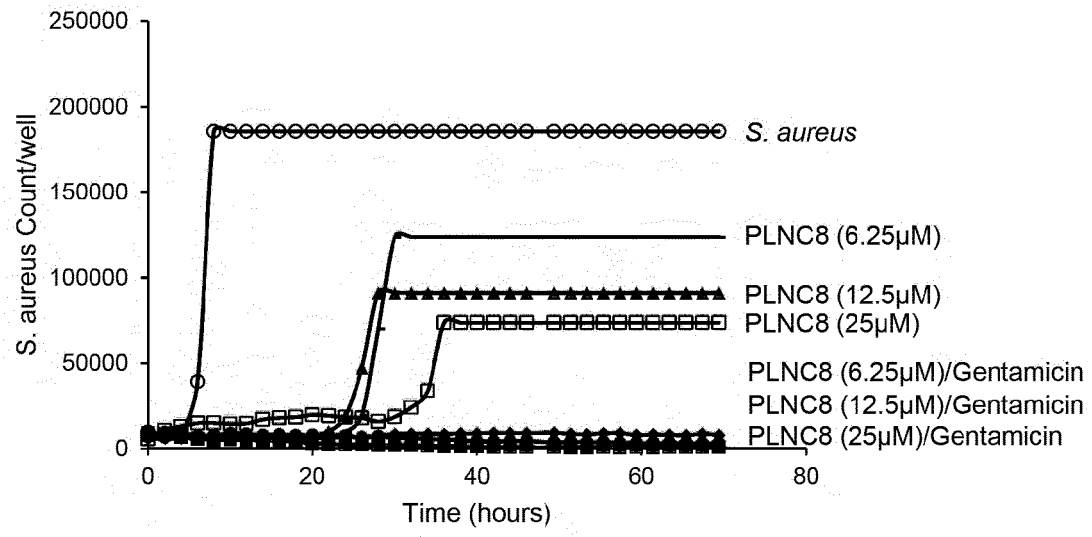

PLNC8(25μM)
PLNC8(12.5μM)
PLNC8(6.25μM)
PLNC8(25μM)/Gentamicin
PLNC8(12.5μM)/Gentamicin
PLNC8(6.25μM)/Gentamicin
S.aureus
Dead Cell Object Count Per Well (Per Well)
30000
27000
24000
21000
18000
15000
12000
9000
6000
3000
0
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
Time (Hours)

Figure 27

A


Dead Cell Object Count Per Well (Per Well)
33000
30000
27000
24000
21000
18000
15000
12000
9000
6000
3000
0
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
Time (Hours)
PLNC8(25μM)
PLNC8(12.5μM)
PLNC8(6.25μM)
PLNC8(25μM)/Gentamicin
PLNC8(12.5μM)/Gentamicin
PLNC8(6.25μM)/Gentamicin
S.aureus

B


S. aureus Count/well
250000
200000
150000
100000
50000
0
0
20
40
60
80
Time (hours)
S. aureus
PLNC8 (6.25μM)
PLNC8 (12.5μM)
PLNC8 (25μM)
PLNC8 (6.25μM)/Gentamicin
PLNC8 (12.5μM)/Gentamicin
PLNC8 (25μM)/Gentamicin

REFERENCES CITED IN THE DESCRIPTION

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Non-patent literature cited in the description

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- **PEARSON.** *Methods in Enzymology,* 1990, vol. 183, 63-98 **[0065]**
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- **BENGTSSON, T. ; ZHANG, B. ; SELEGÅRD, R. ; WIMAN, E. ; AILI, D. ; KHALAF, H.** Dual action bacteriocin PLNC8 αβ through inhibition of Porphyomonas gingivalis infection and promotion of cell proliferation. *Pathogens and Disease,* 12 June 2017 **[0177]**