

Plantaricins markedly enhance the effects of traditional antibiotics against *Staphylococcus epidermidis*

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Aim: Bacteriocins are considered as promising alternatives to antibiotics against infections. In this study, the plantaricins (Pln) A, E, F, J and K were investigated for their antimicrobial activity against *Staphylococcus epidermidis*. **Materials & methods:** The effects on membrane integrity were studied using liposomes and viable bacteria, respectively. **Results:** We show that PlnEF and PlnJK caused rapid and significant lysis of *S. epidermidis*, and induced lysis of liposomes. The PlnEF and PlnJK displayed similar mechanisms by targeting and disrupting the bacterial cell membrane. Interestingly, Pln enhanced the effects of different antibiotics by 30- to 500-fold. **Conclusion:** This study shows that Pln in combination with low concentrations of antibiotics is efficient against *S. epidermidis* and may be developed as potential treatment of infections.

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Staphylococcus spp., including strains that are resistant to multiple antibiotics, are one of the leading causes of community and hospital-acquired bacterial infections [1]. These bacteria can cause superficial infections and severe infections associated with chronic wounds and insertion of medical devices, including catheters and prosthetic joint implants [2]. This may subsequently increase the risk for development of life-threatening conditions, such as sepsis [3,4]. Hand hygiene and infection control strategies, including active surveillance programs, are important to prevent bacterial transmission in hospitals [5]. However, given the rise in antimicrobial resistance, the development of new strategies to combat bacterial infections are urgently needed, and bacteriocins represent a promising alternative [6,7].

Several strains of *Lactobacillus plantarum* are probiotic strains currently used as dietary supplements and have been reported to express several plantaricins (Pln) that belongs to class IIb [8]. Bacteriocins, including plantaricins, are antimicrobial peptides that are secreted by many bacteria as part of their defense mechanism. This group of antimicrobial peptides displays low toxicity toward eukaryotic cells and is active against pathogens and bacteria that have acquired resistance to antibiotics [6,9]. We have recently shown that the plantaricin PLNC8 $\alpha\beta$ efficiently permeabilizes the membrane of the periodontal pathogen *Porphyromonas gingivalis* [10], and antagonizes the cytotoxic effect of this pathogen on host cells [11]. All *pln* genes, including *plnEF* and *plnJK*, are adjacent to each other and located within the *pln* locus of the *L. plantarum* genome. The two-peptide plantaricins, PlnEF and PlnJK, have been suggested to kill microbes through formation of pores [8,12,13]. These mechanisms are difficult to evade and to develop resistance against, compared with conventional antibiotics that usually target metabolic enzymes. A possible approach that may be successful against bacterial infections is combination therapy. It has been suggested that inhibition of multiple bacterial targets is an effective method and could potentially delay selection of resistance while reducing the dosage and thus possible side effects [14,15]. A recent study reported that the bacteriocin garvicin KS acted synergistically with other bacteriocins, including polymyxin B and nisin, against a broad range of Gram-

Table 1. Amino acid sequences and molecular weight of plantaricins.

Plantaricin	Amino acid sequence
A	AYS LQMGATAIKQVKKLFKKWGW
E	FNRRGGYNFGKSVRHVVDAIGSVAGIRGILKSIR
F	VFHAYSARGVRRNNYKSAVGPADWWVISAVRGIHGG
J	GAWKNFWSLRKGFYDGEAGRAIRR
K	RRSRKNGIGYAIGYAFGAVERAVLGGSRDYNK

positive and Gram-negative bacteria [16]. Furthermore, the lantibiotic nisin has been shown to act synergistically with citric acid [17] and with traditional antibiotics, including penicillin and chloramphenicol [18], against several staphylococci strains.

It is important to find alternative antimicrobial candidates against staphylococci, which are responsible for causing the most common bacterial infections in humans. Although bacteriocins have received increased attention for applications in clinical settings, more research is needed to understand their mechanisms of action and effects on resistance development. The aim of the present study was to elucidate the antimicrobial activity of plantaricins, with and without traditional antibiotics, against *Staphylococcus epidermidis*.

Materials & methods

Bacterial culture conditions

Staphylococcus epidermidis ATCC 12228 (ATCC, VA, USA) and a clinical isolate, *S. epidermidis* 126, that was found to have heterogeneous resistance against glycopeptide antibiotics, gentamicin and tetracycline, were grown on Luria–Bertani agar plates and incubated at 37°C overnight. Single colonies were inoculated into 5 ml of Luria–Bertani broth and incubated on a shaker (300 r.p.m.) at 37°C overnight. The bacterial concentration was determined by viable count by culturing on agar plates and was adjusted to correlate with approximately 10⁹ CFU/ml.

Peptide synthesis

All chemicals were bought from Sigma–Aldrich unless otherwise noted and used without further purification. The peptides, PlnA, E, F, J and K (Table 1) were synthesized using conventional Fmoc chemistry on a Quartet automated peptide synthesizer (Protein Technologies, Inc., AZ, USA) in a 100 µmol scale. The C-terminal amino acid of each peptide were attached to a Wang resin (Novabiochem, 1.13 mmol/g) using 5 equivalents (eq) of Fmoc-protected amino acid (Iris biotech gmbh), 5 eq of MSNT and 3.75 eq of Melm in dry dichloromethane (DCM). The reactions were allowed to proceed for 1 h in a N₂ atmosphere. Resins were filtered off and washed with DCM and the loading procedures were repeated once. Peptide elongation was performed using a 4 eq of amino acid and activator (TBTU, Iris biotech gmbh) and using 8 eq of base (DIPEA). The 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, triisopropylsilane 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 semipreparative 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 (UltraflexXtreme, Bruker Daltonics) using α-cyano-4-hydroxycinnamic acid as matrix (Supplementary Figure 1). All peptide stock solutions were diluted with ddH₂O in the same manner to minimize differences in error between samples.

Liposome preparation

Liposomes were formed according to methods that are well established in the field [19,20]. Briefly, 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, AL, USA) were mixed at a molar ratio of 5:95 while dissolved in chloroform. A dry lipid film was formed by evaporation of the chloroform by nitrogen flow and dried overnight at reduced pressure. The film was hydrated with phosphate buffer (10 mM, pH 7.4) containing self-quenching concentration of 5(6)-carboxyfluorescein (CF, 50 mM) for fluorescence leakage assays and phosphate buffer saline (PBS, 10 mM, pH 7.4) for circular dichroism (CD) measurements. The solu-

tions were vortexed for 1 min and put on a shaker for 1 h before extruded 21-times through a 100-nm pore-sized polycarbonate membrane. Liposomes used in fluorescence leakage assays were purified from unencapsulated CF by gel filtration using a PD-25 column (GE Healthcare, Uppsala, Sweden) and eluted with PBS.

Circular dichroism spectroscopy

Bacteriocins are often unstructured in solution but typically adopt a more ordered secondary structure when bound to the bacterial cell membrane (CM) as a result of membrane partitioning [21]. The secondary structure of the bacteriocins (100 μM , PBS) alone and in combinations with and without liposomes (0.5 mg/ml, 660 μM) was investigated using CD spectroscopy. Spectras were recorded using a Chirascan spectropolarimeter (Applied Photophysics, UK) and a 0.1-mm quartz cuvette at 25°C with a sampling interval of 0.5 nm. All measurements were done in triplicates and averaged before converted to mean residue ellipticity and curves were smoothed using Savitzky–Golay algorithm.

Carboxyfluorescein release assay

Leakage of the liposome-encapsulated fluorophore CF due to additions of Pln peptides were recorded using a fluorescence plate reader (Safire 2, Tecan, Austria) with $\lambda_{\text{ex}} = 492$ nm and $\lambda_{\text{em}} = 517$ nm. The 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 Pln peptides (0.005–100 μM), alone and in different combinations and incubated for 30 min prior to measurements. In order to estimate the maximum release from each sample, a final addition of 1% Triton X-100 was made at the end of all measurements and the total amount of CF (100% release) was estimated after 10-min incubation. All data were fitted using a monophasic Hill equation and the concentration of Pln peptides needed to obtain a 50% CF release was extracted.

Microscopy

The fluorescent dye Sytox[®] Green binds to nucleic acids and fluoresces only after having crossed damaged membranes, was used to study the permeabilizing activity of plantaricins on *S. epidermidis*. The bacteria were washed and resuspended in Krebs–Ringer buffer (120 mM NaCl, 4.9 mM KCl, 1.2 mM MgSO₄, 1.7 mM KH₂PO₄ and 8.3 mM Na₂HPO₄, pH 7.3) and incubated in the presence or absence of different combinations of plantaricins in 96-well microtiter plates for 2 min. Images were captured with Olympus BX41 at 40 \times magnification. Fluorescence intensity of Sytox Green was quantified using the software ImageJ.

Transmission electron microscopy was used to visualize the effects of different Pln peptides on *S. epidermidis*. Briefly, the bacteria were pelleted and washed with Krebs–Ringer buffer followed by exposure to different plantaricins at a final concentration of 25 μM for 5 min. Samples were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.3. The samples were then washed in 0.1 M phosphate buffer and postfixed in 2% osmium tetroxide in 0.1 M phosphate buffer for 2 h and embedded into LX-112 (Ladd, VT, USA). Ultrathin sections (~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).

Detection of ATP release

The concentration of extracellular ATP, which was used as a marker of bacterial lysis, was recorded using a luciferin/luciferase bioluminescence assay (Sigma–Aldrich, MO, USA) in bacterial suspensions (2.5×10^8 CFU/ml). The bacteria were exposed to different concentrations of PlnEF and PlnJK, and real-time changes in bioluminescence were recorded in a Chronolog lumi-aggregometer (Chrono-Log, PA, USA) for 10 min. The levels of ATP were calculated based on the bioluminescence signals recorded in response to known concentrations of ATP.

Antimicrobial activity of plantaricins

The broth microdilution method was used to determine MIC and minimal bactericidal concentration (MBC). Twofold serial dilutions of the peptides were used and the final concentrations ranged from 0.097 to 50 μM (~0.34–177 $\mu\text{g/ml}$). Since the activity of PlnEF and PlnJK is dependent on the complementary action of two peptides at equimolar concentrations, a final concentration of 50 μM of, for example, PlnEF is achieved by mixing 50 μM of PlnE with 50 μM PlnF. The final concentrations of the antibiotics vancomycin and teicoplanin ranged from 0.097 to 50 $\mu\text{g/ml}$, while gentamicin ranged from 0.0097 to 5 $\mu\text{g/ml}$ and tetracycline from 0.016 to 8 $\mu\text{g/ml}$.

The antimicrobial effects of plantaricins together with antibiotics were studied by using the same concentration series of antibiotics with a constant concentration of plantaricins in all the wells (12.5 and 6.25 μM equivalent to 44 and 22 $\mu\text{g}/\text{ml}$, respectively). 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.

Statistical analysis

All data were analyzed using GraphPad Prism 5.0 (GraphPad Software, CA, USA). One-way ANOVA with Bonferroni's *post hoc* test was used for the comparisons between the different treatments. The p-values are referred to as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Results

Effects of plantaricins on *S. epidermidis* & model lipid membranes

The antimicrobial effects of plantaricins were tested against one of the most common opportunistic pathogens found in humans, in other words, *S. epidermidis*. The inhibitory and bactericidal concentrations of the different plantaricins were determined by the broth microdilution method. *S. epidermidis* was more susceptible to PlnEF (Figure 1A) than PlnJK (Figure 1B), with a MIC/MBC value of 12.5/25 and 25/50 μM , respectively. However, the individual plantaricin peptides (PlnE, F, J and K) were ineffective at inhibiting bacterial growth. Furthermore, the two-peptide bacteriocins PlnEF and PlnJK, at 25 and 50 μM , caused a significant increase in turbidity immediately after addition to a bacterial suspension, which is indicative of rapid bacterial lysis (data not shown). The PlnA (50 μM) alone displayed antimicrobial activity that suppressed bacterial growth; however, the concentration was not bactericidal.

Plantaricins of *L. plantarum* are short peptides with membrane perturbing properties. By probing the secondary structure with CD spectroscopy, we found that individual Pln peptides adopts a distorted conformation except PlnJ which resides as a distinct α -helix in PBS buffer at pH 7 (Supplementary Figure 2). The presence of a model lipid membrane had little to no influence on individual Pln peptides except PlnA and J. However, the combination of different peptides (PlnEF, AEF, JK and AJK) resulted in more pronounced induced helicity. It has previously been shown that the lipid composition of the model membrane is critical for the formation of helical secondary structure as well as the overall structure of the model membrane, where micelles tend to induce more prominent helices compared with liposomes [22]. We, however, used large unilamellar liposomes with a size of 100 nm composed of the anionic lipid 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS) and zwitterionic 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine (POPC) at a molar ratio of 5:95 as a model system in order to provide a more realistic model membrane. This membrane model system was used to investigate the permeabilizing effect of the Pln peptides by using liposomes loaded with the fluorophore CF at a self-quenching concentration. The relative CF release was recorded 30 min after exposure of liposomes to increasing concentrations of different Pln peptides. The CF release was significantly increased by PlnEF (Figure 1C) and PlnJK (Figure 1D), and reached 50% CF release at 0.33 and 0.20 μM , respectively, compared with each individual peptide alone that caused a 50% CF release at concentration ≥ 30 μM . The PlnA alone was efficient and caused lysis of liposomes at concentrations equivalent to PlnEF. Interestingly, addition of PlnA to EF or JK resulted in a synergistic lytic effect of liposomes with a 50% CF release of 0.028 and 0.025 μM , respectively.

Plantaricins efficiently permeabilize *S. epidermidis* & cause extensive morphological changes

The ability of plantaricins to permeabilize gram-positive *S. epidermidis* ATCC was evaluated with Sytox Green, which only migrates through damaged membranes, binds to nucleic acid and fluoresces. The PlnA alone, but not PlnE, F, J or K, was able to permeabilize the bacteria (Figure 2A & B). The combinations PlnEF and PlnJK caused rapid permeabilization (2 min) in a dose-dependent manner. Interestingly, addition of PlnA to EF or JK caused formation of larger bacterial aggregates, probably due to a synergistic antimicrobial effect of the plantaricins. The antimicrobial effects of plantaricins were verified by quantifying ATP release from *S. epidermidis* after treatment with different concentrations of PlnJK and PlnEF (Figure 2C). Both PlnJK and PlnEF caused rapid lysis that peaked after 2 and 4 min, respectively. The bacteria were lysed in a dose-dependent manner and PlnEF was more effective than PlnJK. As these plantaricins showed different abilities to lyse liposomes and *S. epidermidis*, we studied the ultrastructural changes that are associated with plantaricin-induced bacterial lysis. Transmission electron

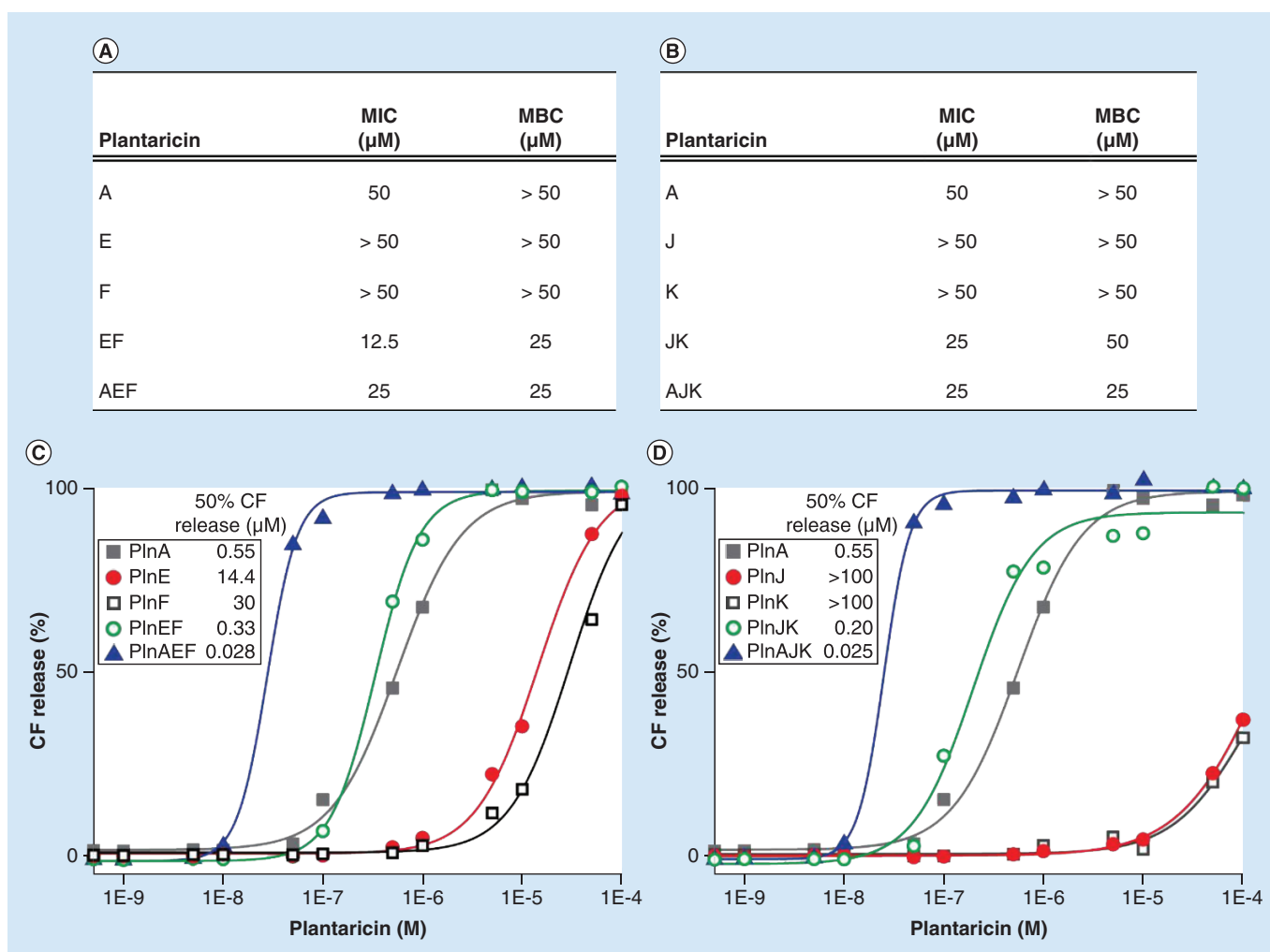


Figure 1. Antimicrobial activity of plantaricins. The antimicrobial activity of plantaricins was investigated against *Staphylococcus epidermidis* and verified using liposome model systems. The MIC and MBC values of (A) different combinations of PInA, E and F and (B) PInA, J and K against *S. epidermidis* ATCC 12228 was investigated after 20 h of incubation. In combination the respective peptides were added at the same concentration, for example, 50 μM of PInEF was achieved by mixing 50 μM of PInE with 50 μM PInF. The PInA alone showed antimicrobial activity, while PInE and F had antimicrobial effect only when combined together. Optimal activity of PInJ and K was observed when the two peptides were combined together. The CF release from liposomes was recorded 30 min after exposure to the same combinations of plantaricins to verify membrane permeabilization. The concentrations required to cause 50% CF release are indicated for (C) different combinations of PInA, E and F; and (D) PInA, J and K. Three independent experiments were performed. CF: Carboxyfluorescein.

microscopy was utilized to visualize the nature of the damage of *S. epidermidis* ATCC following treatment with different PIn peptides at a final concentration of 25 μM for 5 min. Untreated bacteria showed typical morphology, where the outer cell wall (CW) and inner CM could be distinguished (Figure 3). Among the treatments with individual peptides alone, only PInA and to some extent PInE caused leakage of intracellular content. Interestingly, exposure of *S. epidermidis* to PInEF or PInJK, with and without PInA, resulted in a significant alteration of bacterial ultrastructure and severe damage. The thickness of the CW was significantly increased and appeared loosen. Furthermore, the CM was completely detached and no longer tightly associated with the inner zone of the CW.

Plantaricins act in synergy with antibiotics against *S. epidermidis*

The plantaricin combinations EF and JK, respectively, were shown to be efficient against *S. epidermidis* ATCC by rapidly lysing the bacteria. Infections caused by *Staphylococcus* spp. are treated with different classes of antibiotics depending on their resistance patterns. Nonresistant bacteria are treated with, for example, the aminoglycoside antibiotics gentamicin or tetracycline that inhibits protein synthesis, while resistant bacteria are treated with gly-

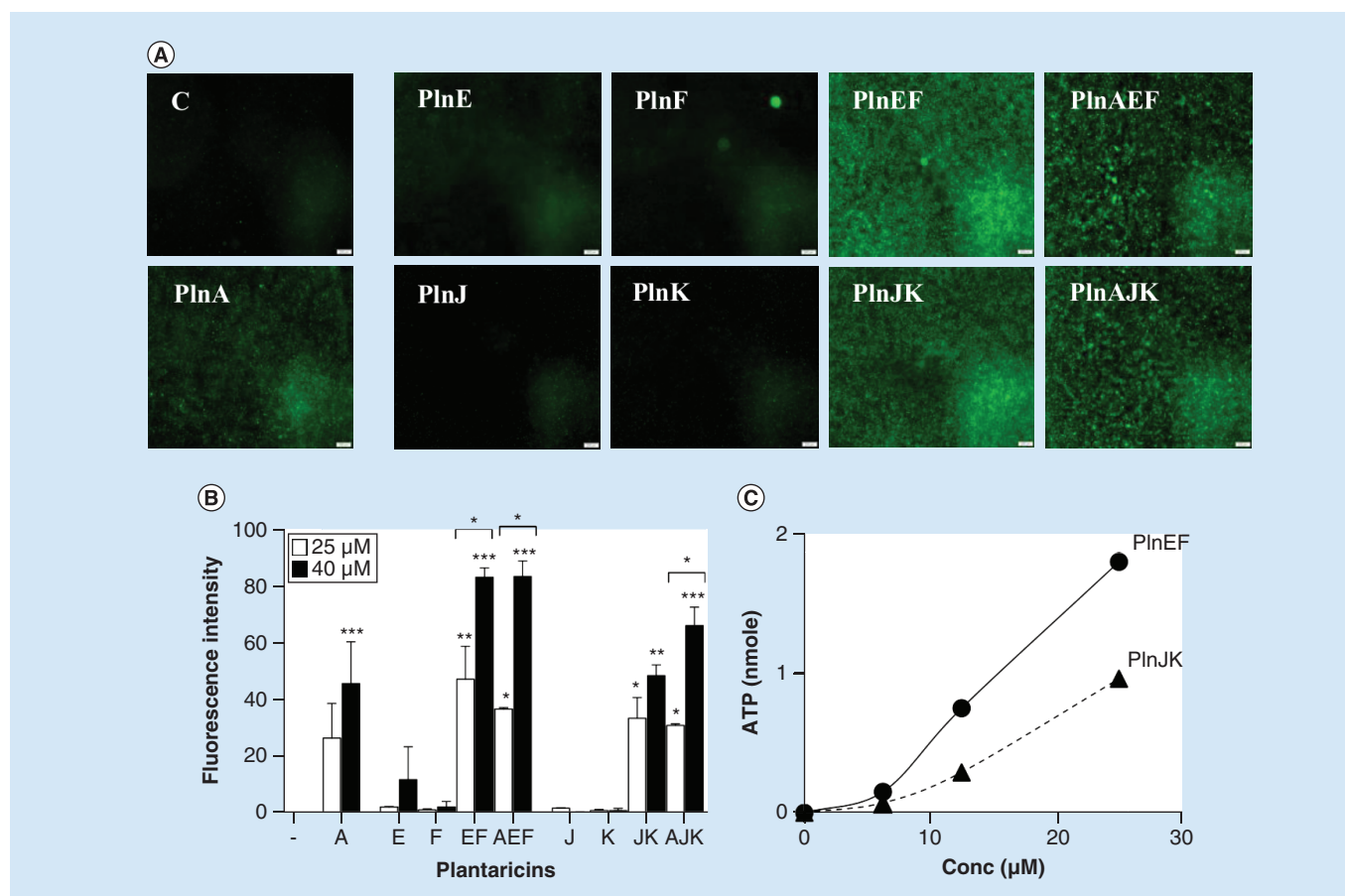


Figure 2. Rapid lysis of *Staphylococcus epidermidis* by plantaricins. *Staphylococcus epidermidis* was incubated with different combinations of plantaricins at a final concentration of 25 or 40 μM for 2 min. **(A)** Representative images of bacteria treated with 25 μM of different Pln peptides are shown. Scale bar is 300 μm. **(B)** Fluorescence intensity of the images was quantified with the software ImageJ and results are presented as mean ± SD. **(C)** Bacterial lysis was verified by quantifying ATP release from *S. epidermidis* ATCC following treatment with PlnEF or PlnJK. The bacteria were rapidly lysed (2–4 min) by PlnEF and PlnJK. The data are from three independent experiments. Pln: Plantaricin; SD: Standard deviation.

copeptide antibiotics, including vancomycin and teicoplanin that interfere with CW synthesis. However, although glycopeptide antibiotics have been used restrictively, resistant bacteria have emerged through production of thicker CWs. This encouraged us to investigate potential synergistic inhibitory and bactericidal effects of plantaricins and antibiotics against *S. epidermidis*. A final and constant concentration of 12.5 μM of PlnEF or PlnJK was tested together with antibiotics. Vancomycin and teicoplanin alone resulted in similar MIC (3.1 μg/ml) and MBC (6.25 μg/ml) values, while a concentration of 0.31 μg/ml of gentamicin was both inhibitory and bactericidal (Table 2A). The presence of PlnEF reduced the MIC values of all three antibiotics by >30-fold, while PlnJK caused a reduction of twofold and approximately sevenfold of teicoplanin and gentamicin, respectively. The bactericidal activity of the combinatorial treatment, primarily with PlnEF, was significantly enhanced compared with antibiotics alone. Similar results were obtained with the laboratory reference strain *S. epidermidis* ATCC 12228 and *S. epidermidis* 126 isolated from an infected prosthetic joint. The latter strain is classified to have heterogeneous resistance to glycopeptide antibiotics is resistant to gentamicin (MIC > 5 μg/ml). Addition of 12.5 μM of PlnEF, but not PlnJK, significantly reduced the MIC and MBC values of gentamicin by 500-fold and 260-fold, respectively. Both *S. epidermidis* strains were found to be resistant to tetracycline (MIC ≥ 2 μg/ml) and PlnEF significantly enhanced the inhibitory effect of tetracycline by 500- and 125-fold, for *S. epidermidis* ATCC and *S. epidermidis* 126, respectively. While PlnEF also lowered the bactericidal concentration of tetracycline, PlnJK was efficient at inhibiting the bacteria, but did not enhance the bactericidal activity. Furthermore, when the final concentration

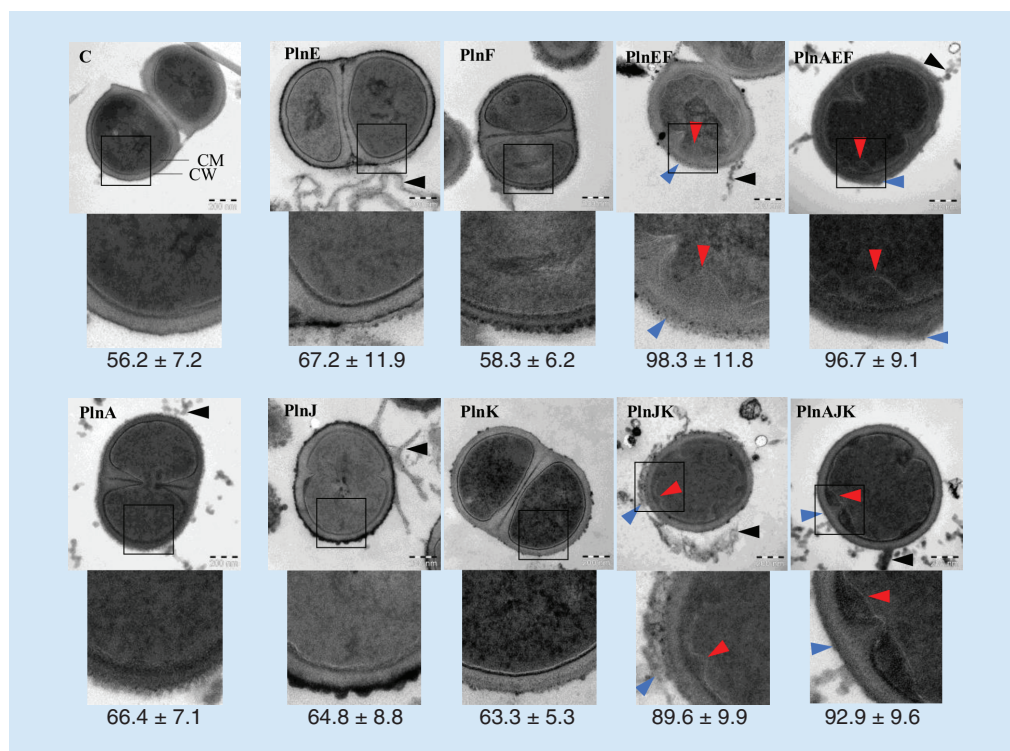


Figure 3. Exposure of *Staphylococcus epidermidis* to plantaricins causes severe ultrastructural changes.

Staphylococcus epidermidis was treated with 25 μ M of different plantaricins for 5 min and bacterial ultrastructure was examined using a Hitachi HT 7700 transmission electron microscope. The bacteria were severely affected, particularly by PlnEF, AEF, JK and AJK. The inner CM was completely detached (red arrow heads) and leakage of intracellular content is observed (black arrow heads). The thickness of the CW was significantly increased (blue arrow heads), which was quantified and is represented by the values (mean \pm SD) under each panel. The lower image in each panel is a magnification of the selected area. Scale bar is 200 nm. CM: Cell membrane; CW: Cell wall; SD: Standard deviation.

of PlnEF was decreased to 6.25 μ M, there was no synergistic effect with vancomycin, while the inhibitory and bactericidal effect with teicoplanin remained on both bacterial strains (Table 2B). A synergistic effect of PlnEF together with gentamicin was obtained only on *S. epidermidis* ATCC, while a synergistic effect of PlnEF with tetracycline was found against *S. epidermidis* 126.

Discussion

Staphylococcus epidermidis is an opportunistic pathogen and considered as one of the major causes of skin and soft-tissue infections in humans [23]. The resistance pattern of *S. epidermidis* includes several classes of antibiotics, such as methicillin, rifampicin, gentamicin and vancomycin [24], which causes limitations in treatment options. Similarly, treatment of MRSA infections with currently available antibiotics has been reported to be unsatisfactory, suggesting other antimicrobials and combination therapies as possible strategies [25]. Alternatives to antibiotics suggest bacteriocins as viable options [6,8]. Corr and colleagues [26] demonstrated that *Lactobacillus salivarius* efficiently protected mice from infections caused by *Listeria monocytogenes*. This inhibitory effect of pathogenic bacteria was directly associated with the ability of *L. salivarius* to produce the bacteriocin Abp118, as a mutation of this gene failed to protect the animals from *L. monocytogenes* infection. Using bacteriocins against skin and soft-tissue infections with less possibility to induce resistance may be advantageous, since bacterial resistance has increased the occurrence of complications in healthcare systems due to overuse of antibiotics [27]. This study shows that plantaricins of *L. plantarum* are effective against *S. epidermidis* when administered in conjunction with low concentrations of conventional antibiotics.

Plantaricins (A, E, F, J and K) are short peptides, ranging from 23 to 34 amino acids that possess antimicrobial characteristics. We show that both peptides of the same bacteriocin, in other words, PlnEF and PlnJK, are required

Table 2. Plantaricins act synergistically with antibiotics.

Antimicrobial agent	<i>S. epidermidis</i> ATCC 12228		<i>S. epidermidis</i> hGISE	
	MIC ($\mu\text{g/ml}$)	MBC ($\mu\text{g/ml}$)	MIC ($\mu\text{g/ml}$)	MBC ($\mu\text{g/ml}$)
A				
Vancomycin	3.1	6.25	3.1	6.25
Vancomycin/PlnEF	<0.097	0.78	<0.097	<0.097
Vancomycin/PlnJK	3.1	3.1	3.1	3.1
Teicoplanin	3.1	6.25	3.1	6.25
Teicoplanin/PlnEF	<0.097	0.19	<0.097	0.39
Teicoplanin/PlnJK	1.5	3.1	1.5	6.25
Gentamicin	0.31	0.31	>5	>5
Gentamicin/PlnEF	<0.0097	<0.0097	<0.0097	0.019
Gentamicin/PlnJK	0.039	0.078	5	>5
Tetracycline	>8	>8	2	4
Tetracycline/PlnEF	<0.016	0.031	<0.016	<0.016
Tetracycline/PlnJK	0.063	>8	0.5	4
B				
Vancomycin/PlnEF	3.1	3.1	3.1	3.1
Teicoplanin/PlnEF	<0.097	<0.097	<0.097	0.39
Gentamicin/PlnEF	0.039	0.078	>5	>5
Tetracycline/PlnEF	4	4	<0.016	<0.016

(A) *Staphylococcus epidermidis* ATCC 12228 or *S. epidermidis* 126, a clinical isolate that was found to have hGISE, were exposed to a serial dilution of different antibiotics alone or in combination with PlnEF or PlnJK at a final concentration of 12.5 μM (44 $\mu\text{g/ml}$). (B) MIC and MBC values of different antibiotics together with PlnEF at a final concentration of 6.25 μM (22 $\mu\text{g/ml}$) toward *S. epidermidis* ATCC 12228 and *S. epidermidis* 126. Three independent experiments were performed. hGISE: Heterogeneous resistance to glycopeptide antibiotics; MBC: Minimal bactericidal concentration.

for efficient lysis of target bacterial membranes. These plantaricins belong to class IIb bacteriocins, and their activity has previously been reported to depend on two complementary peptides at equimolar concentrations [12]. Optimal antimicrobial activity of plantaricins is achieved by interaction of the two amphiphilic α -helical peptides through GxxxG motifs and GxxxG-like motifs. This binding stabilizes the peptides and causes permeabilization of membranes, ultimately leading to death of susceptible bacteria due to alteration in intracellular pH and electric potential [13]. Interestingly, PlnA alone was efficient, both at lysing liposomes and inhibiting *S. epidermidis* growth. The function of PlnA was first described as a peptide pheromone that regulates transcription of genes within the *pln* locus, including *plnEFI* and *plnJKLR*; however, it is now established that this peptide also displays antimicrobial activity [28,29]. Furthermore, we show that the two-peptide bacteriocins PlnEF and PlnJK are remarkably rapid at permeabilizing *S. epidermidis*. Total 2 min of exposure was sufficient to allow Sytox Green to accumulate and significantly fluoresce through DNA-binding, and ATP release peaked at 1–4 min post-treatment. Analysis of bacterial ultrastructure revealed an increased thickness of the CW that appeared loose, while the inner CM was completely detached and distorted. Thickening of the CW has been documented for several conventional antibiotics, such as β -lactam antibiotics, vancomycin, rifampicin and tetracycline (reviewed in [30]). These effects are observed first after ≥ 2 h of treatment with low concentrations of antibiotic. The CW enlargement by antibiotics targeting protein synthesis has been suggested to be due to inhibition of peptidoglycan hydrolases that are needed to loosen the CW, which in addition to continued production and incorporation of peptidoglycan precursor, ultimately results in thickening of the CW [30]. Two-peptide bacteriocins, including PlnEF and PlnJK, target bacterial membranes to be permeable to small molecules [31]. While PlnEF creates pores followed by leakage of monovalent cations, PlnJK causes leakage of anions [32]. This may lead to a perturbed electrolyte balance with accompanying efflux of water that affects the tight association of CM with the inner wall zone, and eventually the thickness of the CW. However, more research is needed to understand the precise mechanisms underlying these dramatic effects. Both PlnEF and PlnJK have been reported to form pores on target membranes, but their mechanism of action and affinity is different toward different microorganisms [13,28].

Staphylococcus spp. are responsible for causing skin and soft tissue infections that may be life threatening. Although vancomycin is an effective treatment, heteroresistance to this antibiotic is increasing [33] and biofilm formation

of staphylococci has been reported to decrease the activity of vancomycin [34]. It would be advantageous to use agents that efficiently permeabilized bacterial CWs in combination with low doses of conventional antibiotics to improve the bactericidal effects. The permeabilizing and antimicrobial activity of plantaricins together with different antibiotics was investigated. The PlnEF dramatically enhanced the effects of vancomycin and teicoplanin by >30-fold, and gentamicin and tetracycline by >500-fold, while PlnJK was less effective. Plantaricin-induced disruption of the CW and CM may facilitate efficient penetration and accumulation of antibiotics, thus rapidly reaching a critical concentration with inhibitory and bactericidal effect. The concept of combination therapy has been implicated to mainly increase the likelihood of providing an effective agent rather than contributing to synergistic effects between two or more agents [14,35]. *In vitro* data may therefore be useful to support the choice of appropriate treatments when providing combination therapy [36]. Gimeno and colleagues showed that the combination of cefotaxime and ofloxacin acted synergistically against 55.8% of the isolated *S. aureus* strains [37]. However, although the mechanism of antimicrobial activity of the combination antibiotic/bacteriocin differs from antibiotic/antibiotic, more research is needed to support potential applications in clinical settings.

Conclusion

In this study, we show that PlnEF act in synergy with gentamicin, tetracycline, vancomycin and teicoplanin against *S. epidermidis*. Sub-MIC concentrations of PlnEF were most effective in significantly reducing the concentrations of gentamicin, tetracycline and teicoplanin. This study suggests that combination therapies composed of plantaricins and conventional antibiotics should be considered and further developed as potential treatment against *S. epidermidis*. Although these results are encouraging, additional antibiotics and bacterial strains have to be tested, and the cytotoxicity and stability of plantaricins have to be fully characterized.

Summary points

- Plantaricin EF and JK of *Lactobacillus plantarum* are efficient at permeabilizing *Staphylococcus epidermidis*.
- The permeabilizing activity of plantaricins was verified using liposome model systems.
- The mechanism PlnEF and PlnJK involves disruption of the bacterial cell membrane.
- Bacteria are rapidly lysed (2 min) as indicated by the instant release of ATP.
- The pheromone peptide PlnA enhances the antimicrobial effect of PlnEF and PlnJK.
- Plantaricins act synergistically with traditional antibiotics, enhancing their effects by 30- to 500-fold.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/full/10.2217/fmb-2018-0285

Author contributions

Design of the work was performed by T Bengtsson and H Khalaf. Acquisition of data, analysis and interpretation, drafting and critical revision, approval of the final version and agreement to be accountable for the work were performed by R Selegård, A Musa, P Nyström, D Aili, T Bengtsson and H Khalaf.

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