Synergistic Antimicrobial Activity Between the Broad Spectrum Bacteriocin Garvicin KS and Nisin, Farnesol and Polymyxin B Against Gram-Positive and Gram-Negative Bacteria

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Abstract  The increasing emergence of antibiotics resistance is of global concern. Finding novel antimicrobial agents and strategies based on synergistic combinations are essential to combat resistant bacteria. We evaluated the activity of garvicin KS, a new bacteriocin produced by Lactococcus garvieae. The bacteriocin has a broad inhibitory spectrum, inhibiting members of all the 19 species of Gram-positive bacteria tested. Unlike other bacteriocins from Gram-positive bacteria, garvicin KS inhibits Acinetobacter but not other Gram-negative bacteria. Garvicin KS was tested in combination with other antimicrobial agents. We demonstrated synergy with polymyxin B against Acinetobacter spp. and Escherichia coli, but not against Pseudomonas aeruginosa. Similar effects were seen with mixtures of nisin and polymyxin B. The synergistic mixtures of all three components caused rapid killing and full eradication of Acinetobacter spp. and E. coli. In addition, garvicin KS and nisin also acted synergistically against Staphylococcus aureus, indicating different in modes of action between the two bacteriocins. Both bacteriocins showed synergy with farnesol, and the combination of low concentrations of garvicin KS, nisin and farnesol caused rapid eradication of all the S. aureus strains tested. Its broad inhibitory spectrum, rapid killing, and synergy with other antimicrobials makes garvicin KS a promising antimicrobial.

Keywords  Synergy effect · Bacteriocin · Garvicin KS · Antimicrobials · Pathogens

Introduction

Infections caused by antibiotics resistant pathogens from both Gram-positive and Gram-negative bacteria have become of global concern. In addition, the slow discovery and development of new antibiotics is unable to catch up the rapid increasing of antibiotics resistance [5, 24]. Searching for alternative antimicrobials for inhibition and elimination of the antibiotics resistant pathogens as well as reducing resistance evolution is essential. Antimicrobial peptides represent a source of unexplored compounds with a potential to kill antibiotic resistant bacteria [27]. Their modes of action are not fully understood, but are different from the commonly used antibiotics. The antimicrobial peptides produced by bacteria are known as bacteriocins. Bacteriocins usually have narrow inhibitory spectra, but from Gram-positive bacteria several bacteriocins with wide inhibitory spectra are known. The bacteriocins of lactic acid bacteria are of particular interest, since many are being used in foods and their bacteriocins may contribute to enhance shelf life and food safety. Based on primary structure, these bacteriocins have been grouped into class I, lantibiotics containing modified residues, and class II without modified residues [6].

The best-studied of these bacteriocins is the lantibiotic nisin, which inhibits most Gram-positive bacteria but not Gram-negative [12, 30]. However, in the presence of compounds that can destabilize the outer membrane, even Gram-negatives are inhibited [14, 28].

We recently described garvicin KS, a bacteriocin produced by Lactococcus garvieae [24]. We report here on its antimicrobial spectrum, which includes Gram-negative bacteria. We show that it can act synergistically with other antimicrobials, including nisin, against several pathogenic bacteria. Moreover, the synergistic mixtures can improve killing kinetics reducing the risk of resistance development.

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Materials and Methods

Bacterial Strains and Growth Conditions

*Lactococci* were grown in M17 broth (Oxoid, Hampshire, UK) supplemented with 0.4% (w/v) glucose (GM17). *Lactobacilli* and *Pediococci* were grown in deMan, Rogosa and Sharpe (MRS) (Oxoid, Hampshire, UK) medium at 30 °C. *E. coli*, *Pseudomonas aeruginosa*, and *Acinetobacter* strains were grown in Lysogeny broth (Oxoid, Hampshire, UK) at 37 °C. *Streptococci* were grown in deMan, Rogosa and Sharpe (MRS) (Oxoid, Hampshire, UK) supplemented with 0.4% (w/v) glucose (GM17), *L. lactis* were grown in M17 broth (Oxoid, Hampshire, UK) or Mueller Hinton Broth (MHB) (BD Difco, MD, USA) at 37 °C with shaking at 225 rpm. Other strains were grown in Brain Heart Infusion (Oxoid, Hampshire, UK) broth at 37 °C.

Antimicrobial Agents

Nisin from *Lactococcus lactis* (2.5% purity), polymyxin B sulfate and farnesol were from Sigma-Aldrich, Co. (St. Louis, MO, USA). Crude garvicin KS was prepared by following procedure. 1 L of GM17 broth was inoculated with 1% of an overnight culture of *L. garvieae* KS1546 and incubated at 30 °C for 8 h. The cells were removed by centrifugation at 12,000× *g* for 25 min and 258 g of ammonium sulfate was dissolved in the cell-free supernatant, and the mixture was left at 4 °C for 24 h. The crude bacteriocin was precipitated by centrifugation at 15,000× *g* for 30 min at 4 °C, dissolved in 20 mL water and kept in a boiling water bath for 10 min.

Inhibition and Checkerboard Assays

Growth inhibition was determined in microtiter plates with 200 µL of growth medium in each well as described by Holo et al. [16]. The minimum inhibitory concentration (MIC) refers to the concentration of antimicrobial agent causing 50% growth inhibition, the MIC was detected once the OD600 of testing strains reaches 0.5. Bacteriocin unit (BU) 

Time-Kill Assays

Time-kill assays were performed using cultures grown overnight and then diluted 100× in MHB with different concentrations of antimicrobial agents. The assays were done in triplicate. Total viable count (TVC) was determined by plating on Mueller Hinton agar. The agar plates were incubated at 37 °C, and the TVC was estimated after 24 h incubation. Interactions between antimicrobial agents were interpreted as synergistic when they in mixture caused ≥2-log decrease in TVC compared to the antimicrobial agents alone [25].

Results

Garvicin KS has a Broad Antimicrobial Inhibition Spectrum

Garvicin KS, produced by *L. garvieae* strain, is composed of three similar peptides [24]. Initially, its inhibitory activity was tested using an agar diffusion assay [24] on 250 indicator strains, of which 240 strains (19 species) were Gram-positive. All the Gram-positive bacteria tested, with the exception of three out of 53 *S. aureus* strains, were sensitive (results not shown) tested. The MICs of garvicin KS against a representative selection of the strains are shown in Table 1. Garvicin KS inhibited pathogens like *Listeria monocytogenes*, *S. aureus*, and vegetative cells of *Bacillus cereus*. The effect of garvicin KS on spores were not tested. The most sensitive bacteria were strains of the lactic acid bacteria *Lactococcus* and *Enterococcus*, with MIC ranging from 5 to 80 BU/mL. 5 BU/mL corresponds to a mixture of 10 nM of each of the pure peptides of garvicin KS [24]. Concentrations to inhibit other Gram-positive strains varied from 320 to 2560 BU/mL.

Notably, garvicin KS was inhibitory to *A. baumannii*, *A. iwoffii*, and *A. calcoaceticus* (MIC = 2560 BU/mL). However, other Gram-negative bacteria, such as *E. coli* and *P. aeruginosa*, did not show any sensitivity to garvicin KS in this assay.

Garvicin KS Acts Synergistically with Other Antimicrobials Against *S. aureus*

*Staphylococcus aureus* LMGT 3242 was the least sensitive Gram-positive bacterium tested in the microtiter assay, and in time-kill assays we were unable to fully prevent growth of this bacterium even with high concentration of garvicin KS. We therefore used this strain to investigate the effects of combining garvicin KS with other antimicrobials, including nisin, another bacteriocin with a wide inhibitory spectrum [1] and farnesol, shown to act as adjuvant to antimicrobials in *Staphylococci* [17]. Using the checkerboard assay, we
found synergy between nisin and garvicin KS (FIC = 0.22) and between bacteriocins and farnesol, (both with FICs of 0.47), and all three compounds in combination (FIC = 0.33) (Table 2). The three components mixture resulted in the reduction of MIC by a factor of 8 for farnesol, and by a factor of 10 for garvicin KS and nisin, respectively.

The effects of garvicin KS, nisin, and farnesol to S. aureus were also studied in time-kill assay. In the experiments, concentrations corresponding to the MIC (Table 2) were used. As shown in Fig. 1, none of the three antimicrobials alone could completely kill the bacteria even after 48 h exposure. When tested alone nisin and garvicin KS both caused an initial 3-log reduction in TVC before growth of survivors was detected. The mixtures nisin + garvicin KS caused a complete killing after about 12 h and no regrowth was seen even after 48 h, demonstrating the strong synergy between the two bacteriocins. Killing using the farnesol + garvicin KS mixture was less efficient, causing 4.5 log reduction of CFU before the growth started. The farnesol + nisin mixture, on the other hand, gave complete killing after 12 h, as did the combination of all three compounds. We tested six other strains of S. aureus, including three MRSA, in the time-kill assay using the same concentrations as shown in Fig. 1. All strains were sensitive to nisin and garvicin KS, but none were eradicated by these bacteriocins alone or in combination. Only one of the six strains was completely killed by the mixture of nisin and farnesol. However, the mixture of farnesol, nisin, and garvicin KS caused complete killing of all six strains.

### Antimicrobial Synergy Against Gram-Negative Bacteria

A unique feature of garvicin KS is that it has antimicrobial effects on Acinetobacter spp., but relatively high amount of garvicin KS was required to inhibit them. Recently, farnesol was found to be a good adjuvant with polymyxin B to A. baumannii [18], and like for S. aureus; we tested farnesol in mixture with garvicin KS for synergy in inhibiting Acinetobacter spp.. No synergy was seen with these combinations (data not shown). We therefore replaced farnesol with polymyxin B in the mixtures.

The A. baumannii and A. calcoaceticus strains tested were both sensitive to polymyxin B with a MIC of 0.63 µg/mL, while A. iwoffii with a MIC of 25 µg/mL is considered resistant [2] (Table 3). Synergy was seen between polymyxin B and garvicin KS against all three Acinetobacter strains tested, even the polymyxin B resistant A. iwoffii. Similar synergistic effects were seen between nisin and polymyxin B. The combinations of three antimicrobial agents also showed synergistic effects against all the Acinetobacter strains. Compared to the individual antimicrobials combining the three compounds caused about 10-fold and 15-fold MIC reductions for garvicin KS and polymyxin B, respectively (Table 3).

The effects on A. baumannii of nisin, garvicin KS, polymyxin B alone and in mixtures were assayed in time-kill

### Table 1

| Bacterial strain            | MIC (BU/mL) |
|-----------------------------|-------------|
| Acinetobacter baumannii B1162 | 2560        |
| Acinetobacter calcoaceticus B1165 | 2560        |
| Acinetobacter iwoffii B1163  | 2560        |
| Bacillus cereus LMGT 2805    | 640–1280    |
| Cellulomonas fimi LMGT3232   | 10–20       |
| Escherichia coli LMGT 3704   | NI          |
| Enterococcus faecalis LMGT 2333 | 40–80        |
| Enterococcus faecium LMGT 2763 | 20–40        |
| Lactobacillus curvatus LMGT 2353 | 80           |
| Lactobacillus plantarum LMGT 2003 | 80           |
| Lactobacillus sakei LMGT 2361 | 320          |
| Lactobacillus salivarius LMGT 2787 | 320–640    |
| Lactococcus garvireae LMGT 3390 | 80           |
| Lactococcus lactis IL1403     | 5           |
| Lactococcus lactis LMGT 2122a | 5            |
| Leuconostoc gelidum LMGT 2386 | 640–1280    |
| Listeria innocua LMGT 2710    | 80          |
| Listeria ivanovii LMGT 2813   | 320         |
| Listeria monocytogenes LMGT 2604 | 160         |
| Pediococcus acidilactici LMGT 2002 | 320         |
| Pediococcus pentosaceus LMGT 2001 | 640         |
| Pseudomonas aeruginosa LMGT 3294 | NI          |
| Staphylococcus aureus LMGT 3242 | 2560        |
| Streptococcus salivarius LMGT 3597 | 320          |

NI no inhibition
Nisin producer

### Table 2

| MIC (FIC) in mixture | Garvicin KS + nisin | Nisin + farnesol | Garvicin KS + farnesol | Garvicin KS + nisin + farnesol |
|----------------------|---------------------|------------------|------------------------|-----------------------------|
| Garvicin KS + nisin  | 312/2.50 (0.22)     | 5.00/0.16 (0.47) | 512/0.16 (0.47)        | 256/2.50/0.08 (0.33)        |

Concentrations are given in BU/mL for garvicin KS, µg/mL for nisin and mM for farnesol
assays using concentrations corresponding to the MIC values shown in Table 3. As shown in Fig. 2, garvicin KS and polymyxin B could both kill the bacteria, but regrowth was seen after about 8 h and an initial 2–3 log killing in either case. Mixing garvicin KS and polymyxin B prevented regrowth of the bacteria, but the nisin + polymyxin B mixture did not. The bacteria were rapidly killed by the mixture of the three components, reaching eradication after 4 h.

Prompted by the results using *Acinetobacter* strains, we extended the synergy studies to other Gram-negative bacteria. Garvicin KS and nisin were unable to kill *E. coli* alone, but the strain tested was sensitive to polymyxin B (MIC = 0.3 µg/mL). Both bacteriocins showed synergy with polymyxin B. A FIC of 0.4 was found for two- and three components mixture with polymyxin B (Table 3). The synergies were also demonstrated in time-kill assays (Fig. 3). Both bacteriocins improved the killing rates compared to polymyxin B alone, but regrowth was seen. The mixture of all three components, however, caused eradication of the bacteria after 8 h exposure.

We also tested synergy effects by using garvicin KS, nisin, and polymyxin B against *P. aeruginosa*. The MIC for polymyxin B against the strain tested was 0.8 µg/mL. However, there were no synergy effects on their combinations (data not shown).

**Discussion**

Garvicin KS is a new bacteriocin comprised of three similar peptides of 32–34 amino acids [24]. The bacteriocin shows a broad inhibitory spectrum encompassing all the Gram-positive genera tested. Such a wide spectrum of activity is uncommon among bacteriocins, and has only been reported for certain lantibiotics like nisin and lactacin 3147 [11]. Of note, unlike the lantibiotics, garvicin KS was able to inhibit Gram-negative bacteria of *Acinetobacter* genus. However, the sensitivity towards garvicin KS varied more than 500-fold between the bacteria tested. Relatively high amounts of garvicin KS were required to kill many of the pathogenic species tested, and these bacteria were not completely killed and regrew after initial killing.

Combining antimicrobial agents offers a potential for increasing antimicrobial treatment efficacy and for reducing resistance evolution, and the use of combination antimicrobial therapy is widely used in the treatment of serious infections [4]. Importantly, the individual bacteriocins may act more effectively in combination with other antimicrobials agents [6], and studies demonstrating such effects have recently been reviewed [19]. Here, we have shown that

| Bacterium | MIC | MIC (FIC) in mixture |
|-----------|-----|----------------------|
|           | Garvicin KS | Nisin | Polymyxin B | Garvicin KS + nisin B | Nisin + polymyxin B | Garvicin KS + polymyxin B | Garvicin KS + nisin + polymyxin B |
| *A. baumannii* B1162 | 2560 | NI | 0.63 | 2560/NI (2.0) | 1.25/0.05 (0.08) | 500/0.15 (0.44) | 150/0.80/0.05 (0.20) |
| *A. iwoffii* B1163 | 2560 | NI | 25.0 | 2560/NI (2.0) | 3.00/5.00 (0.20) | 512/6.25 (0.45) | 250/2.50/1.50 (0.16) |
| *A. calcoaceticus* B1165 | 2560 | NI | 0.63 | 2560/NI (2.0) | 5.00/0.31 (0.50) | 256/0.20 (0.42) | 250/2.50/0.16 (0.35) |
| *E. coli* LMGT 3704 | NI | NI | 0.30 | NI (X) | 10.0/0.12 (0.40) | 3200/0.12 (0.40) | 3200/4.0/0.12 (0.40) |

NI means no inhibition
X means not valid

*Concentrations are given in BU/mL for garvicin KS, µg/mL for nisin and polymyxin B*
garvicin KS is synergistic with other antimicrobial compounds, improving killing kinetics, and eradication hence reducing resistance development. It is notable that garvicin KS could act synergistically with all the three compounds tested, indicating differences in mode of killing.

Importantly, garvicin KS showed synergy with polymyxin B, a drug of last resort in the combat infections by multidrug resistant Gram-negative bacteria but which is avoided due to its toxicity at relevant concentrations [9, 26]. Moreover, the emergence of resistance to polymyxins has been reported [8, 10]. Synergy between polymyxins and nisin and lacticin 3147, both bacteriocins from Gram-positive bacteria, against Gram-negative bacteria has been reported [13, 20, 21]. The synergy can be attributed to disruption of the outer membrane by polymyxin B allowing access of the bacteriocins to their target [7]. Polymyxin B is used for treatment of nosocomial infections mostly caused by *A. baumannii*, but because surviving bacteria are frequently found most patients are recommended to receive combination therapy of polymyxin B with other agents active against *A. baumannii* [23, 29]. The potential benefits of combining with new bacteriocins, like garvicin KS that act synergistically are obvious. Importantly, the concentration of polymyxin B needed to eradicate the bacteria was significantly reduced in combination with garvicin KS and nisin and much lower than the dosage recommended for polymyxin B therapy [29].

Strong synergy between nisin and garvicin KS was also observed against *S. aureus*. The synergy indicates that garvicin KS and nisin have different modes of action, which is different from classical antibiotics. Importantly, against *S. aureus* they were both synergistic with farnesol, a cheap and harmless compound which has been considered a promising adjuvant for antibiotics [15]. Moreover, the mixture of farnesol, nisin and garvicin KS completely killed all the *S. aureus* tested.

In conclusion, garvicin KS is a promising antimicrobial agent. We have demonstrated activity against a wide variety of bacteria, including pathogenic species known to account for a large number nosocomial infections, often with multi-resistant strains. The mode of action of garvicin KS is unknown, but different from many other used antimicrobials. Furthermore, garvicin KS mixtures with other antimicrobial compounds can be highly efficient by improving killing kinetics and eradication hence lower resistance development.

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

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