In vitro and *In vivo* Characterization of Amyloliquecidin, a Novel Two-Component Lantibiotic Produced by *Bacillus amyloliquefaciens*

by

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Declaration

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i

Summary

Antimicrobial resistance is one of the major problems faced by the medical industry today. The ability of bacteria to rapidly acquire resistance against antibiotics and the over prescription and inappropriate use of antibiotics further exacerbate this crisis. Few new antimicrobials are, however, making it through the drug discovery pipeline. The search and development of novel and effective antimicrobials is therefore of the utmost importance.

Lantibiotics are ribosomally synthesized cationic antimicrobial peptides with extensive post-translational modifications. They are active against a wide range of Gram-positive bacteria, including antibiotic-resistant strains. They are characterized by the presence of lanthionine and methyllanthionine rings and have been suggested as alternatives or for use in conjunction with antibiotics against resistant pathogens. *Staphylococcus aureus* is the most common bacteria isolated from skin and soft tissue infections (SSTIs). Strains of *S. aureus* have emerged with resistance against antibiotics with the most common being methicillin-resistant *S. aureus* (MRSA). Several lantibiotics are active against MRSA *in vivo* and have even shown superior activity to traditional antibiotics. Lantibiotics therefore show much promise for the treatment of SSTIs caused by resistant- and non-resistant *S. aureus*.

In this study the bacterially diverse soil of the Fynbos in the Western Cape was screened for novel antimicrobials. Two antimicrobial producing *Bacillus* strains were isolated, *Bacillus clausii* AD1 and *Bacillus amyloliquefaciens* AD2. Both of these strains produce lantibiotics with *B. clausii* AD1 producing a known lantibiotic, clausin. *B. amyloliquefaciens* AD2 produces a novel two-component lantibiotic which was designated amyloliquecidin. The lantibiotic operon of amyloliquecidin was sequenced and annotated. All the genes required for successful production of amyloliquecidin are present in the operon. Amyloliquecidin was characterized *in vitro* and along with clausin is active against clinical strains of *S. aureus* (including MRSA)*, Enterococcus* spp., *Listeria* spp. and beta-haemolytic streptococci. Amyloliquecidin has remarkable stability at physiological pH compared to nisin and clausin. A comparative *in vivo* murine infection model was used to evaluate the effectiveness of amyloliquecidin, nisin, clausin and Bactroban (commercial *S. aureus* topical treatment) in treating wound infections caused by *S. aureus*. All the lantibiotics proved to be just as effective as the Bactroban treatment. Furthermore, the tested lantibiotics did not have a negative influence on the wound closure rates of infected and non-infected wounds. Bactroban had a negative effect on wound healing compared to the lantibiotics.

To our knowledge amyloliquecidin is the third two-component lantibiotic isolated from *Bacillus*. This study represents the first to test the effectiveness of amyloliquecidin *in vivo* and is one of a handful to test lantibiotics as topical treatments.

Opsomming

Antimikrobiese weerstandbiedende bakterieë is op die oomblik een van die grootste probleme in die mediese veld. Die antibiotika krisis word vererg deur die vermoë van bakterieë om vinnig weerstand op te bou teen antibiotika, asook die alledaagse misbruik van antibiotika. Daar is ook 'n tekort in die hoeveelheid antibiotika wat na die finale fases van ontwikkeling gaan. Om die oorhand teen antibiotika-weerstandige bakterieë te kry is dit van uiterste belang dat meer effektiewe antibiotika ontdek word.

Lantibiotika is kationiese antimikrobiese peptiede wat deur die ribosoom gesintetiseer word en bevat 'n verskeidenheid van modifikasies wat na translasie ingebou word. Hulle word gekarakteriseer deur lanthionien en metiellanthionien ringe. Lantibiotika is aktief teen 'n verskeidenheid Gram-positiewe bakterieë en kan in kombinasie met antibiotika, of as alternatief gebruik word. *Staphylococcus aureus* is die mees algemene bakterium wat geassosieer word met vel en sagte weefsel infeksies (VSWIs). *Staphylococcus aureus* met weerstand teen antibiotika is ook al geïsoleer, die mees algemene weerstandige ras is methisillien-weerstandige *S. aureus* (MWSA). Lantibiotika is wel aktief teen MWSA *in vitro* en *in vivo*, met van hulle wat tot beter aktiwiteit as die voorgeskrewe antibiotika het. Lantibiotika kan dus gebruik word as behandeling vir VSWIs wat veroorsaak word deur weerstandige *S. aureus,* asook teen nie-weerstandige rasse.

In hierdie studie was die bakteriese diverse grond van die Fynbos in die Wes-kaap ondersoek vir bakterieë wat antimikrobiese middels produseer. Twee *Bacillus* rasse, *Bacillus clausii* AD1 en *Bacillus amyloliquefaciens* AD2, wat antimikrobiese middels produseer, is geïsoleer. *Bacillus clausii* AD1 produseer 'n bekende lantibiotikum, naamlik clausin. *Bacillus amyloliquefaciens* AD2 produseer 'n nuwe twee-komponent lantibiotikum, amyloliquecidin. Die lantibiotikum operon wat verantwoordelik is vir die produksie van amyloliquecidin is geïdentifiseer en geannoteer. Die operon bevat al die gene benodig vir die biosintese van amyloliquecidin. Amyloliquecidin is *in vitro* gekarakteriseer en het aktiwiteit teen 'n verskeidenheid Gram-positiewe bakterieë. Amyloliquecidin en clausin is aktief teen *S. aureus* (insluitend MWSA)*, Enterococcus* spp., *Listeria* spp. en beta-hemolitiese streptococci wat vanaf infeksies geïsoleer is. Amyloliquecidin is baie stabiel by filologiese pH en aansienlik meer stabiel as nisin en clausin. Die effektiwiteit van nisin, clausin en amyloliquecidin in die behandeling van muis vel infeksies veroorsaak deur *S. aureus* was vergelyk met die kommersiële behandeling Bactroban. Al drie lantibiotika het die verspreiding van *S. aureus*

met die selfde effektiwiteit as Bactroban belemmer. Geen van die lantibiotika het 'n negatiewe effek op wond genesing nie. Bactroban, inteendeel, belemmer wond genesing.

So ver ons weet is amyloliquecidin die derde twee-komponent lantibiotikum wat uit *Bacillus* geïsoleer is. Die studie is ook die eerste om die effektiwiteit van amyloliquecidin *in vivo* te rapporteer, asook ook een van die min studies wat kyk na lantibiotika as behandeling vir topikale infeksies.

Biographical Sketch

Anton Du Preez van Staden was born in Windhoek, Namibia on the 7th of March, 1987. He matriculated at Windhoek High School, Namibia, in 2005. In 2006 he enrolled as B.Sc. student in a Molecular Biology and Biotechnology degree at the University of Stellenbosch and obtained the degree in 2008. In 2009 he obtained his B.Sc (Hons) in Microbiology, also at the University of Stellenbosch. In 2010 he enrolled as M.Sc. student in Microbiology at the University of Stellenbosch, receiving his M.sc (Cum Laude) in 2011. In 2012 he enrolled as a Ph.D student in Microbiology at the University of Stellenbosch.

Preface

This dissertation is represented as a compilation of 5 chapters. Each chapter is introduced separately and is written according to the style of the American Society for Microbiology.

Chapter 1: General Introduction

Chapter 2: Biosynthesis, Mode of Action and Clinical Applications of Lantibiotics

Chapter 3: *In vitro* Characterization of a Novel Two-Component Lantibiotic from *Bacillus amyloliquefaciens*

Chapter 4: Evaluation of the *in vivo* Efficacy of Lantibiotics in the Treatment of *S. aureus*-Induced Skin Infections in Mice

Chapter 5: General Discussion and Conclusions

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__Table of Contents__

Chapter 1   General Introduction

Chapter 2   Biosynthesis, Mode of Action and Clinical Applications of Lantibiotics

Chapter 3 *In vitro* Characterization of a Novel Two-Component Lantibiotic from *Bacillus amyloliquefaciens*

Chapter 4   Evaluation of the *in vivo* Efficacy of Lantibiotics in the Treatment of *S. aureus*-Induced Skin infections in Mice

Chapter 5   General Discussion and Conclusions

Chapter 1
General Introduction

General Introduction

The use of antibiotics to treat bacterial infections is probably the most important contribution to medical sciences in modern times. Hundreds of antibiotics have been described that target cell wall development, DNA- and protein-synthesis (1). The increase in antibiotic resistance is steering science back into the "pre-antibiotic" era and can result in minor infections turning into life threatening diseases (2). Antibiotic resistance is exacerbated by over-prescription and misuse of antibiotics (3, 4). The appropriate use of antibiotics is therefore important in trying to control the spread of resistance. Bacteria become resistant to antibiotics by random mutation, expression of latent resistant genes, or the exchange of genetic material (e.g. horizontal gene transfer). Therefore, despite many attempts to control antibiotic resistance, it is inevitable that bacteria will mutate or adapt. The search and development for novel antimicrobials is thus crucial. It is possible to speed up the discovery of new antimicrobials by using high through-put screening techniques, bioinformatics, structural-, chemical- and synthetic-biology (5, 6). The probability of finding novel antimicrobials can also be increased by screening unexploited environments with diverse bacterial populations.

Lantibiotics are antimicrobial peptides that can be used in combination with antibiotics or as possible alternatives. Lantibiotics are ribosomally synthesized and post-translationally modified peptides. Their main characteristic is that they contain *meso*-lanthionine (Lan) and methyllanthionine (MeLan) residues (7, 8). Lanthionine and MeLan modifications are introduced with the help of modification enzymes that are required for, but not limited to, dehydration and cyclization reactions (7, 8). Lantibiotics are generally only active against Gram-positive bacteria. Several lantibiotics have shown promising activity *in vitro* and *in vivo* against antibiotic-resistant pathogens (9-12). A few lantibiotics are being developed for the treatment of infections caused by Gram-positive bacteria (Novacta Biosystems; Oragenics).

Staphylococcus aureus is the major cause of skin and soft tissue infections (SSTIs). The species has developed resistance against antibiotics, with the most common example being methicillin-resistant *S. aureus* (MRSA). The recommended treatment for SSTIs caused by *S. aureus,* Bactroban, is ineffective against MRSA (13, 14). Resistance of MRSA to one of the last resort treatments, vancomycin, have also started to emerge (15-19). Several lantibiotics are active against MRSA and vancomycin-resistant strains, making them possible alternatives for treatment of *S. aureus* SSTIs (9-12). Little has been published on the *in vivo* treatment of topical infections by lantibiotics. Lantibiotics are, however, effective in the treatment of *S. aureus*

5

infections when administered via subcutaneous, intraperitoneal, intranasal and intravenous routes (9-11, 20). Nisin spun into nanofibers, and applied topically, is also effective in the control of *in vivo* infections caused by *S. aureus* (21). From these results it is evident that lantibiotics have applications in the treatment of SSTIs.

In this study, we set out to isolate and characterize novel antimicrobials from the highly diverse Fynbos soils of the Western Cape, South Africa. A novel two-component lantibiotic, amyloliquecidin, and the lantibiotic clausin were isolated from strains of *Bacillus amyloliquefaciens* AD2 and *Bacillus clausii* AD1, respectively. By making use of an *in vivo* imaging system, we evaluated the effectiveness of the lantibiotics and compared their activity with that of nisin and Bactroban in the treatment of *S. aureus*-induced skin infections in mice.

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Chapter 2
Biosynthesis, Mode of Action and Clinical Applications of Lantibiotics

Introduction to Lantibiotics

Lanthipeptides are ribosomally synthesized and post-translationally modified (PTMs) peptides (RiPPs), that contain *meso*-lanthionine (Lan) and methyllanthionine (MeLan) residues (1). Lanthionine and MeLan modifications are introduced by modification enzymes that are required for, but not limited to, dehydration and cyclization reactions (1, 2). Lanthipeptides are broadly classified into four classes based on their modification machinery. Class I peptides are modified by two enzymes namely LanB and LanC, whereas the other three classes have bi-functional lanthionine synthetases.

Dehydratase enzymes are responsible for the dehydration of Ser to dehydroalanine (Dha) and Thr to dehydrobutyrine (Dhb) (3, 4). Dha and Dhb undergo subsequent cyclization reactions, via a Michael addition of Cys residues to the dehydrated Dha/Dhb, resulting in Lan containing two Ala residues and MeLan containing an additional methyl group, respectively (3, 4). In class I, the dehydration and cyclization reactions are alternating, with reactions following one after another, and for class I and II dehydration generally proceeds in an N- to C-terminal direction (4–6). Additional PTMs can include formation of labionin residues, formed as a result of the initial enolate undergoing a second Michael addition with a second Dha, and oxidative decarboxylation of the lantibiotic C-terminus during aminovinylcysteine formation (7–9). The structural genes of lanthipeptides' form part of a gene cluster containing all the biosynthetic machinery for their modification, export, leader cleavage and regulation (2).

One group of lanthipeptides showing great potential are those with antimicrobial activity, namely lantibiotics. First reported in 1928, nisin is the best studied lantibiotic and is used as preservative in dairy products (10, 11). Since the discovery of nisin the production of lantibiotics by a variety of Gram-positive bacteria has been reported, and with sequenced genomes becoming more readily available, the identification of putative lantibiotics is increasing rapidly (2, 12). Lantibiotics have potent activity against related Gram-positive bacteria, many of which are clinical isolates of *Staphylococcus* spp.*, Enterococcus* spp. and *Clostridium* spp. including some antibiotic-resistant strains (13, 14). The majority of lantibiotics bind to the cell wall precursor lipid II. They can form pores in the cell membrane and prevent cell wall biosynthesis (15). The prototypical lantibiotic nisin binds to the pyrophosphate moiety of lipid II via two of its N-terminal rings, resulting in the inhibition of further cell wall biosynthesis and facilitating formation of the pore complex (16–18). Formation of the pore complex results in cell membrane permeabilization and dissipation of

the proton motive force (16–18). Lantibiotics also have possible roles in the modulation of the innate immune system (19, 20). The lantibiotics gallidermin, Pep5 and nisin induce the release of multiple chemokines at levels similar to that of the human cationic antimicrobial peptide (cAMP) LL-37, with nisin seemingly able to activate multiple signalling pathways, including ERK/MAPK, PKC and PKA (19). At high concentrations nisin is also able to activate neutrophils, resulting in formation of neutrophil extracellular traps (NETs) (20). Neutrophil extracellular traps are known for trapping and killing bacteria (21). Formation of NETs is also induced by a known immune modulating bacterial cAMP, phenol-soluble modulin-γ (PSM- γ; from *Staphylococcus epidermidis*), although at lower concentrations than reported for nisin (22). It is hypothesized that the direct antimicrobial activity of lantibiotics, produced by commensal bacteria, may play a role in the protection of the host from possible pathogenic bacteria (23, 24). Cinnamycin and duramycin indirectly inhibit phospholipase A2 and ancovenin is a potential angiotensin I converting enzyme inhibitor, suggesting that they can also play a role in immune modulation (25–27).

In silico analysis of sequenced genomes reveals the wide-spread occurrence of lantibiotics in several bacteria associated with humans. It is thus possible that they may play an important role in the host-microbe interaction, although very little research has been done on this subject.

Classification, Biosynthesis and Mode of Action of Lantibiotics

Classification

Several classification systems proposed for lantibiotics are based on differences in structure, function, amino acid sequences and biosynthetic machinery. For the purpose of this review, a combination of classification systems based on amino acid sequence similarity of the unmodified precursor peptides (w/o leader sequence) and biosynthetic machinery will be used, as first proposed by Cotter et al. (28).

Classification according to modification machinery separates all lanthipeptides into four groups. Lantibiotics are divided into 13 subgroups (named after the prototypical lantibiotic in that group), based on unmodified precursor peptide amino acid sequences (w/o leader sequence).

Class I

Class I are linear lantibiotics modified by two distinct enzymes to produce precursor peptides. Serine is dehydrated to Dha and Thr is dehydrated to Dhb, which are then cyclized resulting in the Lan/MeLan structures, respectively (3, 4). Gene designations for enzymes involved in this process have been widely used and remain unchanged, e.g. *lanB* for dehydratase and *lanC* for cyclase (1). Khusainov et al. (29) have recently shown interaction between the nisin leader peptide and LanBC complex. By systematically replacing two to four amino acid regions in the leader sequence with Ala, they found that three regions, i.e. STDK (-22-19), FNLD (-18-15) and PR (-2-1) contribute to the interaction of the leader with LanB and LanC, whereas only one region, LVSV (-14-11), contributes to the interaction with LanC (Fig. 1). In all modified leader sequences the interaction with LanC was severely affected, however, LanB is less affected by these changes, suggesting a less specific interaction of the dehydratase with specific leader regions. Changes in the FDLEI-motif of Pep5 also influence its biosynthesis, most likely due to disturbances in its interaction with LanB (30). However, the FDLEI-motif is not essential for biosynthesis, as reported for the FNLD-motif of nisin (30). These results strongly suggest that conserved regions in class I leader sequences are important for the proper initial interaction of class I synthetases and the precursor peptide.

```
Pep5                --------MKNNKNLFDLEIKKETSQ-NTDELEPQ
Planosporicin       MGISSPALPQNTADLFQLDLEIGVEQ---SLASPA
Streptin            --------MNNTIKDFDLDLKTNKKD----TATPY
Nisin               ----------MSTKDFNLDLVSVSKK--DSGASPR
Epidermin           -----MEAVKEKNDLFNLDVKVNAKESNDSGAEPR
                            .  . *:*::     ..         *
```

FIG 1 Leader peptides of prototypical class I lantibiotics. Fully conserved-, strongly- and weakly-conserved residues are indicated by *, : and ., respectively. Bold letters indicates LanP cleavage sites.

Lubelski et al. (4) showed that the presence of lanthionine rings interferes with the dehydrating activity of LanB. The authors have also shown that LanC complex formation (with pre-nisin), and not its cyclase activity is important for proper biosynthesis kinetics of nisin. In the same study they also provided evidence for the alternating and directional activities of LanB and LanC and proposed a new working model for nisin PTM (Fig. 2). In the proposed model LanB and LanC, localized at the cytoplasmic membrane, interacts with a dedicated lantibiotic integral membrane ABC-transporter, LanT (31, 32). It is speculated that LanT pulls the precursor peptide through active sites of the LanBC complex using ATP in the process. This is due to the observations that LanC does not utilize ATP and LanB does not contain motifs that would be able to use ATP. Finally, the fully modified precursor peptide is transported across the membrane and the leader peptide is cleaved off by an extracellular serine protease, LanP (4). This model was proposed with the nisin biosynthetic machinery in mind. However, with few exceptions, modification enzymes of class I lantibiotics have homology and usually cluster closely together, suggesting that this model may be applicable to all class I lantibiotics (33).

Class I is further subdivided into five subgroups based on unmodified precursor peptide amino acid sequence (w/o leader sequence), including, nisin-, epidermin-, streptin-, Pep5- and planosporicin-like lantibiotics. The lantibiotics in these subgroups contain similar N-terminal ring topology that is analogous to the nisin lipid II-binding motif, with the exception of the Pep5-like lantibiotics.

Nisin-like lantibiotics

Nisin-like lantibiotics include all natural nisin variants as well as salvaricin D, subtilin, ericin A and geobacillin I (Fig. 3). Nisin-like lantibiotics have a conserved N-terminal region and similar ring topology. Geobacillin I also has a similar ring topology to that of other nisin-like lantibiotics. However, differences include a single-amino acid linker between rings C and D
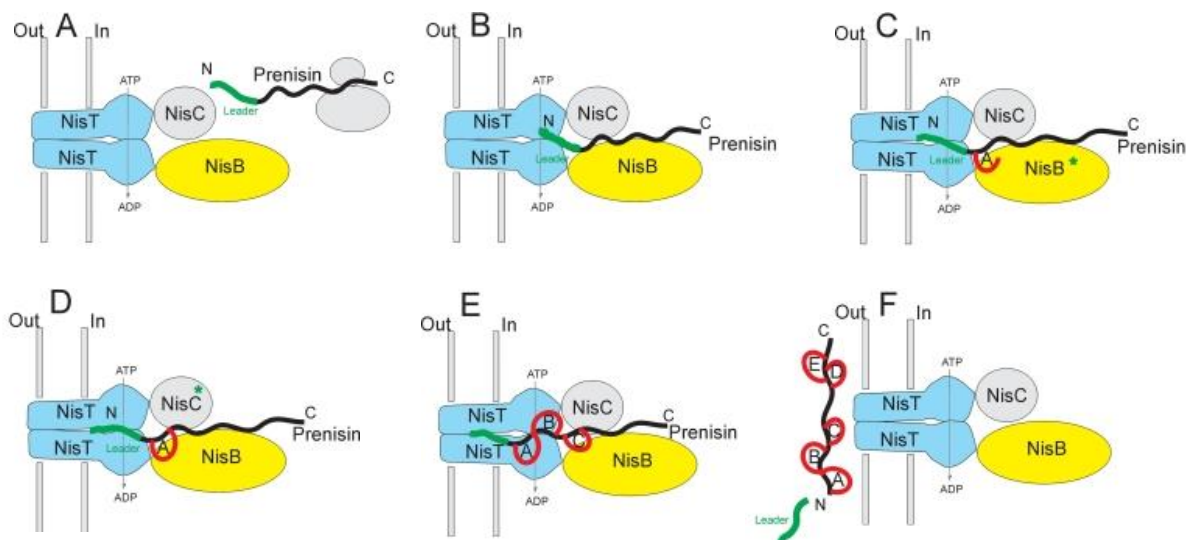
FIG 2 Proposed model for nisin biosynthesis. Ribosomal synthesis of prenisin (A), followed by recognition by NisBTC complex (B). Nisin is subsequently pulled through the active sites of the NisBC complex by NisT (C-E). The alternating actions of NisB and NisC incorporates the Lan/MeLan rings. NisB first dehydrates Ser/Thr followed by cyclization by NisC (indicated by*; C and D, respectively). The pulling action of NisT allows for the consecutive dehydrations/ring formations (E). Finally modified nisin is exported out of the cell and the leader peptide is cleaved by NisP. Figure adapted from Lubelski et al. (4).

and a reduction in the size of ring C (34). Ring E is a Lan, instead of a MeLan and it has two additional C-terminal overlapping rings (34).

Nisin-like lantibiotics bind to the cell wall precursor lipid II with intramolecular hydrogen bonds forming a "pyrophosphate cage" (17). This complex results in inhibition of cell wall biosynthesis and facilitates insertion into the membrane. Insertion results in the leakage of cytoplasmic contents, followed by dissipation of the proton motive force (15, 16, 35, 36). The first two N-terminal MeLan rings play a crucial role in the binding of lipid II (37–39). Ring A of nisin is flexible towards amino acid substitutions. With addition of positive residues correlating with increased antimicrobial activity, and ring disruption resulting in substantial loss of activity (37, 38). The more conserved ring B, is less flexible with regards to amino acid substitutions, with larger amino acids occasionally interfering with the cyclization reaction, resulting in loss of activity (37, 38). Removal or disruption of ring C results in substantial loss of activity (15, 38). The nature of the side chains of ring C amino acids also play a role in its biological activity (15). Substitution of Met at position 17 with Lys does not abolish activity, but reduces pore forming capabilities in lipid II-doped liposomes (15).

Antimicrobial activity is still observed in C-terminal truncated nisin, and non-pore forming lipid II-binding lantibiotics with similar A and B rings. Truncated nisin still binds lipid II, with

14

reduced antimicrobial activity and no pore formation, and acts as an antagonist to full length nisin (37, 38, 40). Nisin also displaces lipid II from its functional locations in Gram-positive bacteria, thereby interfering with cell wall biosynthesis (40, 41). The other antimicrobial action of nisin-like lantibiotics is to produce pores in the cell membrane. Once nisin has been anchored to lipid II, via the pyrophosphate cage, conformational changes result in the formation of stable pores consisting of eight nisin molecules and four lipid II molecules (17, 42). The three amino acid hinge region links the N-terminal A, B, C rings with the C-terminal D and E rings. The hinge provides conformational flexibility which facilitates pore formation (15, 43). Substitution of the three amino acids making up the hinge (positions 20-22) with small chiral amino acids results in increased activity (43, 44). Negative-, aromatic-residues and shortening of the hinge region, has a detrimental effect on activity and pore formation (15, 43, 44). Geobacillin I also forms pores despite only having one amino acid linker between rings C and D (36). Introduction of the Asn-Val-Ala linker, that increases activity in nisin, decreases geobacillin I activity and pore formation (36). Unlike nisin, substitution of the geobacillin I linker with Pro does not have such a detrimental effect on pore formation (36). These results suggest that geobacillin I has a different mechanism of pore formation compared to nisin.

In addition to the antibacterial effect on vegetative cells, nisin and subtilin prevent spore outgrowth in *Bacillus* spp. and *Clostridium* spp. endospores (45-47). Nisin binds to lipid II of germinating spores and prevents spore outgrowth. However, truncated nisin (nisin [1-12] and


```
NisinA        ITSISLCTPGCKTGALMG-CNMKTAT-CHCSIHVSK--
NisinF        ITSISLCTPGCKTGALMG-CNMKTAT-CNCSVHVSK--
NisinQ        ITSISLCTPGCKTGVLMG-CNLKTAT-CNCSVHVSK--
NisinU        ITSKSLCTPGCKTGILMT-CPLKTAT-CGCHFG-----
NisinZ        ITSISLCTPGCKTGALMG-CNMKTAT-CNCSIHVSK--
Subtilin      WKSESLCTPGCVTGALQT-CFLQTLT-CNCKI--SK--
GeobacillinI  VTSKSLCTPGCITGVLM--CLTQNSCVS-CNSCIRC--
SalivaricinD  FTSHSLCTPGCITGVLMG-CHIQSIG-CNVHIHISK--
Entianin      WKSESVCTPGCVTGLLQT-CFLQTIT-CNCKI--SK--
EricinA       VLSKSLCTPGCITGPLQT-CYLCFPTFAKC-------
EricinS       WKSESVCTPGCVTGVLQT-CFLQTIT-CNCHI--SK--
PaenicidinA   VLSIVACSSGCGSGKTAASCVETCG-NRCFTNVGSLC-
```

FIG 3 Nisin-like lantibiotics. Cys residues are shown in red. Bold letters in purple and green designate Thr and Ser that are dehydrated, respectively. Bold black letters indicate Ser that escape dehydration. Lines above letters indicate ring topology of nisin. Figure adapted from Rea et al. (49) and Dischinger et al. (50).

nisin[1-22]) is not able to prevent spore outgrowth, indicating that both modes of action are needed (37, 40). Additionally, nisin hinge mutants unable to form pores, still have antimicrobial activity against vegetative cells but do not prevent spore outgrowth (40). The Dha at position 5 does not seem to be crucial in nisin to prevent spore outgrowth (37, 40). Contradictory results are reported for subtilin, which requires the Dha at position 5 for prevention of spore outgrowth (46, 48). Despite these differences, similarities in the amino acid sequence and similar ring topology leads to speculation that all nisin-like lantibiotics have similar biological activities with regards to vegetative cells and spore out growth.

Epidermin-like lantibiotics

Epidermin-like lantibiotics include, amongst others, mutacin 1140, epidermin, gallidermin and clausin. They have a conserved C-terminal region, which is important for the formation of an additional modification enzyme, LanD (Fig. 4) (51). LanD is responsible for the oxidative decarboxylation of a C-terminal Cys followed by cyclisation by LanC (with Dha/Dhb). This results in the formation of a C-terminal 2-aminovinyl-D-cysteine (AviCys; cyclisation with Dha) or 2-aminovinyl-methyl-D-cysteine (AviMeCys; cyclisation with Dhb) (9, 51, 52).

Epidermin-like lantibiotics bind to lipid II, and have the nisin lipid II binding-motif, composed of rings A and B (16, 41, 53, 54). These peptides are much shorter than nisin-like lantibiotics and cannot form pores in cell membranes exceeding 40 Å (54). Interestingly, wild type gallidermin and gallidermin mutants (A12L) are more potent against *L. lactis* than nisin, even though they lack pore forming capabilities against *L. lactis* (54). This may be explained by the higher affinity gallidermin has for lipid I/II *in vitro*, which has also been observed for epidermin (16, 54). Pore formation is, however, observed in *Micrococcus* spp. and to some extent in *Staphylococcus* spp. possibly due to differences in cell membrane thickness and higher numbers of undecaprenol-linked molecules (54). However, the same A12L gallidermin mutant with increased *L. lactis* activity has significantly less activity against *Staphylococcus* spp. and *Micrococcus* spp. Pore formation is less prominent in *Staphylococcus* spp., however, gallidermin hinge mutants (A12L) still remains active, indicating that further interactions may be involved (54, 56). In a recent study by Chen et al. (55), changes in the size of ring A and its proximity to ring B had major influences on bioactivity of mutacin 1140. This is most likely due to interference with lipid II interaction. As with nisin-like lantibiotics, it seems that the A/B ring motif is crucial for lipid II-binding and bioactivity. Interestingly, introduction of a negatively charged amino acid in the hinge region (R13D) of mutacin 1140 only results in loss

16

of activity when combined with another mutation W4A (55). In contrast, introduction of negatively charged residues in the hinge region of nisin results in decreased activity (43, 44, 57).

Similar to truncated nisin, epidermin-like lantibiotics are able to sequester lipid II from its functional location. This may explain their antimicrobial activity in the absence of pore formation (16, 41, 58). This alternative mode of action is proposed to be conserved in lantibiotics with the nisin lipid II-binding motif (41). Bouhss et al. (53) also showed that clausin binds the pyrophosphate moiety in cell wall lipid intermediates, further illustrating the conserved lipid I/II binding characteristics between nisin- and epidermin-like lantibiotics.

```
Epidermin          IASKFICTPGCA--KTGSFNSYCC
Gallidermin        IASKFLCTPGCA--KTGSFNSYCC
StaphylococcinT    IASKFLCTPGCA--KTGSFNSYCC
MutacinB-Ny266     FKSWSFCTPGCA--KTGSFNSYCC
Mutacin1140        FKSWSLCTPGCA--RTGSFNSYCC
Clausin            FTSVSFCTPGCG--ETGSFNSFCC
MuntacinI          FSSLSLCSLGCTGVKNPSFNSYCC
BsaA2              ITSHSLCTPGCA--KTGSFNSFCC
```

FIG 4 Epidermin-like lantibiotics. Cys residues are shown in red. Bold letters in purple and green indicate Thr and Ser that are dehydrated, respectively. Bold black letters indicate Ser that escape dehydration. Lines above letters indicate ring topology of epidermin. Blue line indicates AviCys. Figure adapted from Rea et al. (49) and Dischinger et al. (50).

Streptin-like lantibiotics

Streptin, produced by *Streptococcus pyogenes,* is the only lantibiotic described in this group (Fig. 5). Little experimental information is published on the structure and activity of streptin (59). Streptin 2 differs from streptin 1 by having three additional amino acids (TPY) on the N-terminal (59). As concluded from data obtained with genome mining, streptin can also be produced by *Bacillus sonorensis* (gi: 657608345) and *Clostridium beijerinckii* (gi: 652493081). The N-terminal ring structure of streptin is similar to that of nisin- and epidermin-like lantibiotics, suggesting that it may also have similar lipid I/II binding properties. As with epidermin-like lantibiotics, streptin is short with only 23 amino acids and if it is capable of pore formation (depending on ring structure) it will most likely not rely on this for antimicrobial activity.

17

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Streptin1     ---VGSRYLCTPGSCWKLVCFTTTVK
Streptin2     TPYVGSRYLCTPGSCWKLVCFTTTVK
```
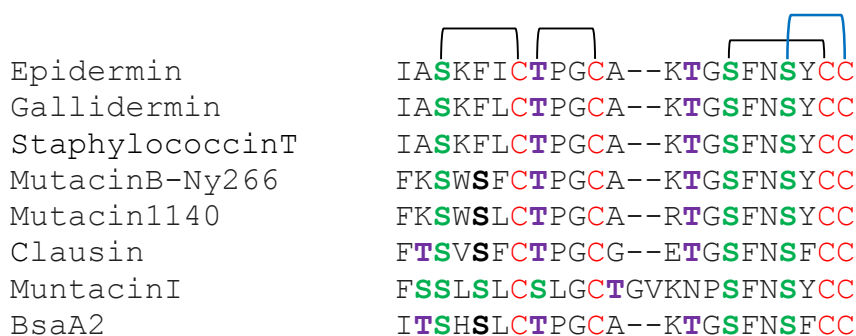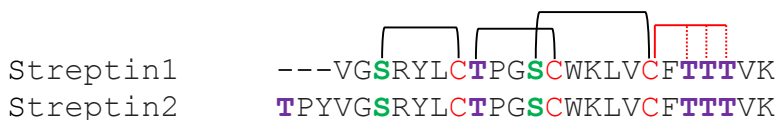
FIG 5 Streptin-like lantibiotics. Cys residues are shown in red. Bold letters in purple and green indicate Thr and Ser that are dehydrated, respectively. Lines above letters indicate proposed ring topology of streptin. Red lines indicates alternative ring C for streptin with dashed lines possible bridging patterns (59). Figure adapted from Rea et al. (49) and Dischinger et al. (50).

Pep5-like lantibiotics

There are four characterized lantibiotics in this subgroup, i.e. Pep5, epicidin 280, epilancin 15X and epilancin K7 (Fig. 6). All four are produced by *S. epidermidis*. All the Pep5-like peptides have an additional modification, i.e. hydration-deamination of Dha/Dhb at position 1. After cleavage of the leader peptide the N-terminal Dha/Dhb at position 1 is exposed and becomes unstable. The unstable Dha (Epicidin 280, Epilancin 15X and Epilancin K7) and Dhb (Pep5) undergoes spontaneous hydration-deamination resulting in the formation of 2-oxopropionyl (Dha) and 2-oxobutyryl (Dhb) (60-64). Furthermore, the N-terminal 2-oxopropionyl undergoes further reduction to form 2-hydroxypropionyl. This reaction is most likely catalysed by the modification enzyme LanO (oxidoreductase), which is found in the operons of epicidin 280 and epilancin 15X, and should also be represented in the operon of epilancin K7.

Pep5-like lantibiotics form pores in sensitive organisms. However, they do not bind to lipid I/II like nisin-, epidermin- and streptin-(possibly) like lantibiotics, suggesting that they use a different recognition/docking mechanism (16, 61). Removal of the N-terminal cap of epilancin 15X does not result in loss of activity, indicating that this modification is not essential for activity. The N-terminal cap may be more essential for stability and protection from cleavage (62). N-terminally truncated epilancin 15X, on the other hand, did display a decrease in activity against *S. cornosus.* This does indicate that there may be an additional non-lipid II docking molecule required for increased activity (62). Flexibility of Pep5-like lantibiotics seems to be important for proper bioactivity as mutations in the hinge region of Pep5 (K18P) were found to be very detrimental for bioactivity (63). Furthermore, the decreased activity observed for epicidin 280, compared to Pep5, may be a result of epicidin 280 being more rigid and not able to properly form pores (64).
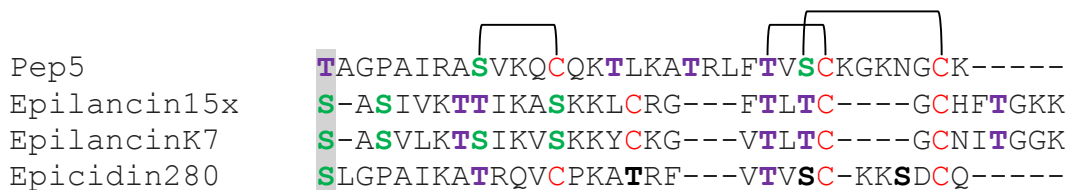
```
Pep5           TAGPAIRASVKQCQKTLKATRLFTVSCKGKNGCK-----
Epilancin15x   S-ASIVKTTIKASKKLCRG---FTLTC----GCHFTGKK
EpilancinK7    S-ASVLKTSIKVSKKYCKG---VTLTC----GCNITGGK
Epicidin280    SLGPAIKATRQVCPKATRF---VTVSC-KKSDCQ-----
```

FIG 6 Pep5-like lantibiotics. Cys residues are shown in red. Bold letters in purple and green indicate Thr and Ser that are dehydrated, respectively. Bold black letters indicate Thr/Ser that escape dehydration. Grey highlighted letters represents Ser/Thr that undergoes hydration-deamination. Lines above letters indicate ring topology of Pep5. Figure adapted from Rea et al. (49) and Dischinger et al. (50).

Planosporicin-like lantibiotics

In the updated classification proposed by Rea et al. (49), planosporicin produced by the uncommon actinomycete *Planomonospora alba* is the only lantibiotic in this group (Fig. 7) (65). However, the lantibiotic microbisporicin also produced by another actinomycete (*Microbispora corallina*) should be included in this group due to high sequence similarity with planosporicin and various genes in its gene cluster, as well as identical thioether bridges (66–70). Microbisporicin does have additional modifications not found in planosporicin, including a chlorinated Trp, hydroxylated Pro and a C-terminal AviCys. These and other amino acid differences may explain the higher antimicrobial activity of microbisporicin (67). The extra modifications in microbisporicin are as a result of two modification enzymes, MibH (tryptophan halogenase) and MibO, which are responsible for the chlorination of Trp and hydroxylation of Pro, respectively (68). It should be noted that the gene clusters of planosporicin-like lantibiotics are not annotated according to the recent recommendations for RiPPs (1). Although there are similarities to the class II mersacidin, they do not have any of the conserved motifs found in this group (65–67). They do, however, have similar N-terminal ring topologies (nisin lipid II-binding motif) found in other class I lantibiotics (66, 67). The C-terminal ring in microbisporicin is almost identical to that of epidermin-like lantibiotics (66, 67). Castiglione et al. (65, 67) showed that microbisporicin and planosporicin are able to block peptidoglycan synthesis in a similar manner to that reported for mersacidin and actagardine. Microbisporicin and planosporicin cause accumulation of UDP-MurNAc-pentapeptide (UDP-*N*-acetylmuramyl-pentapatide), similar to mersacidin and actagardine. Similar accumulation was not observed with nisin, which disrupts the membrane integrity, resulting in cessation of all macromolecular synthesis. It would be interesting to see if epidermin-like lantibiotics also result in this accumulation in a strain where they do not form pores. Due to the short nature of

these peptides, reminiscent of epidermin-like lantibiotics, it is conceivable that they also only form pores under certain conditions. Microbisporicin has minimum inhibitory concentration (MIC) values that are superior to that of nisin for selected vancomycin-intermediate *S. aureus* (VISA), methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant enterococci (VRE) (67). Further research is required to establish how microbisporicin and planosporicin target lipid II and if the nisin lipid II-binding motif has the same function in these lantibiotics.
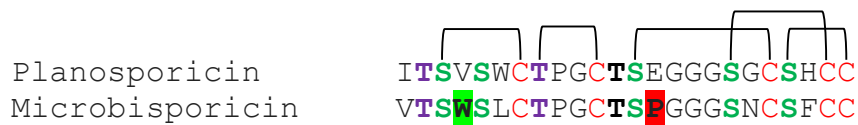
FIG 7 Planosporicin-like lantibiotics. Cys residues are shown in red. Bold letters in purple and green indicate Thr and Ser that are dehydrated, respectively. Bold black letters indicate Thr that escape dehydration. Lines above letters indicate ring topology of planosporicin. Green highlighted Trp is chlorinated to chlorotryptophan. Red highlighted Pro is hydroxylated to dihydroxyproline. Figure adapted from Rea et al. (49) and Dischinger et al. (50).

Class II

Class II lantibiotics often have a more globular structure compared to class I. However, the main difference between class I and class II lantibiotics is the modification machinery they use for dehydration and cyclization. Class I uses LanB and LanC for dehydration and cyclization, respectively, whereas class II lantibiotics have one bi-functional synthetase LanM (1). LanM consists of an N-terminal dehydratase (no sequence homology to LanB) and a C-terminal LanC-like domain. Unlike LanB, LanM utilizes ATP to phosphorylate Ser/Thr to yield the respective dehydrated residues, followed by cyclization by the C-terminal LanC-like domain (2, 33, 71). Lee et al. (6) demonstrated using the LanM enzymes that modify lacticin 481 (LctM) and haloduracin-β (HalM2), process them in an N- to C- terminal direction when the leader peptide is covalently attached. Furthermore, the dehydration and cyclization reactions occur on a similar time scale. Another observation is that the leader peptide is not strictly needed for LctM activity, but when the leader peptide is provided *in trans* enzyme activity significantly increases (5). It was, therefore, suggested that leader peptide binding does not result in a conformational change resulting in active enzyme, but rather stabilizes an active form of the enzyme shifting the equilibrium to more active enzyme (5). Dehydrations do not occur in a specified direction when the leader peptide is provided *in trans* (5). This suggests that attachment of the leader peptide to the core peptide allows for process directionality. As with the leader peptides of class I, class II leader peptides also contain conserved regions that are important for proper modification (Fig. 8) (2, 72, 73). However, in the case of lacticin 481, not all these regions are important for synthetase activity (73). The only single mutation that had a significant effect on activity was at position -7 (L-7K), which resulted in both decreased dehydration and cyclization (73). Similar results were also reported for mutacin II (L-7K) (72). Mutations of the lacticin 481 leader at positions I-4P, D-6P, L-7E/K and E-8A, resulted not only in decreased dehydration but also interfered with proper cyclization. Structural predictive tools calculate a helical motif for stretches of the leader peptide, and helical-breaking mutations, I-4G/P, L-5P, D-6G/P and E-8P, result in decreased dehydratase activity (73). Similar results are also found for the leader of nukacin ISK-1 (74). This suggests that LanM may bind to a secondary structure, and that disruption of this structure results in decreased synthetase efficiency. In addition to nukacin ISK-1 leader mutants (L-7P and E-8P) having reduced/diminished antimicrobial activity, they also have reduced helicity of the leader resulting from the presence of the helix-breaking residue Pro (74). These results further substantiate the possible binding of LanM to a secondary helical structure. The α-helical

21

structure is also found in class I lantibiotic leader peptides and, therefore, may be an important feature in the maturation of all lantibiotics (75). LanM is very promiscuous, with LctM able to process several class II lantibiotics with the lacticin 481 leader attached (73). Additionally, LctM can also process lacticin 481 with a mutacin II leader attached (73).

```
LactocinS              ---------------MKTEK--------KVL-DE---LSLHASAKMGARDVESSMNAD-
BovicinHJ50            ---------------MMNAT------ENQIFVETVSDQEL--EMLIGG-----------
Mersacidin            -MSQEAIIRSWKDPFSRENSTQNP---AGNPF-SELKEAQM--DKLVGAGDMEAA-----
Lacticin3147          ---------------MKEKNMKKNDTIELQLGKYLEDDMI--ELAEGDESHGG------
Lacticin481           ---------------MKEQNSFN------LL-QEVTESEL--DLILGA-----------
CytolysinCLL          ---------------MENLSVVP------SF-EELSVEEM--EAIQGS-----------
CytolysinCLs          MLNKENQ----ENYYSNKLELVGP------SF-EELSLEEM--EAIQGS-----------
Cinnamycin            ---------------MTAS---------ILQQSVVDADFRAALLENPAAFGASAAALPT
Cinnamycin (cont.)PVEAQDQASLDFWTKDIAATEAFA        : .         :
```

FIG 8 Leader peptides of prototypical class II lantibiotics. Strongly- and weakly-conserved residues are indicated by **:** and **.**, respectively. Grey letters indicate site of leader cleavage.

Unlike class I lantibiotics, class II lantibiotics also have a bi-functional membrane protein, LanT that functions as a protease and a transporter (76, 77). Mutations in the leader of mutacin II (G-1A and G-2A) results in accumulation of dehydrated pre-mutacin, suggesting prevention of either cleavage, transport or both (72). In the lacticin 481 leader, the Gly-motif (G-2 and A-1) is important for proteolysis. Furthermore, the protease domain of the bi-functional LctT recognizes the same putative secondary structure that LctM recognizes, upstream of the Gly-motif (73, 76). The recognition by the protease domain of the LctT suggests that substrate recognition takes place in the protease- instead of the transport-domain (76).

Class II is subdivided into eight subgroups based on the unmodified precursor peptide amino acid sequence. These include; lacticin 481-, mersacidin-, ltnA2-, cytolysin-, lactocin S-, cinnamycin-, sublancin- and bovicin HJ50-like lantibiotics.

Lacticin 481-like lantibiotics

This is a large group, made up of more than 19 characterized lantibiotics. Sequence alignment revealed that while some residues differ with high frequency there are several conserved residues throughout this group (Fig. 9) (28). All the lacticin 481-lantibiotics have Ser, Thr and Cys in similar positions, indicating that these are involved in ring formation (50, 78–82). There is also a conserved Glu in most lacticin 481 lantibiotics corresponding to position 13 of lacticin 481 (Fig. 9). Mutation of the Glu13 (E13A) abolishes activity in lacticin 481 (83). Nukacin ISK-1 has an Asp at this site and changing this to Glu does not alter activity, however, other

22

changes at this position result in loss of activity (84). This Glu is also conserved in almost all mersacidin-like lantibiotics, and is important for activity (Fig. 10) (85). In nukacin ISK-1 the Gly at position 5, which is also conserved in lacticin 481-like lantibiotics, is not amendable to change, with only the G5W mutant resulting in activity (84).

Mutants of mutacin II lacking thioether bridges have no or severely diminished activity (86). Similar results are also reported for lacticin 481 and nukacin ISK-1 also does not tolerate changes within its ring regions (84, 87). Collectively these results indicate that all three rings are important for antimicrobial activity.

```
Lacticin481          -KGGSGVIHTISHECNMNSWQFVFTCCS
MutacinII            NRWWQGVVPTVSYECRMNSWQHVFTCC-
MutacinK8            --MGKGAVGTISHECRYNSWAFLATCCS
NukacinISK-1         -KKKSGVIPTVSHDCHMNSFQFVFTCCS
NukacinKQU-131       -KKKSGVIPTVSHDCHMNSFQFMFTCCS
StreptococcinA-FF22  --GKNGVFKTISHECHLNTWAFLATCCS
StreptococcinA-M49   --GKNGVFKTISHECHLNTWAFLATCCS
Macedocin            --GKNGVFKTISHECHLNTWAFLATCCS
MacedocinA1          ---GHGV-NTISAECRWNSLQAIFSCC-
ButyrivibriocinOB247 ---GDGVFRTISHECAMNTWMFIFTCCS
ButyrivibriocinOR36  ---GDGVFRTISHECHMNTWMFIFTCCS
ButyrivibriocinOR79  ---GNGVIKTISHECHMNTWQFIFTCCS
Salivaricin9         ---GNGVVLTLTHECNLATWTKKLKCC-
Salivaricin_A        -KRGSGWIATITDDCPNS----VFVCC-
SalivaricinA1        -KKGSGWFATITDDCPNS----VFVCC-
SalivaricinA2        -KRGTGWFATITDDCPNS----VFVCC-
SalivaricinA3        -KKGPGWIATITDDCPNS----IFVCC-
SalivaricinA4        -KRGPGWIATITDDCPNS----IFVCC-
SalivaricinA5        -KRGPGWIATITDDCPNS----VFVCC-
SalivaricinB         ---GGGVIQTISHECRMNSWQFLFTCCS
SalivaricinG32       ---GNGVFKTISHECHLNTWAFLATCCS
Variacin             ---GSGVIPTISHECHMNSFQFVFTCCS
Ruminococcin_A       ---GNGVLKTISHECNMNTWQFLFTCC-
```

FIG 9 Lacticin 481-like lantibiotics. Cys residues are shown in red. Bold letters in purple and green indicate Thr and Ser that are dehydrated, respectively. Bold black letters indicate Thr/Ser that escape dehydration. Lines above letters indicate ring the topology of lacticin 481. Where Thr/Ser are not bold or coloured the dehydration of Thr/Ser and structure of peptides are unknown. Grey highlighted Gly is conserved throughout group. Figure adapted from Rea et al. (49) and Dischinger et al. (50).

Nukacin ISK-1 and lacticin 481 bind to lipid II, and inhibit the transglycosylation step in peptidoglycan biosynthesis (83, 88, 89). This mode of action is similar to that reported for mersacidin- and planosporicin-like lantibiotics. A C14S ring mutant of nukacin ISK-1 (mutant

lacking ring A) has no interaction with lipid II, and a D13A mutant (same position as the conserved Glu in mersacidin) has less accumulation of UDP-MurNAc pentapeptide, indicating a role of ring A in lipid II binding (88). It would be interesting to see how lacticin 481 Glu13 mutants interact with lipid II, and if there would be less accumulation of cell wall precursors. The absence of activity of mutacin II and lacticin 481 ring A mutants can, therefore, also be due to a lack of interaction with lipid II (86, 87). The conserved sequence of ring A ($\underline{T}$-I$^{/V}$-$\underline{S}^{/T}$-x-E$^{/D}$-$\underline{C}$; x is undefined amino acids, superscript is minority residue changes) resembles the mersacidin-lipid II-binding motif ($\underline{C}$-$\underline{T}$-L$^{/x}$-$\underline{T}^{/S}$-x-E-$\underline{C}$), and is also important for lipid II binding and activity (88, 90).

Mersacidin-like lantibiotics

The mersacidin-like lantibiotics all have similar ring topology, and the putative mersacidin lipid II-binding motif is conserved throughout the group (Fig. 10). Mersacidin is, however, the only one in the group with a C-terminal AviMeCys (90, 91). Lipid II binding and subsequent blocking of peptidoglycan synthesis occurs for several mersacidin-like lantibiotics, including mersacidin, actagardine, lacticin 3147- and haloduracin-alpha (65, 90–94). Mersacidin is able to discriminate between lipid I and II, which suggests that the GlcNAc (*N*-acetyl-$_D$-glucosamine) of lipid II plays a role in recognition (90, 91). Similar to some class I lantibiotics, the pyrophosphate group is also implicated in lipid II binding (90).

The structure-activity relationship of mersacidin has been extensively studied and shows that the peptide is not very amendable to change (85, 95). As expected, the highly conserved lipid II-binding motif (position 12-18) is not flexible with regards to amino acid substitutions, and only conservative amino acid changes are tolerated (85, 95). Actagardine A has a similar reluctance for substitution, with the B ring (lipid II-binding motif) being the least amendable and only conservative changes result in activity (96). The E11D mutant (Glu17 in mersacidin) is produced only at trace levels and is biologically inactive (96). The Gly at position 9 (relative to mersacidin) is also conserved throughout the group (and in lacticin 481-like lantibiotics), and several substitutions at this position are not well tolerated in mersacidin, actagardine and lacticin 3147-α (Fig. 10) (95–97). Mersacidin, actagardine and lacticin 3147-α have additional modifications. Mersacidin has an N-terminal AviMeCys and the accompanying LanD to catalyse the modification (90, 91, 98). Actagardine has an N-terminal sulfoxide group with formation catalysed by LanO (GarO) (99). Lacticin 3147α contains $_D$-alanine residues which

24

arise from the hydrogenation of Dha (i.e. L-serine to D-alanine) by a dedicated modification enzyme LanJ (LtnJ) (100, 101).

The alpha peptides of two-component lantibiotics also fall into this group. Two-component lantibiotics consist of two separate peptides (alpha and beta) that are inactive on their own and need to be combined for activity. As with mersacidin and actagardine, amino acid substitutions of the conserved Glu are not well tolerated. Ring A of the alpha peptides are more tolerant towards amino acid changes, compared to actagardine and mersacidin, and is not essential for activity (77, 95, 96, 102, 103). It has, therefore, been suggested that the A ring plays a possible role in protection from proteolytic cleavage, rather than activity (102). The conserved Gly at position 13 of lacticin 3147-α (conserved in alpha peptides) does not handle substitution well with mutants displaying reduced activity (97). Substitution of the conserved Glu (E22Q; Glu17 in mersacidin) in the B ring of haloduracin-α containing the conserved lipid II-binding motif does not result in complete loss in activity (102). Follow up studies reported that the destruction of the B ring (corresponds to mersacidin C ring) only reduces the ability of haloduracin-α to inhibit lipid II polymerization (5-fold reduction), while an E22Q (corresponds to Glu17 in mersacidin) substitution abolished inhibition (up to 100 μM) (92, 104). These results are interesting, as haloduracin-α E22Q added to the beta peptide, still retained low levels of activity (92, 104). Disruption of the C ring (equivalent to haloduracin-α B-ring) in lichenicidin-α also does not abolish activity, but does result in a significant reduction (77). An E26A (corresponds to Glu17 in mersacidin) substitution abolishes activity (77). These results are in contrast to those reported for lacticin 3147-α, where destruction of the C ring (containing lipid II-binding motif) results in loss of activity (103). It should be mentioned that both lichenicidin and haloduracin were heterologously produced in *Escherichia coli.* In the case of lichenicidin, total extract (cell and supernatant) was used for activity assays, and for haloduracin, His-tagged purified peptide was obtained from cell extracts and processed *in vitro* with LanM. Lacticin 3147 assays used cell-free supernatant (i.e. secreted peptide). Therefore, the lack of activity observed for the ring mutants may be due to peptide that is not exported, but accumulates in the cell. Small quantities of peptide with the correct mass were detected by colony mass spectrometry of lacticin 3147-α C19A and C25A mutants (103). This may also be the case in some experiments performed with other lantibiotics, where low excreted yields may obscure possible activity of un-secreted/low-secreted variants.

Haloduracin-α with its leader attached, retains activity when combined with haloduracin-β, albeit significantly less than the wild type (92). The alpha peptide with the leader attached

shows potent lipid II-binding (*in vitro*) activity. The leader peptide and the bacterial cell are both negatively charged, which could interfere with the lipid II interaction, and would explain the reduced antimicrobial activity. These results suggest that the N-terminal length does not influence lipid II binding. It would be interesting to evaluate if the same result is observed with other mersacidin- and lacticin 481-like lantibiotics.

FIG 10 Mersacidin-like lantibiotics. Cys residues are shown in red. Bold letters in purple and green designate Thr and Ser that are dehydrated, respectively. Bold black letters indicate Thr/Ser that escape dehydration. Lines above letters indicates the ring topology of mersacidin (blue line indicates AviMeCys) and haloduracin-α, respectively. Dashed line indicates a disulphide bridge with underlined Cys residues taking part in disulphide bridge formation. Grey highlighted residues are conserved throughout group. Yellow highlighted Ser are converted to D-Ala. Green highlighted letters indicate lanthionine sulfoxide bridge. Grey highlighted Thr is converted to 2-oxobutyryl. Red arrow indicates site of second proteolytic cleavage. Figure adapted from Rea et al. (49) and Dischinger et al. (50).

LtnA2-like lantibiotics

This group only consists of the beta peptides of the two-component lantibiotics, which have little N-terminal homology, but a conserved C-terminal motif (C-P-T-T-K$^{/A}$-C-T$^{/S}$-x-x-C) (Fig. 11). Geobacillin II, which is not part of a two-component lantibiotic system, can also be included in this group (34). The ring topologies of peptides in this group are highly similar, with identical C-terminal rings (50, 77, 105, 106). As with lacticin 3147-α, lacticin 3147β has

26

an additional modification, namely the reduction of Dha to D-Ala by the dedicated reductase LanJ (100, 101). The beta peptides of lichenicidin and lacticin 3147 are not amendable to disruption of any of their C-terminal Lan/MeLan rings, as disruption either results in low- or no-production variants (77, 103). Disruption of the C and D rings of haloduracin-β also results in significant loss of activity (102). However, no conclusions can be made for the disruption of ring B of haloduracin, as its disruption affects the formation of multiple other rings (102). Once again, the haloduracin-β variants were produced in *E.coli* and were processed *in vitro* by LanM, whereas lichenicidin and lacticin 3147 were produced and processed in their heterologous hosts, *E. coli* and *L. lactis*, respectively. The use of *in vitro* processing overcomes any limitations that may be faced by complex *in vivo* systems, such as no- or low production. Despite these differences, it is evident that the C-terminal Lan/MeLan rings of the beta peptides are required for proper synergistic activity with their corresponding alpha peptides. Similar to the alpha peptides, beta peptides have either a ring structure or a cap (e.g. oxobutyryl) at/near their N-terminal (77, 102, 103). The N-terminal cap may serve more of a protective role, as they are not essential for activity (77, 102, 103). The way in which the beta peptide recognizes the alpha peptide is unknown. However, experimental data shows that the alpha peptide must first bind to lipid II, followed by recruitment of the beta peptide which binds to the alpha peptide-lipid II complex, which results in pore formation (104, 107). The conserved nature of the C-terminal rings of the beta peptide does, however, suggest that the C-terminal may play a role in the recognition of the alpha peptide (103). It is also interesting to note that alpha and beta peptides seem to be promiscuous and are able to show synergistic activity when combined with different partners, as shown for combinations of lacticin 3147 and staphylococcin C55 peptides (108).

Some of the beta peptides undergo a secondary cleavage reaction to remove an additional six amino acid hexapeptide after the initial cleavage by LanT (77, 105, 109, 110). The protease involved in this reaction is not always identifiable in the gene cluster and may not be a dedicated protease. However, a serine protease, LicP, is involved in the cleavage of the hexapeptide from lichenicidin-β (77). LicP shows similarity to CylA, which is an extracellular protease responsible for N-terminal trimming of cytolysin peptides (77). Similarly, a putative extracellular protease was identified to be responsible for the cleavage of the hexapeptide from haloduracin-β (102). Geobacillin II could not be produced *in vivo*, it is thus not known whether a secondary cleavage reaction takes place (34). However, removal of at least two amino acids

after the GG-motif is required for activity and it has been proposed that the entire heptapeptide (YTEVSPQ) is removed after initial cleavage (34).
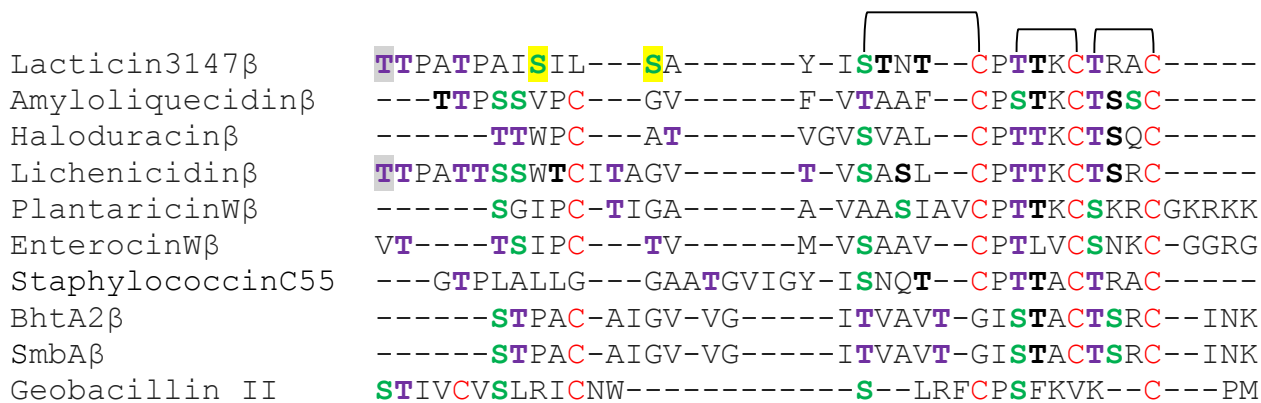
```
Lacticin3147β      TTPATPAISIL---SA------Y-ISTNT--CPTTKCTRAC-----
Amyloliquecidinβ   ---TTPSSVPC---GV------F-VTAAF--CPSTKCTSSC-----
Haloduracinβ       ------TTWPC---AT------VGVSVAL--CPTTKCTSQC-----
Lichenicidinβ      TTPATTSSWTCITAGV------T-VSASL--CPTTKCTSRC-----
PlantaricinWβ      ------SGIPC-TIGA------A-VAASIAVCPTTKCSKRCGKRKK
EnterocinWβ        VT----TSIPC---TV------M-VSAAV--CPTLVCSNKC-GGRG
StaphylococcinC55  ---GTPLALLG---GAATGVIGY-ISNQT--CPTTACTRAC-----
BhtA2β             ------STPAC-AIGV-VG-----ITVAVT-GISTACTSRC--INK
SmbAβ              ------STPAC-AIGV-VG-----ITVAVT-GISTACTSRC--INK
Geobacillin_II     STIVCVSLRICNW-----------S--LRFCPSFKVK--C---PM
```

FIG 11 LtnA2-like lantibiotics. Cys residues are shown in red. Bold letters in purple and green indicate Thr and Ser that are dehydrated, respectively. Bold black letters indicate Thr/Ser that escape dehydration. Lines above letters indicate the ring topology of lacticin 3147β. Grey highlighted Thr are converted to 2- oxobutyryl. Yellow highlighted Ser are converted to D-Ala. Figure adapted from Rea et al. (49) and Dischinger et al. (50).

Cytolysin-like lantibiotics

This group consists of the two-component lantibiotics cytolysin and the newly isolated carnolysin (A1 and A2) and cerecidin (Fig. 12). Unlike the other two-component lantibiotics, the two peptides have substantial N-terminal homology to each other (111, 112). Recently, cerecidins (A1 and A7) were discovered in the genome of *B. cereus* As 1.1846, with homology to cytolysin L_s, carnolysin A2 and some LtnA2-like lantibiotics (113). Upon closer examination of the amino acid sequences the most similarity, with regard to cytolysin, is within the six amino acids cleaved off during the second cleavage (Fig. 12). Similar observations can be made for carnolysin and cerecidins. Cerecidins are not two-component lantibiotics and the two characterized peptides only differ by two amino acids, and have potent individual activity (113). The ring topologies of carnolysin and cytolysin are similar, both with only two Lan/MeLan rings (114–116). Cerecidins have a similar ring topology to cytolysin L_s and canrnolysin A2, with only two Lan rings and the DhxDhxXxxXxxCys (where Dhx = Dha/Dhb) motif at ring A (113). Cytolysin, carnolysin and most likely cerecidins contain the unusual stereoisomers LL-Lan and LL-MeLan in certain positions and the more common DL-Lan stereoisomer in others (114, 116). Furthermore, the LL- and DL-stereoisomers are in the same

positions in carnolysin and cytolysin (114, 116). The $_{LL}$-Lan/MeLan configuration is formed from the DhxDhxXxxXxxCys motif. This motif is also found in other lantibiotics, including haloduracin and lichenicidin, and is proposed to be responsible for the unusual $_{LL}$-configuration (116). Using chiral GC/MS, it is observed that haloduracin-β has a 1:2 ratio of $_{LL}$:$_{DL}$-MeLan (116). This means that the $_{LL}$-configuration is not so unusual. The formation of only two non-overlapping rings in cytolysin L_s, carnolysin A2 and cerecidins make this group unique in lantibiotics (113, 114, 116). Carnolysin is even more unusual in that it contains $_D$-Ala and $_D$-Abu, making it the first ribosomally synthesized peptide to contain $_D$-Abu, and the first lantibiotic to contain $_{LL}$-Lan/MeLan in combination with $_D$-amino acids (114). The reductase, CrnJ, is responsible for the reduction of Dha and Dhb to $_D$-Ala and $_D$-Abu, respectively (114). Therefore making it more flexible than LtnJ (114). Cytolysin, in addition to antibacterial activity, also has lytic activity against erythrocytes and other eukaryotic cells, making it a virulence factor (116–118). The same lytic activity is not observed with mature carnolysin, and may be due to differences in amino acid sequence or to the additional PTMs found in carnolysin (114). Unlike the other two-component lantibiotics, both of the cytolysin peptides undergo a secondary proteolysis reaction to remove the hexapeptide (sequence for both L_S and L_L are the same), this cleavage reaction (performed by CylA) is essential for activity (116). The same secondary cleavage is required for carnolysin to be active, with eight amino acids removed from carnolysin A1 (114). Carnolysin is not secreted in an active form and needs to be cleaved *in vitro* with GluC (both peptides) (114). There is a gene for a protease homologous to CylA (i.e. CrnP) in the carnolysin gene cluster. However, it is not known if the protease is active or if it is expressed under experimental conditions (114).

The mode of action of these peptides is unknown and whether there is a docking molecule, such as lipid II, still needs to be established. In the absence of target cells (phosphatidylcholine: cholesterol lipid bilayers), CylL$_S$ and CylL$_L$ forms a complex, inhibiting CylL$_S$ from autoinducing the cytolysin operon (119). When CylL$_L$ binds to target cells (with a 6.5 fold higher affinity than CylL$_S$) CylL$_S$ is released and able to autoinduce the operon (119). Cerecidins do not need an additional component for antimicrobial activity, and present the simplest architecture required for potent lantibiotic activity. Alanine scanning of cerecidin A7 also revealed that the peptide does not tolerate Ala substitutions, with the exception of T13A, which resulted in increased activity (113). It would be interesting to see if cerecidins have the same haemolytic activity as cytolysin. These peptides may have evolved from the same ancestor and that cerecidins may have once been part of a two-component system. Another

possible introduction to this group, although not fully characterized, is a putative lantibiotic produced by *Bifidobacterium longum* DJO10A. This peptide is reported to have a broad antimicrobial spectrum, with activity against Gram-negative- and -positive bacteria (120, 121). Judging from the amino acid sequence, the N-terminal does not have any thioether rings. However, the C-terminal may have possible similarities in ring topology to carnolysin A1 and cytolysin L_L. This is only speculation and further characterization of this peptide needs to be done to confirm structural and activity properties (120, 121).
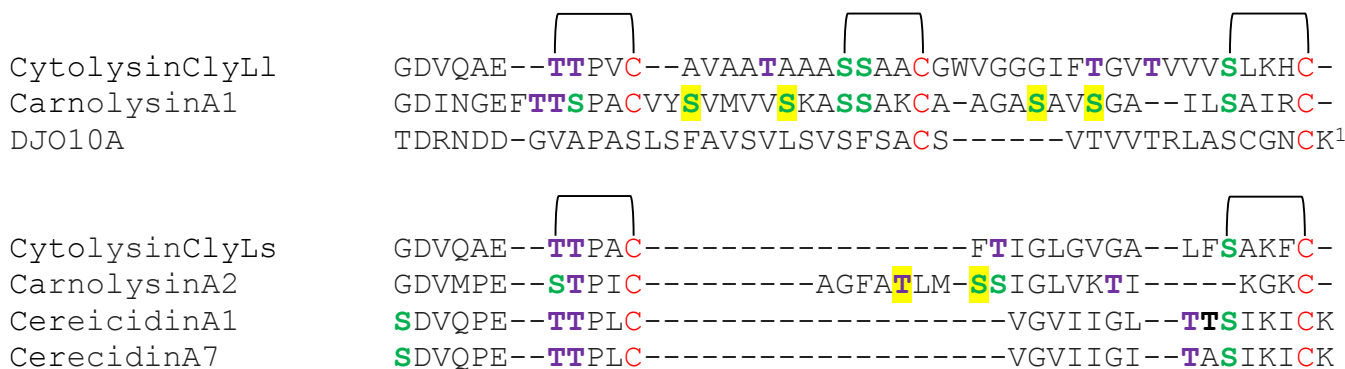
```
CytolysinClyLl   GDVQAE--TTPVC--AVAATAAASSAACGWVGGGIFTGVTVVVSLKHC-
CarnolysinA1     GDINGEFTTSPACVYSVMVVSKASSAKCA-AGASAVSGA--ILSAIRC-
DJO10A           TDRNDD-GVAPASLSFAVSVLSVSFSACS------VTVVTRLASCGNCK[1]


CytolysinClyLs   GDVQAE--TTPAC----------------FTIGLGVGA--LFSAKFC-
CarnolysinA2     GDVMPE--STPIC---------AGFATLM-SSIGLVKTI----KGKC-
CereicidinA1     SDVQPE--TTPLC-----------------VGVIIGL--TTSIKICK
CerecidinA7      SDVQPE--TTPLC-----------------VGVIIGI--TASIKICK
```

FIG 12 Cytolysin-like lantibiotics. Cys residues are shown in red. Bold letters in purple and green indicate Thr and Ser that are dehydrated, respectively. The bold black Thr escapes dehydration. Lines above letters indicate the ring topology of cytolysinC_L and cytolysinC_S, respectively. Yellow highlighted Ser and Thr are converted to D-Ala and D-Abu, respectively. [1]Predicted structure, cleavage site and PTMs unknown. (113, 114). Figure adapted from Rea et al. (49) and Dischinger et al. (50)

Lactocin S

Lactocin S does not have significant homology to any of the other lantibiotics in class II, and belongs to its own subgroup (Fig. 13). It is the least modified lantibiotic and belongs to the few lantibiotics that contain D-amino acids (122, 123). Unlike lacticin 3147 and carnolysin, a LanJ has not yet been identified for the formation of D-alanine in lactocin S. The protein designated LasN (gi:1150481) has sequence similarity to CrnJ, suggesting its involvement in D-alanine formation (124, 125). Lactocin S has been synthesized using solid state synthesis and in doing so the stereochemistry of the ring structures was confirmed (126). The exact mode of action has not been elucidated, but it is known that activity is pH dependent (127).When *Pediococcus acidilactici* was grown in the presence of lactocin S at an initial pH of 6.8, growth commenced normally and declined steadily as the pH started to drop below 6. At pH values below 5.8 the turbidity of the culture started to decrease, suggesting cell lysis (127). When the initial pH was less than 5.5 no growth was detected (127). All experiments were corroborated with cell counts.

These experiments suggest that at higher pH lactocin S is bacteriostatic and at lower pH, increasing the positive charge of the peptide and thus, the interaction with the bacterial membrane, results in bactericidal activity. The length of the peptide also suggests that it is possible for the peptide to form pores to result in membrane destabilization (54, 128).

FIG 13 Lactocin-like lantibiotics. Amino acid sequence of lactocin S. Cys residues are shown in red. Bold letters in purple and green indicate Thr and Ser that are dehydrated, respectively. The bold black Thr escapes dehydration. Yellow highlighted Ser are converted to ᴅ-Ala. Lines above letters indicates ring topology. Figure adapted from Rea et al. (49) and Dischinger et al. (50).

Cinnamycin-like lantibiotics

The cinnamycin-like lantibiotics, that include cinnamycin, ancovenin, duramycin, duramycin B and C, are short globular lantibiotics with very high homology (Fig. 14). Cinnamycin rings are formed bi-directionally, such that the Cys involved in MeLan formation is located on the N-terminal side and the Cys involved in Lan formation is located at the C-terminal end (as with other lantibiotics) (129). This is also observed for the other lantibiotics in this group (129). Cinnamycin and duramycin peptides contain additional PTMs, namely a hydroxylated Asp (Asp15) and a lysinoalanine link between the C-terminal Lys and Dha (Dha6) (27). The biosynthetic enzymes involved in these modifications have been identified; LanX (hydroxylase) for the hydroxylation of Asp15, and the small protein, Cinorf7, is important for the formation of lysinoalanine in cinnamycin (129). The structures for all the cinnamycin-like peptides are known, revealing identical ring topology, with the exception of ancovenin, in that it lacks a lysinoalanine cross-link (27, 130, 131).

Cinnamycin-like lantibiotics are not known for their antimicrobial activity, and are only active against a few bacterial strains. They interfere with membrane permeability, calcium uptake and protein transport (26, 132–136). Cinnamycin and duramycin bind ethanolamine phospholipids and phosphatidylethanolamine (PE), preferring smaller vesicles with a high membrane curvature (137–139). Bacterial cells with little or no PE prove to be resistant to these peptides (137). The lysinoalanine cross-link is important for activity, heterologously expressed cinnamycin, lacking lysinoalanine has no activity (129). Nuclear magnetic resonance of

31

cinnamycin:LysoPE complexes also suggest interaction of lysinoalanine with the phosphate group of LysoPE (140). Phosphatidylethanolamine is a substrate for phospholipase A2, which is involved in inflammatory responses (e.g. vascular inflammation). Therefore, the sequestering of PE by cinnamycin and duramycin may result in immune modulation through the indirect inactivation of phospholipase A2 (139, 141, 142). Little is known of the antimicrobial activity of ancovenin and more focus has been placed on its role in the modulation of angiotensin I converting enzyme (ACE) (25, 130).
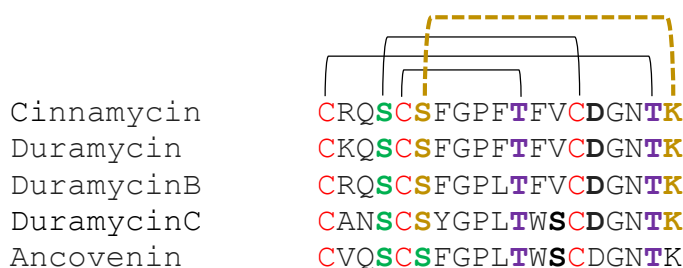

Cinnamycin	CRQSCSFGPFTFVCDGNTK
Duramycin	CKQSCSFGPFTFVCDGNTK
DuramycinB	CRQSCSFGPLTFVCDGNTK
DuramycinC	CANSCSYGPLTWSCDGNTK
Ancovenin	CVQSCSFGPLTWSCDGNTK

FIG 14 Cinnamycin-like lantibiotics. Cys residues are shown in red. Bold letters in purple and green indicate Thr and Ser that are dehydrated, respectively. Bold black Asp and Ser undergoes hydroxylation and escape dehydration, respectively. Lines above letters indicate the ring topology of cinnamycin. Dashed gold line and letters indicates lysinoalanine bridge and residues involved, respectively. Figure adapted from Rea et al. (49) and Dischinger et al. (50).

Sublancin-like lantibiotics

Sublancin 168, produced by *B. subtilis*168, was grouped as a lantibiotic due to the assumed presence of Dha and one MeLan bridge (143). However, it was recently shown that sublancin 168 is not a lantibiotic, but rather an unusual S-linked glycopeptide (144). This is supported by the absence of the lantibiotic biosynthetic modification machinery in the *B. subtilis*168 genome. There is some similarity between the leader peptide of sublancin and that found in other class II lantibiotics (same putative LanM/T recognition motifs), which may be required for recognition by the ABC-transporter SunT, which has some homology to PepT (143). The leader peptide was not required for the S-glycosyl transferase activity, which catalyses the attachment of glucose to Cys22 (required for bioactivity) (144). Based on these results sublancin-like lantibiotics should be removed from the updated classification system (49).

Bovicin HJ50-like lantibiotics

In previous classification schemes bovicin HJ50 was grouped as a lacticin 481-like lantibiotic (49). However, it has recently been suggested that bovicin HJ50, along with several other similar lantibiotics, be placed in their own subgroup (145, 146). The basis for this, in addition to sequence homology and identical ring topology, is a disulphide bridge found in all these lantibiotics and is essential for their activity (Fig. 15) (145). The disulphide bridge forms the C ring of bovicin-like lantibiotics, whereas the corresponding ring in lacticin 481-like lantibiotics is formed by a thioether bridge. Characterized peptides in this new group include bovicin HJ50, suicin, perecin, cerecin and thuricin (145).

Residues important for activity in bovicin HJ50 correspond to conserved amino acids in lacticin 481-like lantibiotics, which is not surprising due to their high sequence similarities (145, 147). The N-terminal of bovicin HJ50 first interacts with the membrane, most likely through the lipid II-binding motif (T-L-T-K-D-C), followed by insertion of the C-terminal (147). In contrast to lacticin 481-like lantibiotics and similar to nisin-like lantibiotics, bovicin HJ50 can form pores and does not result in the accumulation of UDP-MurNAc pentapeptide (147). The pore-forming capability is proposed to be due to the presence of β-sheets and turns in the B- and C-ring regions when in the presence of a hydrophobic membrane environment (147). The disulphide bridge of the C ring does not contribute to the secondary structure of bovicin HJ50, but does contribute to the hydrophobicity of the peptide (145). Substitution of the C ring of bovicin HJ50 with that of other bovicin HJ50-like lantibiotics does not influence activity, taking into account that residues not involved in ring formation are not very conserved (145). Therefore, it is proposed that similar to the retention of the conserved lipid II-binding motif, the disulphide bond of ring C evolved in this subgroup for the retention of biological activity (145).


```
BovicinHJ50    ADRGWIKTLTKDCPNVISSICAG-T-IITA--CKNCA
Suicin         AGSGFVKTLTKDCPGFLSNVCVN-IGFISG--CKNC-
Perecin        AGAGFIKTLTKDCPEVVSQVCGSFFGWVSA--CKNC-
Cericin        AGPGWVETLTKDCPWNMPVACVT---ILGQRICKKCY
Thuricin       AGPGWVETLTKDCPWNVPVACVT---IMGQRICKKCY
```

FIG 15 Bovicin HJ50-like lantibiotics. Cys residues are shown in red. Bold letters in purple designate Thr that are dehydrated. Bold black letters indicates Ser/Thr that escape dehydration. Lines above letters indicate the ring topology of bovicin-HJ50. Dashed line indicates a disulphide bridge (145).

Class III and IV

Class III and IV are not strictly lantibiotics as they generally do not have antimicrobial activity, but rather have other bioactivities. Therefore the modification enzymes and selected peptides will be briefly discussed.

Class III and IV peptides are modified by bi-functional enzymes LanKC and LanL, respectively, and are responsible for the formation of lanthionines and labionines (LanKC) (1, 148). These enzymes have lyase-, kinase- and cyclase-domains, which are responsible for the respective PTMs (1, 149, 150). The generation of dehydroamino acids occurs via independent phosphorylation and elimination steps catalysed by the central kinase- and N-terminal lyase-domains, respectively (7, 149, 151–153). In LanKC, the cyclase domain is responsible for the respective cyclase reactions (formation of lanthionine and labionine), and in LanL the C-terminal cyclase domain is responsible for cyclization resulting in Lan/MeLan rings (149, 153). Labionin results from a double Michael addition. Briefly, as with normal Lan-formation a Cys thiol is added to Dha and instead of protonation the subsequent enolate adds to a second Dha resulting in the labionin motif (7, 148). In contrast to class I and II modification enzymes, the direction of dehydration of LabKC (LanKC of labyrinthopeptins) proceeds in a C- to N-terminal direction (150). Cyclization by AciKC and CurKC (LanKC of catenulipeptin and curvopeptin, respectively) also occurs in this direction (153, 154). CurKC has been extensively studied with regard to the directionality of all reactions (i.e. phosphorylation, elimination and cyclization) (154). The proposed biosynthetic route for curvopeptin is very interesting and complex. Ser1 is phosphorylated first followed by consecutive phosphorylation and elimination of Ser15 and Ser14 (resulting in Dha). The pSer1 undergoes elimination first (resulting in Dha), followed by C-terminal Lan formation. This is followed by the phosphorylation and elimination of Ser4 and Ser2, with the formation of the N-terminal Lan (i.e. Dha1) occurring last (154). Although biosynthesis occurs preferably in a C- to N-terminal direction alternative routes are also observed for CurKC, suggesting some flexibility in the processing order.

Labyrinthopeptin-like lanthipeptides

Labyrinthopeptins A1/A3 and A2 were the first lanthipeptides discovered that are modified by LanKC to form labionin (8). The three peptides have high sequence homology, with the only difference between A1 and A3 being an additional N-terminal Asp (Fig. 16). Longer

fermentation times of the producing strain revealed that the Asp is lost and A3 is converted to A1 (8). Although labyrinthopeptins do not have significant antimicrobial activity, A2 and A1 have activity against neuropathic pain and antiviral activity, respectively (8, 155). Catenulipeptin has a similar ring topology to labyrinthopeptin, and also has no antimicrobial activity (153). The leader peptide is essential for proper modification, and in the case of labyrinthopeptin the conserved ILELQ is important for recognition and dehydration (cyclization was not evaluated) (156). The leader peptide in catenulipeptin is important for both dehydration and cyclization (153). The leader peptide has a helical structure, which represents an important secondary structure for lanthipeptide modification machinery (156).

Although labyrinthopeptins are unique in that they only have labionin motifs, several labyrinthopeptin-like lanthipeptides do not contain this motif exclusively and have both Lan and Lab rings (151, 157, 158). Erythreapeptin can contain an N-terminal Lan and a C-terminal Lab. A similar mixture of Lan/Lab is also found for other labyrinthopeptin-like lanthipeptides (151, 157). However, it is not known whether Lan/Lab is present in one peptide, or if there is a mixture of peptides containing either Lan or Lab being produced (157). The ability of some LanKC enzymes to produce peptides with Lan and only trace amounts of Lab-containing peptides is interesting, with all of them harbouring the $SerXXSerX_nCys$ motif (assumed to be a precursor for Lan/Lab ring formation) at similar positions. The mechanism by which LanKC enzymes are able to select between Lan or Lab formation is unknown. It would be interesting to investigate if LabKC (LanKC of labyrinthopeptin) would be able to exclusively form Lab rings, in other Lan ring dominant labyrinthopeptin-like lanthipeptides.

```
LabyrinthopeptinA1    --------SNASVWE-CC--STGSWVPFTCC-
LabyrinthopeptinA2    --------SDWSLWE-CC--STGSL--FACC-
Erythreapeptin        --------SNLSLLASCAN STVSLLT -CH-
Avermipeptin          --------STVSLLS-CV--SAASV--LLCL-
Griseopeptin          --------SQVSLLV-CEY-SSLS-VVL-CTP
Curvopeptin           --------SSASLLL-CDKFSAFSTLL--CL-
Catenulipeptin        --------SGLSVTG-CNGHSGISLL---CDL
SapB                  ------TGSRASLLL-CGD-SSLS-ITT--CN
SapT                  YTQGGCS-GLCTIVI-CAT-----VVI--CG-
```
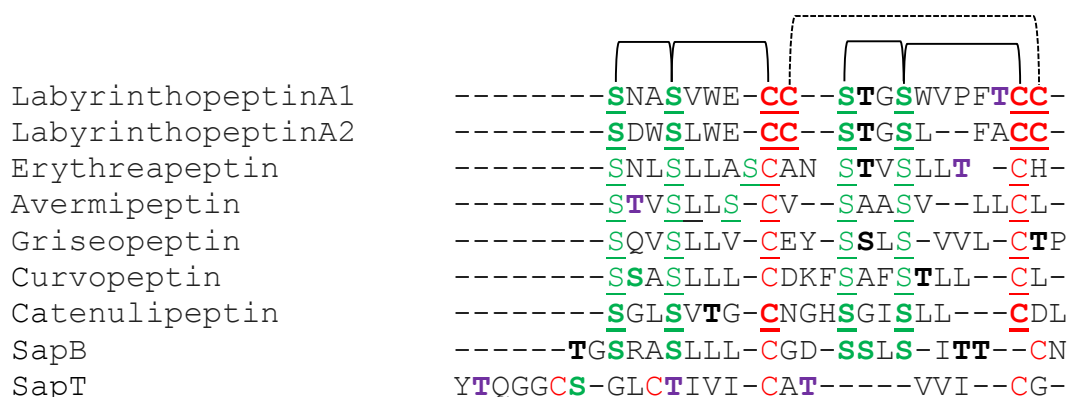
FIG 16 Labyrinthopeptin-like lantibiotics. Cys residues are indicated in red. Purple and green letters indicate dehydrated Thr and Ser, respectively. Bold and underlined letters indicate residues that take part in labionin formation (Ser/Thr must, therefore, be dehydrated). Letters underlined and not in bold indicate residues that can be involved in lanthionine or labionin formation. Bold black letters indicate Thr that escape dehydration. Lines above letters indicate the ring topology of labyrinthopeptinA1 (labionin rings) and dashed line indicates a disulphide bridge (151, 153, 157). Figure adapted from Dischinger et al. (50).

Venezuelin-like lanthipeptides

Venezuelin is the only characterized peptide in this group and the only characterized peptide modified by LanL, making it the only peptide in class IV (149). Similar to class I and II LanC-like domains, and unlike LanKC, LanL has a zinc binding site. The zinc ion is proposed to activate Cys in the precursor peptide for nucleophilic attack (149, 159). Each of the domains of VenL is responsible for their respective modifications, namely phosphorylation (central kinase domain), elimination (N-terminal lyase domain) and cyclization (C-terminal LanC-like cyclase domain) (149, 152). Furthermore, mutations in the lyase domain of VenL showed that some of the conserved domains of LanL and LanKC are functionally important, and that similar conserved regions found in LanM are not essential for dehydratase activity (152).

Venezuelin has a globular structure, with four overlapping rings, reminiscent of cinnamycin-like lantibiotics (Fig. 17) (149). Venezuelin was discovered using a bioinformatic approach (149). Production could not be detected by the host, *Streptomyces venezuelae,* or after cloning the gene cluster into a heterologous host. Therefore, it was reconstituted *in vitro* with *E. coli* as host, and using VenL to incorporate the various modifications (149). The gene cluster does not contain an obvious protease or protease cleavage site. An amino acid sequence (PFA) in the leader of venezuelin is similar to the AXA motif required for type I signal peptidases, and a similar motif is found in cinnamycin (AFA) (149, 160, 161). Several peptides were constructed with cleavage sites for commercial proteases in this position, and after modification and cleavage these constructs were tested for activity against several strains known to be sensitive to lantibiotics (149). As with class III lanthipeptides, no antimicrobial activity was observed for venezuelin (149). It should be noted that the cinnamycin-like lantibiotics are also not known for their antimicrobial activity. This suggests a possible structure-function relationship shared among venezuelin- and cinnamycin-like lanthipeptides (149).

FIG 17 Venezuelin-like lantibiotics. Cys residues are shown in red. Bold purple and green letters indicates dehydrated Thr and Ser, respectively. Lines above letters indicates ring topology (149).

36

Conclusion

Lantibiotics are a diverse group of post-translationally modified peptides, with differences in their biosynthesis, structure and biological activities. The current classification system attempts to encompass the diverse and complex nature of lantibiotics, and group them into designated classes and subclasses. Lantibiotics are first divided into two major groups based on the modification enzymes that install Lan/MeLan residues. However, classification into two groups based on modification machinery is not sufficient. By using the amino acid sequences of the unmodified core peptides, lantibiotics can be placed in respective subgroups. In most cases the lantibiotics in a particular subgroup also share similar structural properties (e.g. similar rig topology). This classification system can account for almost all characterized lantibiotics. Furthermore, it is also used to group and subgroup other lanthipeptides, namely those modified by bi-functional enzymes, LanL and LanKC. The main drawback of this classification system is that it does not account for biological activity. Although most lantibiotics are active against Gram-positive bacteria, some have alternative biological activities (e.g. labyrinthopeptin-like lantibiotics). Investigation into structure-function relationships may prove useful in further subdividing lantibiotics (and lanthipeptides in general) according to their biological activities.

The identification of novel lantibiotics, with new and complex structures is aided by advances in *in silico* analysis of genomic data. With this increase in characterization of novel lantibiotics, the classification system will inevitably have to evolve to accommodate the increase in complexity and diversity.

Clinical Applications of Lantibiotics

Nisin has been used as preservative in dairy products for over 40 years (162). However, due to the diversity and complex nature of lantibiotics (and lanthipeptides in general) they have also been explored for use in various medical applications. An increase in the number of sequenced genomes has also lead to an increase in *in silico* analyses of genomic data to find possible lantibiotic/lanthipeptide precursors (163). With the help of *in vitro/in vivo* engineering, these putative lantibiotics/lanthipeptides can be functionally expressed, further increasing the arsenal of candidates for medical application (148). Several medical applications have been proposed and some are in clinical trials (50, 109, 164). With the increase in discovery of novel lantibiotics this number will surely increase.

Treatment of Infections

Due to their antimicrobial properties, lantibiotics have found a place as potential alternatives or additions to antibiotics. However, there is already a substantial arsenal of antibiotics currently available for the treatment of a wide range of infections (165–170). Nonetheless, antibiotics are becoming ever more ineffective against once treatable infections (165–172). A pessimistic view may be that we are treating ourselves back into a "pre-antibiotic era". The most recent world health organization (WHO) report on antimicrobial resistance revealed that, in most cases, more than 50% of *E. coli, Klebsiella pneumonia* and *S. aureus* isolates are resistant to specified antimicrobial drugs (170). Another report by the Centers for Disease Control and Prevention (CDC) indicated that in the United States more than 2 million people contract infections that are caused by microorganisms that are resistant to one or more of the prescribed antibiotics (166). In addition to morbidity and mortality experienced by patients, antibiotic resistance carries a significant economic burden resulting from increased societal- and medical-costs (166, 170, 171, 173). The over prescription, or prescription of ineffective antibiotics, is wide spread and contributes to the prevalence of resistance (167). Resistance can be seen as endogenous, arising inside the organism by mutation and selection, or exogenous, arising by horizontal gene transfer (HGT) (168). In a recent example of endogenous resistance, sub lethal concentrations of antibiotics resulted in resistance to multiple drugs (174). Resistance correlates with bactericidal antibiotics that induce formation of reactive oxygen species by bacteria that in turn causes mutations, in some cases resulting in resistance (174). Mutations that confer resistance, and are maintained in the population, can possibly then result in exogenous resistance via transfer of mutated genes to non-resistance strains by HGT (174).

Carbapenemase-resistance plasmids are transferred via HGT, not just between strains or species but across genera (175). This is an example of how wide-spread HGT is, and how the overuse of antibiotics may aid in the transfer of various antibiotic resistance elements to otherwise low-virulence organisms (175). In addition to resistance caused by direct antimicrobial use, the natural "resistome" of the environment also plays a role in the transfer of resistance elements to potential pathogens (172, 176).

Although antibiotic resistance is on the increase, challenges faced by drug discovery programs (e.g. target selection and cost of research) have led to an antibiotic discovery void, with very few new antibiotic classes introduced over the past 25 years (168, 177). However, the search for new antibiotics has not come to a complete stand-still. With new technologies in high through-put screening techniques, bioinformatics, structural-, synthetic- and chemical biology, novel antibiotics are making it into the drug discovery pipeline (168, 177). The discovery of novel antibiotics is limited by several steps, including target selection, which is important if a novel antibiotic is to remain effective for a prolonged time period. Vancomycin is a good example of an antibiotic that has been used effectively for a prolonged time period (168). This is mainly due to its target, the lipid II MurNAc-pentapeptide end terminal motif D-Ala-D-Ala (178). Vancomycin resistance would, therefore, require the alteration of an end product, produced by multiple steps in the peptidoglycan pathway. In the case of VanA- and VanB-type glycopeptide resistance, operons containing several genes have to be imported and expressed, resulting in the end terminal D-Ala being replaced with D-lactate, conferring resistance to vancomycin (179–181). Even if a potential high-value target is found, such as lipid II, it should lack structural/functional homology to human targets so as to reduce toxicity. The antibiotic should also have desirable pharmacokinetic/pharmacodynamic properties. These are only some problems encountered in the drug discovery pipeline, which is also hampered by the high cost of research and development, compared to possible financial gains.

Lantibiotics are attractive antimicrobials as they are active at low concentrations and mostly target lipid II, which is a high-value target. Additionally pore forming lantibiotics have a dual mode of action which can possibly reduce development of resistance. Furthermore, the availability of sequenced genomes, and use of bioinformatics make it possible to identify not only new lantibiotics, but also new classes of lanthipeptides, which may have novel and more effective modes of action (163). Development of more efficient *in vitro/in vivo* strategies for expressing these peptides also make large scale production a realistic possibility (148).

Lantibiotics are, however, limited by their narrow spectrum of activity and are limited to Gram-positive bacteria, and under some circumstances activity against Gram-negatives is reported.

Lantibiotic activity against **Staphylococcus aureus** *and other staphylococci*

Skin and soft tissue infections (SSTIs) are wide spread and are some of the most common infections. This is not surprising, as the skin is the largest organ in the body and comes in constant contact with the outside environment (182, 183). Skin and soft tissue infections caused by bacteria, can be defined as invasion of the dermis, epidermis and subcutaneous tissue by bacteria, which subsequently results in inflammation (183). The skin has its own unique microbiota, which maintains a symbiosis with the body, and usually does not cause infections or adverse inflammatory responses (184). However, host-microbe dysbiosis can occur and result in a disease state. Dysbiosis is mainly as a result of exogenous factors, such as infection and injury, but can also be caused by polymorphisms in host innate/adaptive immune system (184).

Staphylococci are one of the main genera represented on the skin, either as commensals or pathogens, with *S. aureus* most commonly presenting as a pathogen (182, 184–186). The vast majority of SSTIs are caused by *S. aureus* and it is usually associated with boils, abscesses, carbuncles and localised wound sepsis (182, 183, 185). Pathogenic staphylococci have various virulence factors that make them very dangerous, including phenol soluble modulins (PSMs), produced by several pathogenic and commensal staphylococci. Phenol soluble modulins are small cationic antimicrobial peptides (cAMPs) that have direct antimicrobial- and proinflammatory-activities (187, 188). They can either play a role in immune defence or pathogenesis (22, 189, 190). Phenol soluble modulins produced by pathogenic *S. aureus* (PSMα peptides) lure neutrophils via chemotaxis and then induce lysis of the infiltrate neutrophils by a high dose of PSMs (22, 189, 190). Phenol soluble modulins and several other virulence factors, and the prevalence of *S. aureus,* make it a pathogen that can escalate from a minor to a major infection in a short time period (191, 192).

It's not surprising that *S. aureus* has developed resistance to antibiotics, with the most common being MRSA (170, 185). Methicillin resistance is due to a penicillin binding protein, known as PBP2a, which has low-affinity to β-lactams and is produced by the *mecA* gene (193). The *mecA* gene is found on a mobile genetic element known as the staphylococcal chromosomal cassette *mec* (SCC*mec;* i.e. resistance island), and encodes resistance to several other antibiotics in

addition to methicillin (193, 194). Cassette chromosome recombinases (ccr) are unique site specific recombinases that are found in SCC*mec* elements, and are used as one of the criteria to group SCC*mec* elements into types, along with the class of the *mec* gene complex (193). Currently there are eleven SCC*mec* types that are a combination of eight *ccr* gene- and six *mec* gene-complexes (194). J-regions are gene complexes which contain non-essential components, such as additional antibiotic resistant determinants, and are used to further group SCC*mec* into subtypes based on polymorphisms/variations in this region (193). There are SCC*mec* elements that do not contain the *mecA* gene but do contain other genes associated with SCC*mec* (193). Additionally, pseudo-SCC elements that do not have *ccr* genes have also been identified (193). A cytolytic PSM peptide, PSM-mec, has been linked to SCC*mec* elements. Transcription and translation of *psm-mec* may regulate the virulence properties of MRSA (195).

According to the WHO report, MRSA proportions exceed 12% of *S. aureus* infections, and some regions report that between 60-70% of *S. aureus* strains are methicillin-resistant and that more than 90% are resistant to penicillin (170). The CDC reports on more than 80,000 severe MRSA infections per year in the United States, with more than 11,000 deaths, and is characterized as a serious threat (166). Infections caused by MRSA can be characterized as either being hospital acquired MRSA infections (nosocomial infection; HA-MRSA) or community acquired MRSA infections (CA-MRSA) (166, 196). There are genetic differences between HA- and CA-MRSA, in that they contain different SCC*mec* types (usually I-III and IV-VII, respectively). Infections caused by CA-MRSA are usually easier to treat due to less antibiotic resistance, however, they are more virulent due to increased toxin production (189, 195–197). The more virulent CA-MRSA are more frequently being isolated from hospitals (197).

A region in the SCC*mec* of HA-MRSA (SCC*mec* type II-III) strains called the F-region is linked to decreased virulence and colony spreading, and is not found in CA-MRSA (SCC*mec* type IV) (198, 199). This decrease in virulence is as a result of the transcription and translation products of *psm-mec,* which have a negative influence on the production of PSMα (causes neutrophil lysis) (198, 200). Contradictory results were obtained in another study that showed that PSM-mec has a positive effect on virulence (195). However, this discrepancy may be due to concentration differences of PSM-mec and PSMα produced by the different strains used, as well as differences in their genetic background (195, 200).

In addition to MRSA strains, strains with resistance to other antibiotics not associated with SCC*mec* have also been identified. This includes vancomycin-resistant *S. aureus* (VRSA), which can either have intermediate resistance (hVRSA) or high level resistance (VRSA). Resistance in hVRSA strains is due to adaptation due to prolonged exposure, whereas high level VRSA resistance is due to the acquisition of vancomycin resistance genetic elements, such as *vanA* (201, 202). Vancomycin-resistant enterococci and VRSA are usually co-isolated, and *in vitro* and *in vivo* HGT of *vanA* occurs between VRE and *S. aureus,* which is most likely the mechanism by which MRSA acquires vancomycin-resistance (202–206). Resistance to vancomycin is a serious threat, as vancomycin is usually the drug of choice for treatment of MRSA infections.

Several lantibiotics are effective against antibiotic-resistant *S. aureus in vitro* and *in vivo* (13, 104, 207, 208). Although lantibiotics have potential as treatment for SSTIs, there are limited published studies evaluating lantibiotics as topical treatments. Nisin incorporated into nanofibers is effective in the treatment and prevention of *S. aureus* in mice, using a topical infection model (209). The nisin nanofibers not only treat infection, but also significantly improves wound healing and closure. Nisin is also currently being used for the prevention of mastitis in cows and a treatment option is under development (162). Other lantibiotics, such as mutacin 1140 (Oragenics) and NVB333 (Novacta Biosystems), are currently either in development or preclinical trials for the treatment and prevention of Gram-positive infections.

In addition to their application in the treatment of topical *S. aureus* infections, several lantibiotics have potential for use in other areas related to staphylococcal infections. *Staphylococcus* spp. can colonize medical devices such as catheters, cardiac devices and prosthetic implants, with formation of biofilms that further complicate treatment. Infections of these implants leads to increased morbidity and cost for patients (210, 211). Nisin and gallidermin incorporated into bone cement remains active against *S. aureus* and *S. epidermidis in vitro* (212, 213). Nisin-eluting cement also prevents infection by *S. aureus* when implanted subcutaneously in mice, as determined by bioluminescence (212). Additionally, no viable cells are isolated from implants after removal (212). Nisin in combination with RNAIII-inhibiting peptide (RIP) also prevents methicillin-resistant *S. epidermidis* colonization on grafts, when implanted into subcutaneous pockets on the back of rats (214). A nisin-RIP combination proved to be very effective in preventing colonization, with less than 10 CFU/cm^2 remaining after seven days (214). Nisin, RIP and rifampin, individually, resulted in 7.8×10^3, 6.1×10^2, and

5.5×10^4 CFU/cm^2, respectively (214). Teflon catheters coated with nisin and implanted intravenously in sheep maintains bactericidal properties for more than five hours after implantation, and polyvinyl chloride tracheotomy tubes coated with nisin has activity for 1-2 hours after implantation in ponies (215). Gallidermin is effective in preventing biofilm formation by staphylococci *in vitro* and nisin incorporated into polymer films is effective in preventing *S. epidermidis* biofilm formation (216, 217).

In murine infection models, microbisporicin has equivalent or superior activity when compared to reference treatments (e.g. penicillin, vancomycin and linezolid) in the treatment of MRSA and glycopeptide-intermediate *S. aureus* (67, 208). Interestingly, microbisporicin is more effective when administered intravenously compared to subcutaneous administration, suggesting better bioavailability when injected intravenously (67, 208). Lacticin 3147 is also able to control the systemic spread of *S. aureus* in mice, vancomycin still, however, more effective in reducing *S. aureus* spread (218). Mersacidin completely eradicates *S. aureus* in a murine rhinitis model, with no reports of mucosal lesions or morphological changes in the liver (219). Nisin is able to inhibit the growth of *S. aureus* in the respiratory tract of rats and does not have negative effects on the trachea, lungs and bronchi. (220). Mersacidin has superior activity against MRSA when compared to vancomycin in an *in vivo* infection model, where mice were injected intraperitoneally with lethal amounts of *S. aureus* (MRSA and methicillin sensitive *S. aureus*) and treated subcutaneously (221). In contrast to these results, nisin is ineffective in the treatment of *S. aureus* when mice are infected subcutaneously and in the peritoneal cavity (222, 223). However, in these studies infections were left longer before treatment, and a crude extract of nisin was used which may have resulted in insufficient amount of active peptide being available to prevent or treat infection (222, 223). It is also likely that there is an interaction between the peptide and host components when administered subcutaneously or directly into the peritoneal cavity. This is similar to microbisporicin, which showed better activity when injected intravenously (208). However, in the case of microbisporicin, some activity was still present when administered subcutaneously (208). Mersacidin also has reduced bioavailability when injected subcutaneously, which only improves after using the more water soluble potassium mersacidin (221).

Problems such as production, solubility and enzyme degradation have held back lantibiotic use. However, nisin is produced commercially as a food additive and is effective *in vitro* and *in vivo*, illustrating that these hurdles can be overcome. It is highly likely that many of the lipid

II-binding lantibiotics would make good candidates for the treatment of multiple drug-resistant *S. aureus.* Based on their diversity, lantibiotics with desirable pharmacokinetic/dynamic properties are bound to be found. The continued advancement in synthetic biology and heterologous expression systems should also make large scale production more feasible.

Lantibiotic activity against other Gram-positive bacteria

Lantibiotics have activity against a wide range of Gram-positive bacteria, including several pathogens associated with human infections.

Streptococci include several pathogenic strains and are divided into alpha- and beta-haemolytic streptococci. Alpha-haemolytic streptococci contain *S. pneumonia,* which is the cause of pneumococcal infections, including otitis media, sinusitis, pneumonia and meningitis. Several lantibiotics are active against *S. pneumonia* including actagardine, nisin and microbisporicin (208, 224, 225). Mice infected intraperitoneally with *S. pneumonia,* at concentrations sufficient to result in death, were treated with either nisin or vancomycin. Two intravenous treatments with nisin of 0.16 mg/kg each resulted in 100% survival, whereas the survival of mice treated with 1.25 mg/kg vancomycin was only 83% (224). After five minutes the nisin concentration in serum was 50 mg/l with a serum half-life of 0.9 hours and was not detectable after three hours. The low blood and tissue levels of nisin thus seem to be sufficient to prevent death of the mice (224). In another study carboxamides of actagardine were generated (225). The monocarboxamides were more active compared to the other variants *in vitro* and a more water soluble derivative was effective in a murine septicaemia model, with effective dose (ED_{50}) values comparable to the reference antibiotics used (225). Compared to nisin, the actagardine derivative is eliminated faster with a serum half-life of 0.3 hours (224). The ED_{50} of microbisporicin is at least half that required for linezolid and penicillin G treatment of *S. pneumonia*, when compared in a murine lethal infection model (208).

Beta-haemolytic streptococci are divided into groups A and B. Group A is found on the skin and inside the throat, and is responsible for most beta-haemolytic streptococcal infections. Common infections caused by group A beta-haemolytic streptococci (GAS; *S. pyogenes*) include impetigo, cellulitis, pharyngitis and scarlet fever. Several lantibiotics including microbisporicin, planosporicin, mersacidin, actagardine and lantibiotics produced by streptococci (e.g. salivaricin9 and streptin) are active against GAS (59, 65, 67, 221, 225, 226). A water soluble derivative of mersacidin has comparable activity to that of vancomycin, when

it is used for treatment of *S. pyogenes* in mice (221). The same water soluble actagardine derivative used against *S. pneumonia* is also effective *in vivo* against *S. pyogenes* (225). Planosporicin protects mice in a septicaemia model from *S. pyogenes* and has no toxicity up to 100 mg/kg. However, compared to teicoplanin the ED_{50} of planosporicin is much higher at 3.75 mg/kg compared to 0.18 mg/kg for teicoplanin (65).

Enterococcal infections, especially those caused by VRE, are becoming an increasingly important problem (166). Vancomycin-resistant enterococci are characterized as a serious threat by the CDC and results in over 20,000 cases in the United States each year (166). Several lantibiotics, including lacticin 3147, nisin, mersacidin, epidermin, haloduracin and microbisporicin have promising *in vitro* activity against enterococci, with some active against VRE (13, 67, 104, 207, 227). Microbisporicin administered intravenously and subcutaneously, in mice, has the lowest ED_{50} against two VRE strains compared to linezolid (208). Although VRE is a threat the emergence of carbapenem-resistant enterococci (CRE) which are resistant to all treatments is even more serious (166). To date lantibiotics have not been specifically tested against CREs, which would make investigating lantibiotic activity against these strains imperative.

Probionibacterium acnes is a member of the normal skin flora, mainly found in the sebaceous follicles, and under certain conditions can become an opportunistic pathogen. *P. acnes* is implicated in several disease states such as acne vulgaris, folliculitis, and sarcoidosis and can also cause postoperative infections (228). Most lantibiotics are active against *P. acnes* including mutacin B-Ny266, gallidermin, epidermin, microbisporicin, nisin and lacticin 3147 (67, 227, 229, 230).

Listeria monocytogenes is a foodborne pathogen and can cause sepsis, fetal loss and gastroenteritis (231, 232). Several lantibiotics are active against *L. monocytogenes,* and the extensive use of nisin in the food industry assists in the prevention of food spoilage caused by listeria. Nisin controls the spread of listeria in mice for three days after only one treatment (infected and treated intraperitoneally) (233). In addition, a nisin hinge variant, nisin V (M21V), is more effective than nisin A (233).

Another important foodborne and nosocomial pathogen, involved in severe gastrointestinal tract (GIT) infections, is *Clostridium difficile. Clostridium difficile* associated diarrhoea (CDAD) is one of the major causes of hospital associated diarrhoea, with 250,000 infections per year in the United States alone, with at least $ 1 billion in excess medical costs (166).

45

Current treatment of CDAD includes oral administration of vancomycin and metronidazole, however, vancomycin treatment can lead to colonization of VRE in the GIT or even the spread of vancomycin resistance within a hospital environment. Nisin and lacticin 3147 are effective against *C. difficile.* Prevention of spore outgrowth by these and other lantibiotics, in *Clostridium* spp. and *Bacillus* spp. have also been demonstrated (14, 40, 47, 104, 234). Prevention of spore outgrowth can help in curbing the growth and spread of *C. difficile* which may contribute to the successful treatment of CDAD. Actagardine (NVB-302) is currently being developed for treatment of *C. difficile* and is undergoing phase I clinical trials. In an *in vitro* GIT model actagardine compares well with vancomycin in the treatment of *C. difficile,* with less deleterious effects on *Bacteriodes fragilis* (a GIT commensal) (235). Combination of actagardine and ramoplanin is especially effective against multiple *C. difficile* strains (236). Lacticin 3147 in a faecal fermentation model completely eliminates *C. difficile* within 30 min, with minimal effects on anaerobes, bacteroides and other Gram-negative bacteria. The enterococci, lactobacilli and bifidobacteria populations were, however, negatively affected (14). When lacticin 3147 is fed to pigs at similar concentrations it has no effect on the microbiota including lactobacilli and enterococci, with neither of the peptides detected in diagesta of pigs after 2 hours (237). This suggests that one or both of the peptides are either degraded during intestinal transit or already passed through to the stomach (237).

Mycobacterium tuberculosis is the causative agent of the respiratory tract infection known as tuberculosis (TB). Worldwide 8.7 million people contracted and 1.3 million died of the disease in 2012 (170). Cases of multiple- and extensively-drug resistant *M. tuberculosis* place an immense burden on the efforts to control the spread of TB. The unique cell wall and slow growing nature of *M. tuberculosis* may make it difficult for lantibiotics (and other treatments) to exert antimicrobial activity. However, the ability of lantibiotics to bind to lipid II gives it an advantage over treatments such as rifampicin, which need to be transported across the plasma membrane. The lipid II structure of mycobacteria does differ from other bacteria, due to modifications on both MurNac and the peptide side chain (238). Despite these differences, nisin has activity against the non-pathogenic *M. smegmatis* and *M. bovis*, with intracellular ATP leakage and dissipation of the PMF (43, 239, 240). Nisin hinge mutants have improved activity against *M. smegmatis* (43). Nisin and lacticin 3147 are active against clinical mycobacteria isolates, with lacticin 3147 showing the best activity against *M. tuberculosis* (241). These lantibiotics have potential, however, an appropriate delivery system should be developed in order to reach *M. tuberculosis,* which reside in macrophages found in the distal

lung. Further research is required to establish the feasibility and use of lantibiotics as an anti-mycobacteria treatment.

Lantibiotic activity against Gram-negative bacteria

Although lantibiotics are not known for their activity against Gram-negative bacteria, there are some exceptions.

Helicobacter pylori causes a very difficult to treat GIT infection and is associated with several GIT disorders, including, peptic ulcer disease, chronic gastritis and gastric cancer (242). Treatment of *H. pylori* is difficult due to its location in the GIT and requires treatment with multiple drugs to be effective (242). Nisin has completed phase I clinical trials for the treatment of *H. pylori*, however, initiation of phase II trials have not been reported (243).

Neisseria meningitides and *Neisseria gonorrhoeae* are the causative agents of meningococcal meningitis and gonorrhoea, respectively. *Neisseria* spp. are susceptible to lantibiotics such as mutacin B-Ny266, microbisporicin and nisin (67, 230). *Neisseria meningitides* and *N. gonorrhoeae* are especially sensitive to microbisporicin (0.5 and 0.25 μg/ml, respectively), compared to nisin (8 and 4 μg/ml, respectively) (67).

Haemophilus influenza is an opportunistic pathogen and can cause bacteraemia, pneumonia- and bacterial-meningitis. Nisin, mutacin B-Ny266 and microbisporicin have *in vitro* activity against *H. influenza*. However, only microbisporicin (32 μg/ml) and mutacin B-Ny266 (13 μg/ml) demonstrate moderate activity, while nisin is required at high concentrations (>66 μg/ml) to be effective (67, 230).

Campylobacter jejuni is a common cause of gastroenteritis and is identified as an important enteric pathogen, and is the leading cause of food borne-illness (244). Nisin and mutacin B-Ny266 have *in vitro* activity against *C. jejuni* at relatively low concentrations (230).

A lantibiotic from *B. longum* DJO10A has Gram-negative activity, including activity against *Serratia marcescens, Proteus vulgaris* and *E. coli*. However, this is observed using a crude lantibiotic preparation, and activity needs to be studied with purified peptide (120, 121).

Other therapeutic applications of lantibiotics

Lantibiotics as immune modulators

Several cAMPs play a crucial role in modulating the immune system during infection and injury, including, LL-37 and α- and β-defensins (245, 246). Most complex species have cAMPs, which interact with the innate immune system using several mechanisms. These peptides are generally short, gene encoded, overall positively charged, with a large proportion of hydrophobic residues, making them very similar to lantibiotics (245-247).

Gram-positive bacteria, including staphylococci, are able to, or at least have the genetic potential to, produce lantibiotics. Several lantibiotic gene clusters can be identified in many pathogenic and commensal staphylococci (50, 248). It is hypothesized that lantibiotics produced by commensal bacteria may play a role in maintaining microbial balance, through direct antimicrobial activity (23, 24, 184). This has not yet been shown *in vivo*, however, a *S. aureus* strain with its lantibiotic gene cluster knocked-out does result in a growth-attenuated strain in a mouse abscess model (249).

Nisin, gallidermin and Pep5 are able to induce the release of multiple chemokines at levels similar to that of LL-37 (19). Furthermore, nisin uses multiple signalling pathways including the ERK/MAPK, PKC and PLA pathways (19). Mice pre-treated with nisin followed by infection with *Salmonella typhimurium* and *E. coli* showed a significant reduction in bacterial counts compared to the control (19). Nisin does not have activity against these Gram-negative bacteria, suggesting that nisin is able to provide protection via an immunomodulatory mechanism.

Phospholipase A2 plays an important role in inflammatory responses resulting from its role in the release of arachidonic acid. The oxidative metabolism of arachidonic acid results in eicosanoids, such as prostaglandins and leukotrienes, which are strong mediators of the immune system. Cinnamycin-like lantibiotics are able to indirectly inactivate phospholipase A2 by sequestering PE (substrate for phospholipase A2), thereby having potential to indirectly mediate inflammatory responses (139, 140-142). Another cinnamycin-like lantibiotic, ancovenin, is an inhibitor of ACE, which is important in regulating blood pressure by catalysing the conversion of angiotensin I to angiotensin II (25, 130).

These results suggest that lantibiotics are capable of interacting and modulating the immune systems, potentially using similar mechanisms employed by human and other cAMPs.

Lantibiotics as ion channel regulators

Duramycin has potential in the treatment of cystic fibrosis which is caused by abnormal chloride ion transport. Duramycin elevates intracellular calcium thereby activating an alternative chloride channel and increases chloride permeability in nasal epithelium of cystic fibrosis patients (250, 251). In a phase II clinical trial duramycin was shown to be safe and resulted in overall positive results on the pulmonary function of cystic fibrosis patients (252). Duramycin is also under development for the treatment of dry eye syndrome.

Lantibiotics as treatment for neuropathic pain

Labyrinthopeptin-like lantibiotics do not have antimicrobial activity, however, labyrinthopeptin A2 has been shown to be effective against neuropathic pain in mice (8). Tactile allodynia is measured in mice using a spared nerve injury model (8). Labyrinthopeptin A2 administered intravenously at concentrations ranging from 0.01 - 3.0 mg/kg results in significant attenuation of tactile allodynia (ED_{50} 50ug/kg). Efficacy reaches 100% and remains stable over the six hours with loss of efficacy after 24 hours (8).

Lantibiotics as contraceptives

Nisin can completely immobilize sperm. The immobilization effect is time- and dose-dependent with 50 μg/ml, 200 μg/ml and 300-400 μg/ml nisin required to immobilize rat, rabbit, human or monkey spermatozoa, respectively, in 20 seconds (253). Seminal fluid significantly affects nisin potency with up to 4x reduction in potency. Nisin (200 μg via intravaginal administration) was tested *in vivo* in rats for its contraceptive potential (253). None of the animals treated with nisin intravaginally before mating became pregnant, whereas all rats in the control group become pregnant. Nisin does not have any cytotoxic effects nor any effect on the oestrus cycle or fertility of rats (253). Nisin is also effective when used in rabbits as a contraceptive, with 1 mg nisin being sufficient and safe (254).

Conclusion

The diversity of lantibiotics, in terms of structure and bioactivity, make them ideal candidates to be investigated for clinical use. The obvious use of lantibiotics as antimicrobials has been extensively studied, with research focusing on clinical and food preservation applications. Several problems plagued the large scale roll out of lantibiotics in the medical industry, which include production of insufficient quantities of lantibiotics, reduced solubility at higher concentrations/pH and protease degradation. However, several lantibiotics can be produced on a large scale, including nisin and lacticin 3147. Companies such as Novacta Biosysoltems and Oragenics are developing large scale fermentation and recovery processes for the lantibiotics they are pushing towards clinical use. Another company spearheading the development of lanthipeptides is LanthioPharma, that focus on the discovery and development of lanthipeptide based drugs for various clinical (other than antimicrobial) applications. By using lanthipeptides, LanthioPharma are developing novel peptides, and incorporating lanthionines into existing peptides (e.g. apelin), that are more stable and resistant to protease degradation. Lanthionine bridges in lantibiotics make them more stable than other linear peptides, and can provide protection against protease degradation. With such a diverse group of lantibiotics it is possible to find alternative naturally occurring lantibiotics that are more stable under required conditions. In addition to high natural diversity, the bio-engineering of lantibiotics and improvements in production processes, make it possible to tailor lantibiotics for specific needs. The solubility concerns that are raised for lantibiotics can be overcome by making derivatives that are more soluble, such as potassium mersacidin that is more water soluble but retains its antimicrobial activity. Incorporating lantibiotics, such as nisin, into nanofibers at high concentrations is also effective in delivery of nisin to sites of infection. By constraining lantibiotic release, using a delivery system, issues of low solubility can possibly be circumvented.

Therefore, due to the diverse application potential of lantibiotics, and other lanthipeptides, it is inevitable that their clinical use will increase.

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Chapter 3
In vitro Characterization of a Novel Two-Component Lantibiotic from *Bacillus amyloliquefaciens*

Chapter Summary

Lantibiotics are ribosomally synthesized and post-translationally modified antimicrobial peptides, with potential applications in the medical industry. They have been isolated from a variety of bacteria from diverse environments. *Bacillus clausii* AD1 and *Bacillus amyloliquefaciens* AD2 were isolated from the bacterially diverse soils of the Fynbos. Both bacteria produced potent antimicrobials active against Gram-positive bacteria. Purification of the antimicrobials by HPLC revealed a single active peak for the antimicrobial produced by *B. clausii* AD1. The antimicrobial produced by *B. clausii* AD1 was identified as clausin according to its mass and the presence of the flavoprotein ClausD. Only after combination of two HPLC purified peaks could activity be observed for the antimicrobial produced by *B. amyloliquefaciens* AD2. A complete lantibiotic operon could be identified in the genome of *B. amyloliquefaciens* AD2. Sequence comparison in the NCBI database revealed that the operon contains two putative genes encoding the individual precursor peptides of a two-component lantibiotic. The individual peptides have similarity to other two-component lantibiotics and was designated amyloliquecidin. In addition to the two-component lantibiotic genes, the operon also harboured all the required genes for biosynthesis, transport and amyloliquecidin immunity. Clausin and amyloliquecidin has activity against clinical Gram-positive isolates including methicillin-resistant *Staphylococcus aureus*. Amyloliquecidin has superior stability when stored at physiological pH compared to nisin and clausin. Amyloliquecidin is also heat stable at temperatures up to 100˚C. This is the first report of the two-component lantibiotic amyloliquecidin. The antimicrobial spectrum and stability suggests amyloliquecidins' possible use in the treatment of Gram-positive infections.

Introduction

Lantibiotics are post-translationally modified (PTM) peptides and require several enzymes to produce a mature active peptide. Their characteristic modification, the lanthionine bridges, are formed between dehydroalanine/dehydrobutyrine (Dha and Dhb, respectively) and cysteines (1, 2). Dehydratase enzymes are responsible for the dehydration of Ser to Dha and Thr to Dhb (1-3). Subsequent cyclization reactions between Dha/Dhb and cysteines, results in the formation of the lanthionine (Lan) and methyllanthionine (MeLan) rings, respectively (3). Class I lantibiotics utilize two separate enzymes for the dehydration and cyclization reactions, namely LanB (dehydratase) and LanC (cyclase). Class II on the other hand, have one bi-functional enzyme, LanM, which has dehydratase and cyclase domains (1). In addition to these modifications, lantibiotics can have several other PTMs, including labionin residues formed by a second Michael addition with a second Dha, and oxidative decarboxylation of the lantibiotic C-terminus during aminovinylcysteine formation (4–6). The genes required for lantibiotic production are found in gene clusters. Genes required for introduction of modifications, transport, regulation and lantibiotic immunity are usually found in the same cluster (1, 7). Lantibiotics can be used as antimicrobials and nisin, the prototypical lantibiotic, is widely used as a preservative in dairy products (8). Lantibiotics also have potential applications in the medical industry due to their activity against clinical pathogens, including antibiotic-resistant strains (9–14).

Lantibiotics are a diverse group of peptides that are found in several genera, including *Staphylococcus* spp., *Streptococcus* spp., *Lactobacillus* spp., *Lactococcus* spp. and *Bacillus* spp. Numerous lantibiotics have been isolated from *Bacillus* spp. representing both class I and II lantibiotics, including the well-studied mersacidin (Table 1). *Bacillus* spp. are well-known producers of not only lantibiotics, but also hydrolytic enzymes, surfactants and a variety of non-ribosomally synthesised peptides (15–17). Phenotypic properties, such as the ability to survive on a variety of substrates, withstand harsh conditions (spore formation), and antimicrobial production, provide *Bacillus* spp. with unique advantages. It is therefore not surprising that *Bacillus* species are so ubiquitous in nature.

Fynbos forms part of the South African Cape floral kingdom, which is considered one of the worlds' diversity hotspots (18). Fynbos is characterized by a richness of plant species with a high proportion of the plants being endemic (18). Recently, a study examined the bacterial diversity in four soils across the western hemisphere and reported on an estimated 10 000-50

74

000 bacterial species per gram of soil (19). The bacterial community of the Fynbos soil is highly diverse, with the high plant diversity contributing to the diversity of the microbial communities (20, 21). Molecular techniques indicated that more than 80% of bacteria associated with the rhizosphere of plants (Proteaceae family) are novel genospecies (21). This diverse and complex relationship with the bacterial communities of soil and plants in the Fynbos biome provides an ideal setting to isolate bacteria that may produce novel antimicrobial compounds (20). Furthermore, Fynbos is a largely prestine unexploited niche that increases the probability of isolating novel antimicrobial-producing bacteria.

The current study aimed to characterize novel antimicrobials produced by bacteria isolated from the highly diverse Fynbos soils of the Western Cape, South Africa.

TABLE 1. Lantibiotics Produced by *Bacillus* spp.

Lantibiotic	Producer Strain
Mersacidin	[1]*Bacillus amyloliquefaciens* HIL-Y85
Subtilin	[2]*Bacillus subtilus* ATCC6633
Entainin	[3]*Bacillus subtilus* DSM15029T
EricinS and A	[4]*Bacillus subtilus* A1/3
Clausin	[5]*Bacillus clausii* OC
Haloduracin	[6]*Bacillus halodurans* C-125
Lichenicidin	[7]*Bacillus licheniformis* DSM13/VK21
Amylolysin	[8]*Bacillus amyloliquefaciens* GA1

[1](22); [2](23); [3](24); [4](25); [5](26) [6](27); [7](28, 29); (30)

Materials and Methods

Materials

All bacterial growth media were from Biolab Diagnostics (South Africa), unless otherwise stated. Trifluoroacetic acid (TFA) and XAD-16 beads were from Sigma-Aldrich (Germany). Acetonitrile and isopropanol were from Merck-Millipore (USA). All reagents used in Southern hybridization, including restriction enzymes, were from Roche Life Sciences (Germany). PureYield™ Miniprep kits used for plasmid DNA isolation were from Promega (USA) Nisaplin was from Danisco (Denmark). SepPak C18 columns were from Waters (USA). The bicinchoninic acid (BCA) protein assay was from Pierce Biotechnology (USA).

Isolation and Identification of Antimicrobial Producing Bacteria from Fynbos Soil

Soil and plant samples collected from the Jonkershoek nature reserve (Stellenbosch, South Africa) were suspended in sterile distilled water. Flower samples were homogenized using a blender, and incubated at 30°C for 24 h. After incubation, samples were plated onto selected media such as brain heart infusion (BHI) agar, Luria (LB) agar, nutrient (NB) agar and de Man Rosa Sharp (MRS) agar. Bromocresol purple (0.025 g/l) was added to all growth media to observe changes in pH. Fungal growth was inhibited by adding cycloheximide (0.001%, wt/vol) to all media. Plates were incubated aerobically and anaerobically at 37°C until visible colonies appeared (24-48 h). Colonies were overlaid with BHI soft agar (1% wt/vol agar) seeded with *Staphylococcus aureus* Xen 36 and incubated for a further 24 h at 37°C. Colonies surrounded by a clear zone of inhibition were selected, inoculated into respective media and incubated at 37°C. Pure cultures were obtained after streaking onto agar plates.

DNA was isolated from pure cultures according to standard methods (31). Genes encoding *16s-rDNA* and *gyraseA* were amplified using primer sets listed in Table 2. Amplified partial-*16s rDNA* and -*gyraseA* fragments were cloned into pGEM-T (Promega) and transformed into *Escherichia coli* BL21. Transformants were selected on LB agar supplemented with ampicillin (100 µg/ml). Plasmid DNA was isolated from positive clones and the DNA was sequenced (central analytical facility, Stellenbosch University, South Africa). Sequences were used in BLASTn searches of NCBI databases (http://www.ncbi.nlm.nih.gov/blast.cgi) to identify strains. ClausD primers were designed to amplify *lanD* present in putative lantibiotic gene

clusters (Table 2). Amplified *lanD* was cloned into pJET (Thermo Scientific) and *E. coli* BL21, and transformants were selected on LB agar supplemented with ampicillin (100 μg/ml). Degenerative *lanM* primers were used to amplify a region of the *lanM* gene from the putative lantibiotic gene clusters (Table 2). Positive amplicons were cloned into pGEM-T, transformed into *E. coli* BL21 and transformants were selected on LB agar supplemented with ampicillin (100 μg/ml). Plasmid DNA was isolated from positive clones, sequenced and used in BLASTn searches of the NCBI databases to identify *clausD* and partial *lanM* sequences.

TABLE 2. Primers used in this study

Primer Name	Primer Target	Primer Sequence (5'-3')
F8	*16s rDNA*	CAGGCATCCAGACTTTGATYMTGGCTCAG
R1512	*16s rDNA*	GTGAAGCTTACGGYTAGCTTGTTACGACTT
[1]pGyrAF	*gyraseA*	CAGTCAGGAAATGCGTACGTCCTT
[1]pGyrAR	*gyraseA*	CAAGGTAATGCTCCAGGCATTGCT
[2]LanMDegF	*lanM*	ATGCWAGWYWTGCWCATGG
[2]LanMDegR	*lanM*	CCTAATGAACCRTRRYAYCA
AmyA2F	*amyAβ*	ATAATTTAGCGGCCGCTTAGCAGCTTGATGTACATTTGG
LicR1R	*licR1*	CAATTAATTGGGTTGGCAGATCCAT
ClausDF	*lanD*	GATCAAGCTTTTAGCTTTTTTCTTCTACAGCC
ClausDR	*lanD*	CTAGATGGATCCATGCCGGAAAAGGAAGAA

[1](31); [2](32)

Production and Purification of Lantibiotics

Mueller Hinton broth (MH; Sigma-Aldrich) was clarified with activated-XAD-16 beads before autoclaving. XAD-16 beads were activated using 80% isopropanol containing 0.1% trifluoroacetic acid (Iso-TFA) (vol/vol/vol). Activated beads were collected and rinsed with distilled water. Mueller Hinton broth (10 g/l) was added to activated-beads and incubated at 26˚C for 30 min. Beads were removed and MH broth was autoclaved as per manufacturer's instructions.

A single colony of *B. clausii* AD1 was inoculated into 10 ml of sterile XAD-16-treated MH broth, placed on an orbital shaker (100 rpm) at 30˚C for 24 h. The culture was subsequently re-inoculated (1% vol/vol) into 500 ml of the same MH broth and incubated on an orbital shaker (100 rpm) at 30˚C for 8-10 days (26). Cells were removed by centrifugation at $10,000 \times g$ for 25 min (4˚C). Cell-free supernatant (CFS) was added to activated-XAD-16 beads (10 g/500 ml) and placed on an orbital shaker (50 rpm) at 4˚C for 2 h. Beads were collected and washed with 30% ethanol (vol/vol), followed by several double-distilled water wash steps. Peptide bound to beads was eluted using 80% Iso-TFA (vol/vol) and filtered through a 45 μM cellulose acetate filter. Isopropanol was removed using rotary evaporation (RotaVapor®, Buchi).

To the cell-pellet 80% Iso-TFA (vol/vol) was added and vigorously shaken at 4˚C for 24 h to retrieve any peptide bound to the bacterial cells. Cells were separated by centrifugation at $10,000 \times g$ for 25 min (4˚C). The CFS was filtered and the Iso-TFA removed by rotary evaporation, as previously described.

Samples from the cell-pellet and XAD-16 beads were combined and loaded onto a Sep-Pak C18 column. The column was washed with double-distilled water and manually eluted using a stepwise acetonitrile (containing 0.1% TFA (vol/vol/vol)) gradient (10-80%, 10% increments at 1 ml/min). Fractions were tested for activity using the agar-well diffusion assay. Briefly, BHI soft agar (1 % wt/vol) was seeded with a dense overnight (18 h) culture of *S. aureus* (1% vol/vol) (Table 3). Wells (5 mm in diameter) were made into the solidified agar and filled with 50 μl aliquots of each fraction. Activity was observed as a clear zone of growth inhibition. Active fractions were frozen at -80˚C, freeze-dried and stored at -20˚C until further use.

Media (BHI) were treated with XAD-16 beads as previously described. *Bacillus amyloliquefaciens* AD2 was streaked onto BHI agar and grown for 24 h at 37˚C. A single colony was used to inoculate 5 ml LB broth, placed on an orbital shaker (100 rpm) at 37˚C and incubated for 24 h. Luria broth containing *B. amyloliquefaciens* AD2 was mixed with sterile, activated XAD-16 beads (5 g/5 ml LB). This suspension was spread over BHI agar (2% wt/vol agar; Ø150 mm plates) and incubated for 96 h at 30˚C. XAD-16 beads were removed from agar using double-distilled water and incubated on an orbital shaker (50 rpm) for 2 h at 4˚C. The beads were washed with double-distilled water to remove bacteria. The peptide was eluted from the beads and Iso-TFA removed as previously described. Samples from XAD-16 beads were combined and loaded onto a Sep-Pak C18 column. Peptide was eluted from Sep-Pak C18

columns and antimicrobial activity was tested as previously described. Active fractions were frozen at -80˚C, freeze-dried and stored at -20˚C until further use.

Nisaplin was dissolved in 25% acetonitrile containing 0.1% TFA (vol/vol/vol). Undissolved particles were removed by centrifugation. The supernatant was loaded onto a C18 Sep-Pak column and eluted as previously described. Active fractions were frozen at -80˚C, freeze-dried and stored at -20˚C until further use.

Peptides were further purified to homogeneity using high-performance liquid chromatography (HPLC). Freeze-dried samples were dissolved in 10% acetonitrile containing 0.1% TFA (vol/vol/vol) and applied to a Discovery BIO Wide Pore C18 HPLC column (10 μm, 250×10 mm; Sigma-Aldrich). Clausin and amyloliquecidin (AmyA) were eluted in an increasing gradient of 25% to 60% B over 28 min (A: MilliQ water containing 0.1% TFA (vol/vol) and B: acetonitrile containing 0.1% TFA (vol/vol)). Nisin was eluted in an acetonitrile gradient of 10% to 60% B over 38 min. Sample peaks were detected by measuring the absorbance at 230 and 254 nm. Peaks were collected and tested for activity against *S. aureus* using the agar-well diffusion assay as previously described. Fractions with active peaks were freeze-dried as well as analyzed by electrospray ionization-mass spectrometry (ESI-MS; central analytical facility, Stellenbosch, South Africa).

Identification of the *Bacillus amyloliquefaciens* Lantibiotic Operon

Southern- and colony-hybridization were performed according to standard methods (34). Total DNA from *B. amyloliquefaciens* AD2 was digested with *Sal*I, *Eco*RI, *Sac*I, *Nco*I, *Pst*I as well as combinations of these restriction enzymes. By using degenerative *lanM* primers, a partial *lanM* sequenced could be amplified. The partial *lanM* sequence was used to prepare probes labeled with DIG-11-dUTP. Random priming was performed according to the manufacturer's instructions (DIG DNA labeling mix). Appropriate restriction enzyme combinations were chosen after Southern blotting and hybridization, in order to clone the region containing the desired operon. DNA fragments were digested with selected restriction enzymes and ligated into pRSFDeut-1 (Novagen) and transformed into *E. coli* BL21. Transformants were plated onto LB agar containing kanamycin (50 μg/ml). Colony hybridization was performed using the same *lanM* probe. Plasmid DNA isolated from positive clones was sent for sequencing analysis. Sequences were used in BLASTn searches of NCBI databases to identify genes related to the lantibiotic gene cluster. Primers were generated to amplify the regions not identified with

79

Southern hybridization and are indicated in Table 2 (AmyA2 and LicR1). Amplified fragments were cloned into pJET, and *E. coli* BL21 transformants were plated onto LB agar containing ampicillin (100 μg/ml). Plasmid DNA was isolated from positive clones, sequenced and used in BLASTn searches of NCBI databases to identify amplified sequences. This resulted in the elucidation of the sequence of one of the structural genes for the antimicrobial peptides from *B. amyloliquefaciens* AD2.

DNA was sent for whole genome sequencing on the Ion Proton (central analytical facility, Stellenbosch, South Africa). Ion Proton DNA preparation and system settings were done according to manufacturer's instructions (Life Technologies). Velvet *de novo* genome assembler (version 1.2.10), CLC genomics workbench (version 7.0.3; Qiagen) and MIRA (version 4.0) were used for *de novo* assembly of contigs (35, 36). The partially identified lantibiotic operon obtained from the Southern hybridization approach was used to identify contigs containing the putative lantibiotic operon.

Antimicrobial Spectrum and Determination of MIC against *S. aureus*

An agar-well diffusion assay was used to determine the antimicrobial spectrum of lantibiotics. Indicator strains were grown for 18 h in appropriate media and under optimal conditions (Table 3). Peptide concentration was determined by using the BCA protein assay according to the manufacturer's instructions. Peptides were made up to a concentration of 50 μM in sterile double-distilled water (containing 0.1% TFA (vol/vol)). In the case of AmyA, the alpha and beta peptides were combined in a 1:1 molar ratio. Peptides were dispensed in wells (25 μl) and plates were incubated for 24 h at the appropriate temperatures (Table 3). Antimicrobial activity was qualitatively determined by the presence or absence of growth inhibition.

Microtiter plates were used to determine the minimal inhibitory concentration (MIC) of peptides against *S. aureus* Xen strains (Table 3). Bacteria were grown overnight (18 h), inoculated into fresh MH broth and incubated at 37°C until an OD_{600} of 0.1 was reached. Peptide concentrations were determined and adjusted as previously described. Each well was composed of 150 μl bacteria and 50 μl peptide. Controls were as follows: 150 μl media with and without bacteria with 50 μl double-distilled water containing 0.1% TFA (vol/vol). The

TABLE 3. Bacteria and Culture Conditions

Bacteria	Media	Temp (˚C)	Reference
Bacillus clausii AD1	BHI/NB	30	This study
Bacillus amyloliquefaciens AD2	BHI/NB/LB	30/37	This study
Bacillus cereus LMG13569	NB		Lab culture collection
Beta-hemolytic streptococci (3 strains)	TSB	37	This study*
Enterococcus spp. (5 strains)	BHI/MRS	37	This study*
Lactococcus lactis QU2	MRS	30	Lab culture collection[1]
Lactobacillus plantarum 423	MRS	30	Van Reenen et al., 1998[2]
Listeria spp. (2 strains)	BHI	37	This study*
Listeria monocytogenes EDGE	BHI	37	Caliper Life sciences, Hopkinton, MA, USA
Staphylococcus epidermidis	BHI/MH	37	This study*
Staphylococcus aureus Xen29, 30 (MRSA), 31 (MRSA), 36	BHI/MH	37	Caliper Life sciences, Hopkinton, MA, USA
Staphylococcus aureus (5 strains)	BHI/MH	37	This study*
Streptococcus mutans	BHI/MH	37	Lab culture collection
Escherichia coli BL21	LB	37	Lab culture collection

BHI: Brain heart infusion; NB: Nutrient Broth; LB: Luria broth; MRS: Man de Rosa Sharp; MH: Mueler Hinton; TSB: Tryptic Soy Broth. [1]Gift from Prof. Sonomoto; [2](36). * Clinical isolates, Pathcare, South Africa.

initial OD_{600} reading was taken with another at 5 h and the end point reading at 18 h. The MIC was determined as the lowest peptide concentration where no bacterial growth was observed after 18 h.

Stability Assays

Protease Stability Assay

Trypsin and proteinase K were dissolved in 10 mM Tris-HCl (10 mg/ml; pH 8.0) and pepsin was dissolved in double-distilled water (containing 0.1% TFA (vol/vol); 10 mg/ml). All proteases were added to peptides (final peptide concentration of 50 μM) to a final concentration of 1 mg/ml. Peptide-protease combinations were incubated at 37˚C for 4 h. The alpha and beta peptides were incubated separately. Untreated peptides were dissolved in 10 mM Tris-HCl or

double-distilled water (containing 0.1% TFA (vol/vol)). After incubation the proteases were inactivated by heating at 95˚C for 5 min. Protease treated peptides were added to their respective untreated partner (alpha or beta) peptides and used in agar-well diffusion assays by dispensing 20 µl combined peptide into prepared wells. Wells were created in MH soft agar by using a 48-well PCR plate, with *S. aureus* as indicator strain. Digital photos were taken of the plates after incubation and activity was quantitatively measured by measuring the zone areas using the software program ImageJ (version 1.48).

Heat Stability Assay

The alpha and beta peptides of AmyA were made up to a concentration of 70 µM in double-distilled water containing 0.1% TFA (vol/vol). The peptides were subsequently combined and incubated at 100˚C for 30 min and 121˚C for 15 min (autoclaved). The respective AmyA peptides were also separately treated at 121˚C and added to the corresponding untreated peptide after incubation. After incubation, treated peptides were spotted directly onto MH soft agar (10 µl spots) seeded with *S. aureus* and incubated for 18 h at 37˚C. Digital photos were taken of the plates after incubation and activity was quantitatively measured using the software program ImageJ as previously described.

Storage Stability Assay

To determine the stability of peptides after storage in a neutral buffer, a stability assay similar to that described by Oman and van der Donk (13) was used. Stock solutions of nisin, clausin, AmyAα and AmyAβ were dissolved in phosphate buffered saline (PBS; pH 7.4) with final peptide concentrations of 50 µM. The peptide stocks were aliquoted into glass HPLC vials, sealed and incubated at 26˚C. Aliquots were removed at selected time intervals and stored at -80˚C until analysis. Peptide aliquots were analysed by HPLC. Each aliquot (20 µl) was applied to a Hypersil Gold C18 HPLC column (5 µm, 100 × 4.6 mm; Thermo Scientific) and separated by maintaining the mobile phase at 10% B for 2 min followed by an increase to 100% B in 9 min at a flow rate of 1 ml/min. For each peptide the peak area corresponding to intact peptide was determined and plotted as a percentage relative to the peak area of intact peptide at day 0. Agar-well diffusion assays were used to determine the antimicrobial viability of the peptides. A total of 40 µl was dispensed into each well. Nisin and clausin were added as 20µl peptide combined with 20µl sterile PBS (pH 7.4). The alpha and beta peptides of AmyA were added as 20µl AmyAα (day *n*) combined with 20µl AmyAβ (day *n*). Digital photos were taken of the

plates after incubation and activity was quantified using the software program ImageJ as described previously.

Results

Isolation and Identification of *Bacillus amyloliquefaciens* and *Bacillus clausii* from Fynbos Soil

The overlay assay used to identify potential antimicrobial producing bacteria provided for high throughput screening of hundreds of colonies. Activity was only observed with bacteria obtained from soil samples. The colony morphology of bacteria with antimicrobial activity were different from other isolated colonies and were further investigated (Fig. 1). One strain of
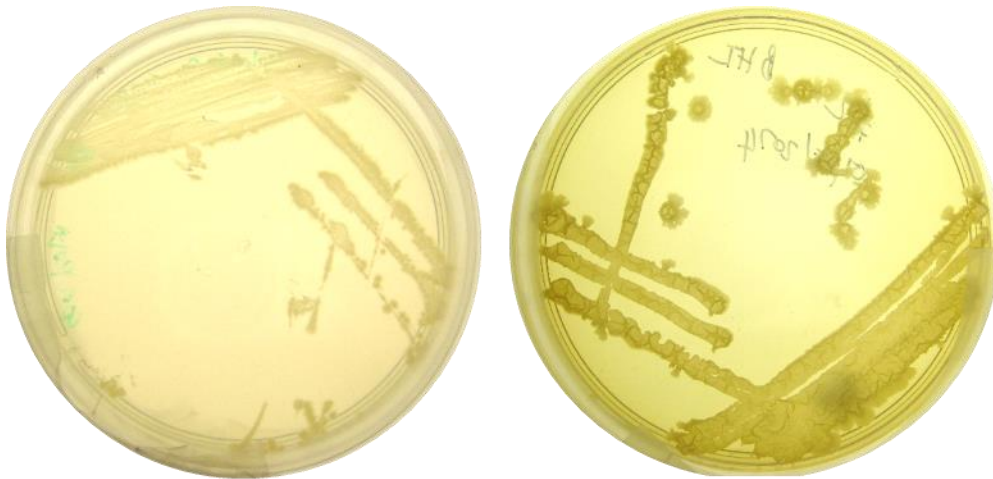
FIG 1 *Bacillus clausii* AD1 (left) and *Bacillus amyloliquefaciens* AD2 (right) grown on BHI

the antimicrobial producing bacteria could be identified by its partial *16s rDNA* sequence and was identified as a *B. clausii* strain (designated *B. clausii* AD1). However, this was not sufficient to properly identify the second bacterial strain which could only be isolated to species level (i.e. *Bacillus* spp.). The sequence of the partial *gyraseA* identified the second bacterial strain as a novel *B. amyloliquefaciens* with only 91% nucleotide- and 99% amino acid-identity to the nearest *B. amyloliquefaciens*. This novel strain was designated as *B. amyloliquefaciens* AD2. The *lanD* gene in *B. clausii* AD1 could be amplified using the selected primers and had 100% amino acid and nucleotide identity (100% sequence coverage) to *clausD* (BAD66090.1) in *B. clausii* KSM-K16 (AP006627.1). A partial *lanM* sequence could be amplified from *B.*

amyloliquefaciens AD2 and had the closest homology to a putative *lanM* from *B. licheniformis* 9945A (GenBank: CP005965.1).

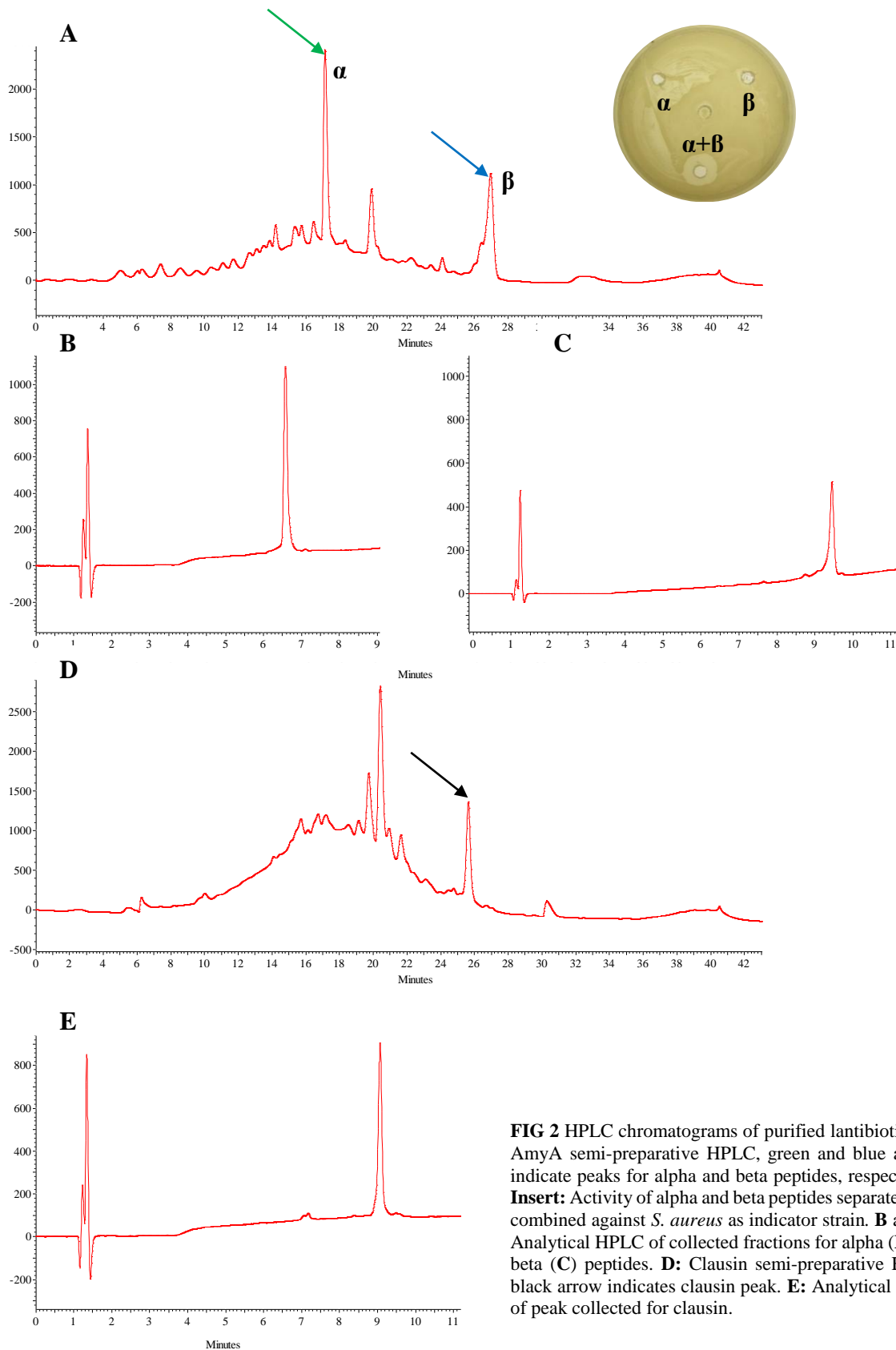## Production and Purification of Lantibiotics

Bacillus clausii AD1 and *B. amyloliquefaciens* AD2 had activity on solid media. However, activity could not be observed in supernatants of cultures grown in liquid broth. In the case of *B. clausii* AD1, activity could be observed in supernatant after prolonged incubation of the culture. Even after prolonged incubation in liquid broth activity could not be observed in the supernatant of *B. amyloliquefaciens* AD2. Activity could only be observed in isopropanol extracts of *B. amyloliquefaciens* AD2 cells after being scraped off solid agar. Solid media was subsequently used to produce AmyA. The initial purification process was significantly enhanced by using autoclaved activated XAD-16 beads to capture peptide being released by bacteria growing on solid media. Additionally, no residual peptide remained on the surface of the bacteria when they were grown in the presence of XAD-16 beads. The treatment of media with XAD-16 beads before autoclaving also significantly reduced the background of crude samples when run on HPLC.

Clausin could be separated easily from crude extract using HPLC, with the active fraction eluting at 25.6 min. It had a size of 2,108.79 Da, corresponding to the known size of clausin (Fig. 2D, E and 3C). Multiple peaks were observed for AmyA, none of which exhibited any antimicrobial activity on their own (Fig. 2A-C). By combining different peaks antimicrobial activity was achieved, specifically when the peaks eluting at 17 and 27 min were combined (Fig. 2A insert). Electrospray ionization-MS revealed that the sizes of the peaks were 3,027.27 and 2,438.04 Da for AmyAα (17 min) and Amyβ (27 min), respectively (Fig. 3A and B).

Identification of *Bacillus amyloliquefaciens* Lantibiotic Operon

Genomic DNA was digested with *Eco*RI plus *Sal*I and using the partial *lanM* sequence as a probe in Southern hybridization, a ~4 kb fragment of the lantibiotic operon could be identified. The partially identified operon included the sequence for *amyAβ* (Fig. 4B). Based on the homology to *B. licheniformis* 9945A, primers were designed to amplify the *amyAα* structural gene. Forward and reverse primers were designed to the sequences of *amyAβ* and *licR1* (from

B. licheniformis 9945A), respectively (Table 2). Using PCR, the structural gene of *amyAα* was obtained (Fig. 4A).
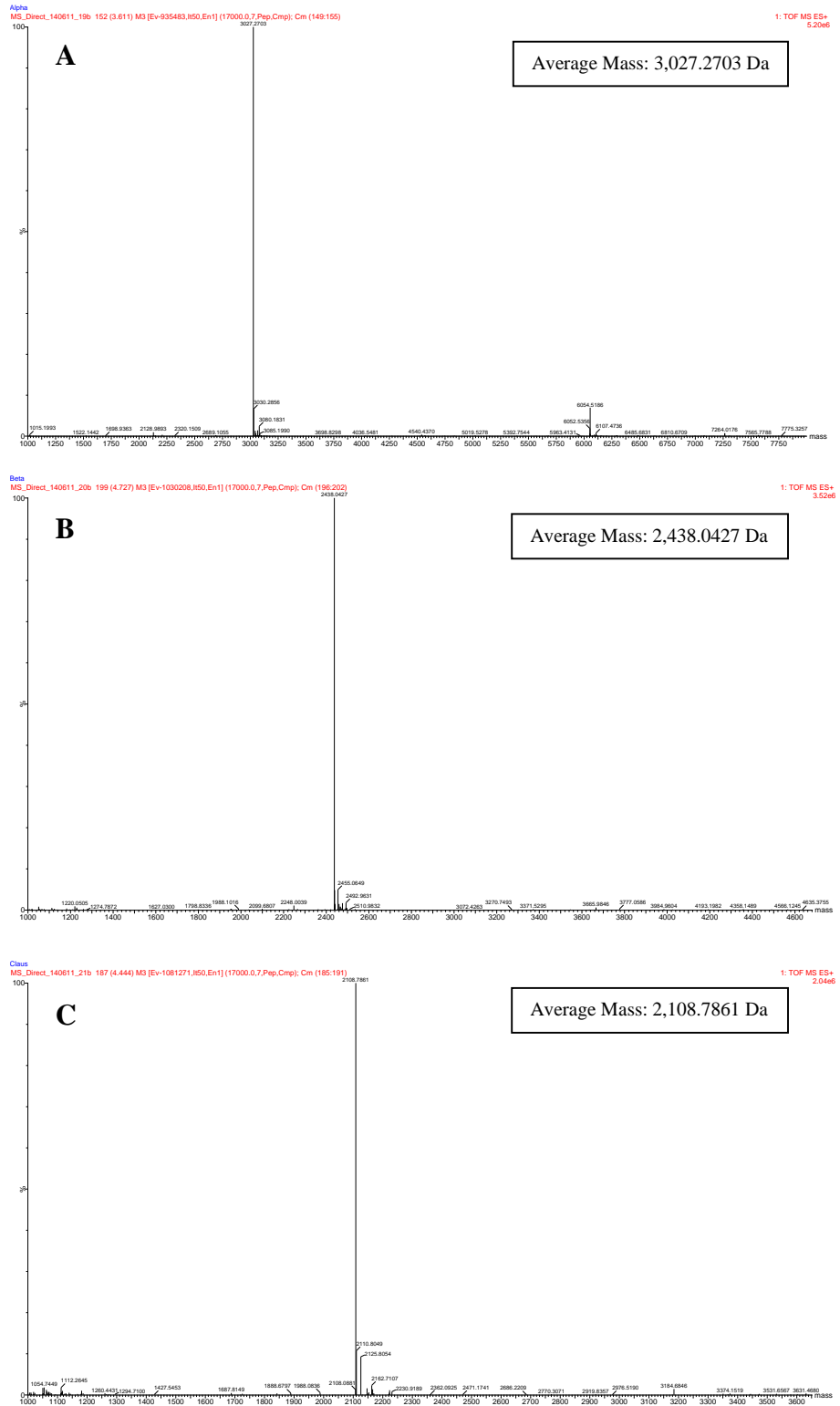

FIG 2 HPLC chromatograms of purified lantibiotics. **A:** AmyA semi-preparative HPLC, green and blue arrows indicate peaks for alpha and beta peptides, respectively. **Insert:** Activity of alpha and beta peptides separately and combined against *S. aureus* as indicator strain. **B** and **C:** Analytical HPLC of collected fractions for alpha (**B**) and beta (**C**) peptides. **D:** Clausin semi-preparative HPLC, black arrow indicates clausin peak. **E:** Analytical HPLC of peak collected for clausin.

FIG 3 ESI-MS (direct injections) of purified lantibiotics, AmyAα (**A**), AmyAβ (**B**) and clausin (**C**).

A

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AmyAα    ------------CA--WYDISCKLGNKGAWCTLTVECQSSCN
HalAα    ------------CA--WYNISCRLGNKGAYCTLTVECMPSCN
LicAα    -------TITLSTCA--I--LSKPLGNNGYLCTVTKECMPSCN

LtnAα    ------------CSTNTFSLSDYWGNNGAWCTLTHECMAWCK
PlwAα    --------K----CK--WWNISCDLGNNGHVCTLSHECQVSCN
SacAα    ------------CSTNTFSLSDYWGNKGNWCTATHECMSWCK
BhtA1    IGTTVVNST----------FSIVLGNKGYICTVTVECMRNCQ
SmbB     IGTTVVNST----------FSIVLGNKGYICTVTVECMRNCSK
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AmyAα leader peptide: MKMSIIETWKNPALRMNSQVVNPAGDLMEELSDNEMEMLAGG

B

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AmyAβ    ADVTPH ---TTPSSVPC---GV------F-VTAAF--CPSTKCTSSC
HalAβ    GDVHAQ ------TTWPC---AT------VGVSVAL--CPTTKCTSQC
LicAβ    NDVNPE TTPATTSSWTCITAGV------T-VSASL--CPTTKCTSRC

LtnAβ           TTPATPAISIL---SA------Y-ISTNT--CPTTKCTRAC
PlwAβ    GDPEAR ------SGIPC-TIGA------A-VAASIAVCPTTKCSKRCGKRKK
SacAβ           ---GTPLALLG---GAATGVIGY-ISNQT--CPTTACTRAC
BhtA2           ------STPAC-AIGV-VG----ITVAVT-GISTACTSRC--INK
SmbA            ------STPAC-AIGV-VG----ITVAVT-GISTACTSRC--INK
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AmyAβ leader peptide: MSNREKAELYRNAGKRTELGFVNPVGEVSEDELRNLAGA

FIG 4 Sequence alignment of selected two-component lantibiotics. **A:** Sequence alignment of AmyAα core peptide with core peptides of haloduracin (HalAα, WP_010896632), lichenicidin (LicAα, Q65DC4) lacticin 3147 (LtnAα, O87236), plantaricin W (PlwAα, AAG02567), staphylococcin C55 (SacAα, BAB78438), BHT (BhtA1, AAZ76603), Smb (SmbB, BAD72777). **B:** Sequence alignment of AmyAβ core peptide with core peptides of haloduracin (HalAβ, WP_010896631), lichenicidin (LicAβ, P86720), lacticin 3147 (LtnAβ, O87237), plantaricin W (PlwAβ, AAG02566), staphylococcin C55 (SacAβ, BAB78439), BHT (BhtA2, AAZ76602), Smb (SmbA, BAD72776). Lines above alignments indicate bridging pattern of haloduracin, solid lines: Lan/MeLan rings and dashed line: disulphide bridge. Red arrow indicates secondary cleavage of hexapeptide from beta peptides. Bold purple and green letters indicate Thr/Ser that are dehydrated. Underlined letters indicate Ser/Thr that escape dehydration. Green highlighted Ser/Thr in AmyAα and AmyAβ indicates residues that possibly escape dehydration. Yellow highlighted Ser are converted to D-Ala. Grey highlighted letters indicates GG/GA cleavage site in AmyA leader sequence. Grey highlighted Thr are converted to 2-oxobutyryl.

However, after this initial success further elucidation of the operon was unsuccessful. Therefore, the genome of *B. amyloliquefaciens* AD2 was sequenced. Using the partially identified lantibiotic operon in BLASTn searches of the assembled contigs, the entire lantibiotic operon of AmyA could be identified and annotated. The AmyA lantibiotic operon

is located on both strands and covers approximately 18 kb and contains all the components necessary for the biosynthesis of AmyA (Fig. 5).

Analysis of the lantibiotic operon revealed the presence of two putative LanM modification enzymes, AmyM1 (40% and 36% identity to HalM1 (BAB04174.1) and LicM1 (AAU42941.1), respectively) and AmyM2 (34% and 34% identity to HalM2 (BAB04171.1) and LicM2 (AAU42939.1), respectively). The two peptides, corresponding to those found in initial experiments, were found following each of the *lanM* genes. The leader sequence of AmyAα and AmyAβ peptides had the characteristic GA (alpha) or GG (beta) cleavage sites also found in other two-component lantibiotics (Fig. 4). Using the molecular mass of purified peptides it was observed that AmyAα should undergo three dehydration reactions and AmyAβ eight dehydrations. There is an additional cleavage reaction to remove a hexapeptide (ADVTPH) after the GA cleavage site of AmyAβ (Fig. 4). When comparing AmyA to other two-component lantibiotics from *Bacillus* spp. it was hypothesized that the two C-terminal Ser residues of AmyAα possibly escape dehydration and that two Thr and/or Ser residues escape dehydration in AmyAβ (Fig. 4A and B).The two peptides with their leaders attached has a length of 70 and 71 amino acids for AmyAα and AmyAβ, respectively. After all cleavage reactions the mature peptides are composed of 28 and 26 amino acids for AmyAα and AmyAβ, respectively (Fig. 4A and B). The two precursor- and core-peptides have limited homology to each other with only 28% and 29% identity, respectively. The core-peptides of AmyA has high amino acid sequence identity with other two-component lantibiotics including haloduracin (HalA), lichenicidin (LicA) and lacticin 3147 (LtnA; Fig. 4). The identity of AmyAα to HalAα, LicAα and LtnAα was 82%, 63% and 71%, respectively. Similarly, AmyAβ also has high identity to these two-component lantibiotics, HalAβ (62%), LicAβ (50%) and LtnAβ (70%). Open reading frames were identified that have identity to genes found in other lantibiotic gene clusters, with putative roles in transport, regulation and processing (Fig. 5). AmyP is classified as a putative serine protease with high identity to the putative LicP (AGN34600.1) found in *B. licheniformis* 9945A (67%) and lower identity to LicP (AAU25563.1) in *B. licheniformis* DSM13 (29%). LanE'F'G'EFG were identified as putative membrane proteins, typically
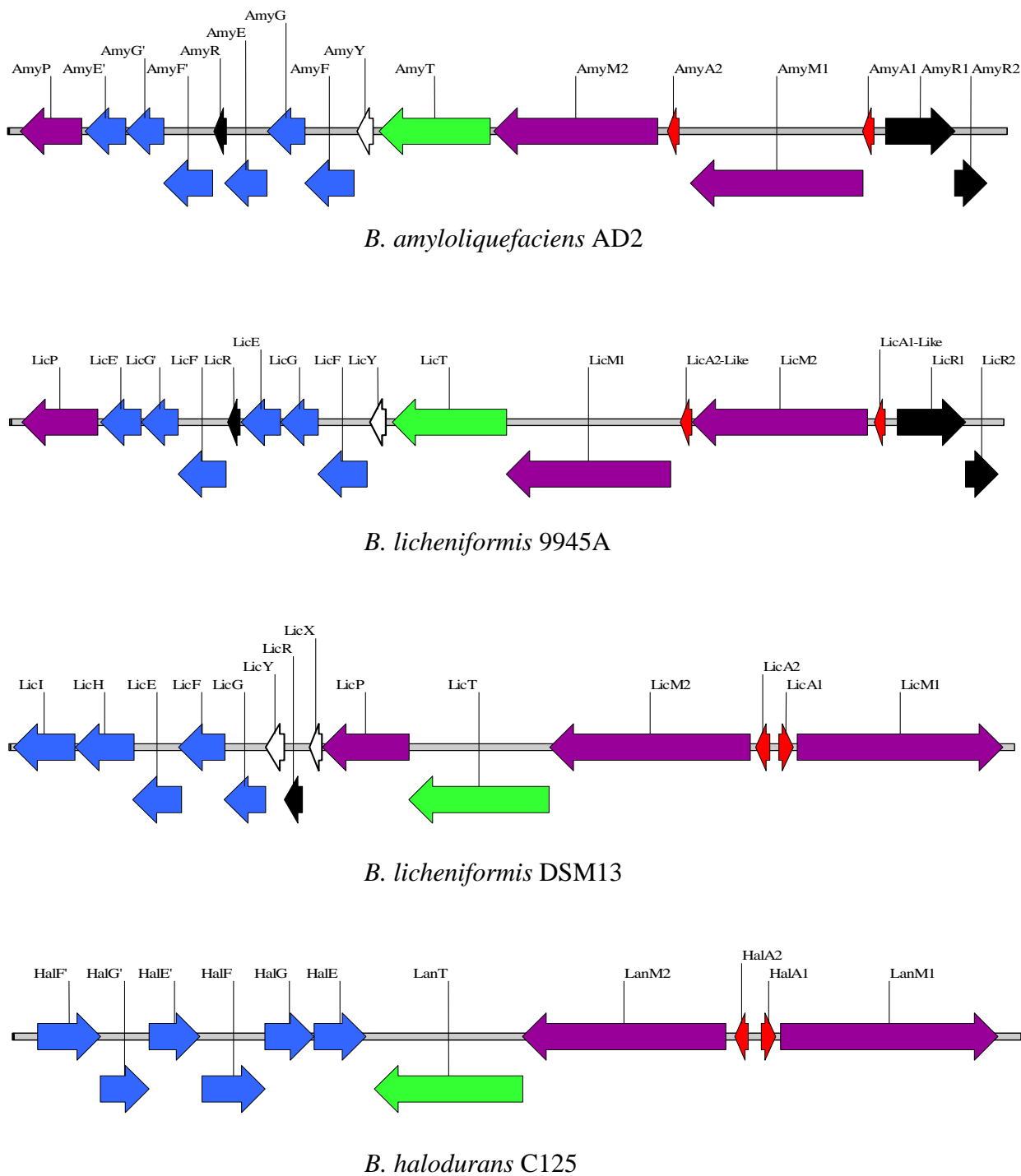
88

FIG 5 Organization of gene clusters involved in the biosynthesis of *Bacillus* spp. two-component lantibiotics. Genes coding for transport proteins involved lantibiotic immunity are indicated in blue and those coding for proteins involved in regulation are indicated in black. Genes involved in maturation and transport of lantibiotics are indicated in purple and green, respectively. Genes coding for the precursor peptides are indicated in red. Genes with unknown or putative functions are indicated in white.

associated with immunity. They also have low sequence identity to related proteins in *B. licheniformis* (9945A and DSM13) and *B. halodurans* C-125, as well as other putative transmembrane proteins in other *Bacillus* spp. AmyT was identified as a lantibiotic transporter

with identity to HalT (54%; BAB04170.1) and LicT (81% and 49% identity to 9945A (AGN34609.1) and DSM13 (AAU25564.1), respectively) and belongs to the ABC transporter superfamily with an integrated peptidase domain. AmyY has identity to LicY (30% and 71% identity for DSM13 (AAU25560.1) and 9945A (AGN34608.1), respectively) and may be important in biosynthesis of one or both peptides, with a possible role as a membrane protein (38). AmyR was identified as a putative lantibiotic transcriptional regulator with identity to LicR (85% and 52% identity to 9945A (AGN34604.1) and DSM13 (AAU25561.1), respectively). AmyR1 has identity to multiple histidine kinases with 65% identity to LicR1 from 9945A (AGN34614.1) and 76% C-terminal identity to a signal transduction histidine kinase from *B. sonorensis* (WP_006640356.1). AmyR2 was identified as a putative accessory gene regulator with 54% and 55% identity to LicR2 (AGN34615) from 9945A and AgrB from *B. sonorensis* (WP_006640357.1), respectively. LicR1/R2 possibly acts as a two-component regulatory system.

Antimicrobial Spectrum and Determination of MIC against *S. aureus*

All peptides had activity against clinical isolates of *S. aureus,* beta-haemolytic streptococci and *Listeria* spp. with nisin and clausin showing activity against all enterococci tested (Table 4). MIC values were determined for lantibiotics against an additional four *S. aureus* Xen strains, including two methicillin-resistant *S. aureus* (MRSA) strains, Xen 30 and 31. Clausin had the lowest MIC against the respective strains, followed by nisin and AmyA (Table 5).

TABLE 4. Inhibitory Spectra of Lantibiotics in Agar Well-Diffusion Assay

Bacteria	Nisin	Amy	Clausin
S. aureus (5 strains)	+	+	+
Enterococcus spp. (4 strains)	+	+	+
Enterococcus spp. 5	+	-	+
L. lactis	+	+	+
L. plantarum 423	+	+	+
Listeria spp. (2 strains)	+	+	+
B. cereus	+	-	+
S. epidermidis	+	-	+
S. mutans	-	-	-
[1]BHS (3 strains)	+	+	+

[1]BHS: beta-haemolytic streptococci. + and − indicates presence or absence of growth inhibition, respectively.

TABLE 5. MIC against *S. aureus* Xen strains

Peptide	MIC (µM)			
	Xen36	Xen31	Xen30	Xen29
Nisin	5	7	5	5
AmyA	5	10	10	7
Clausin	5	5	5	5

Stability Assays

Protease Stability Assay

Both AmyA peptides were degraded by proteinase K and both peptides were resistant to degradation by pepsin (Fig. 6). Only the alpha peptide was degraded by trypsin (Fig. 6).
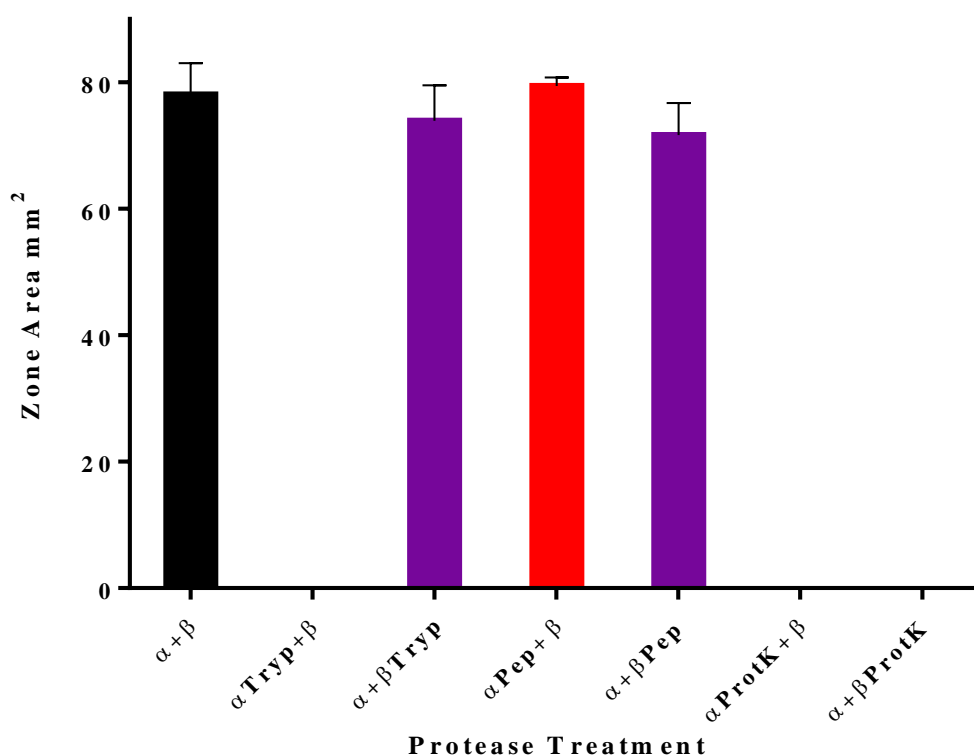

FIG 6 Stability of AmyA peptides after treatment with trypsin (Tryp), pepsin (Pep) or proteinase K (ProtK). Error bars indicate standard deviation from three replicates.

Heat Stability Assay

Marked stability was observed for AmyA when heated at 100˚C for 30 min with more than half of the activity remaining (Fig. 7). However, a substantial loss in activity was observed when AmyA was treated at 121˚C (Fig. 7A). When AmyAα was treated separately at 121˚C no activity could be observed when added to untreated AmyAβ, however, when AmyAβ was treated separately at 121˚C and added to untreated AmyAα almost all antimicrobial activity was retained (Fig. 7B).
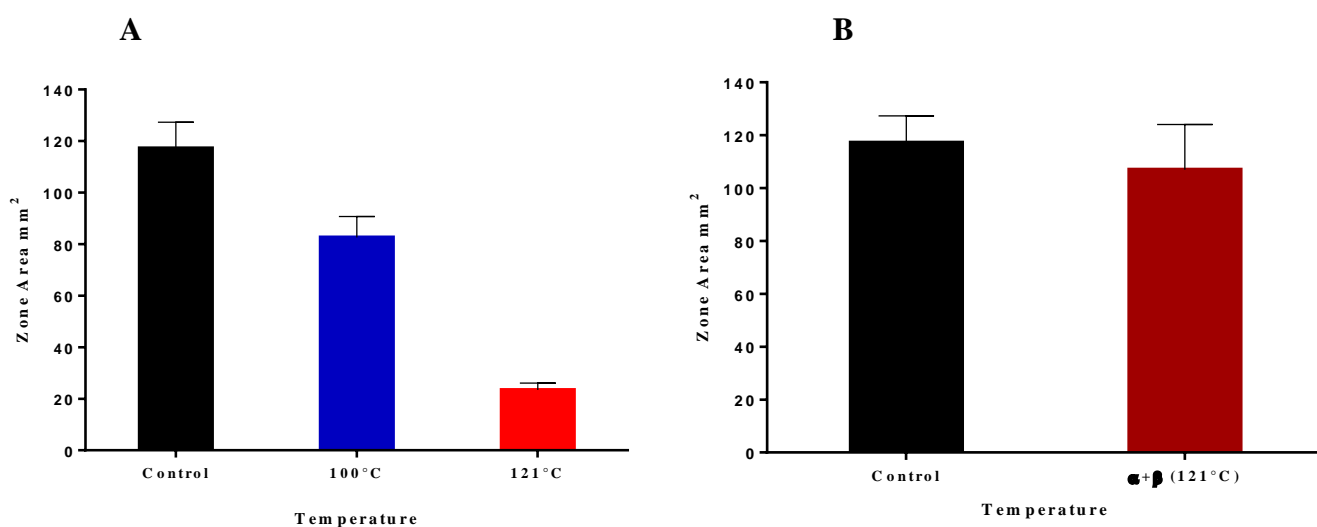
FIG 7 Stability of AmyA peptides after heat treatment. **A:** AmyAα and AmyAβ combined and incubated **B:** AmyAα and AmyAβ incubated separately at 121˚C (AmyAα (121˚C) + AmyAβ (untreated) not shown). Error bars indicate standard deviation from three replicates.

Storage Stability Assay

Stability of AmyAα and AmyAβ were observed throughout the 12 day period (at 26˚C) with the best stability observed for AmyAα (Fig. 8). The peak areas of AmyAα and AmyAβ on day 12 was 85.1% and 71.5%, respectively. Nisin and clausin showed a steady decrease in peak area with clausin having a sharp decline after day 6. Nisin had a minimum peak area of 38.8% on day 12. Clausin had a peak area of 73.1% on day 6 which dropped to 17.1 and 10.4% on days 9 and 12, respectively. The activity results for AmyA and clausin resembled that recorded by HPLC, with almost all activity being retained up to day 12 for AmyA. Clausin activity decreased steadily up to day 6 with a substantial loos in activity observed on days 9 and 12. In contrast to the decrease in peak area recorded by HPLC results, nisin retained almost all of its activity up to day 12.
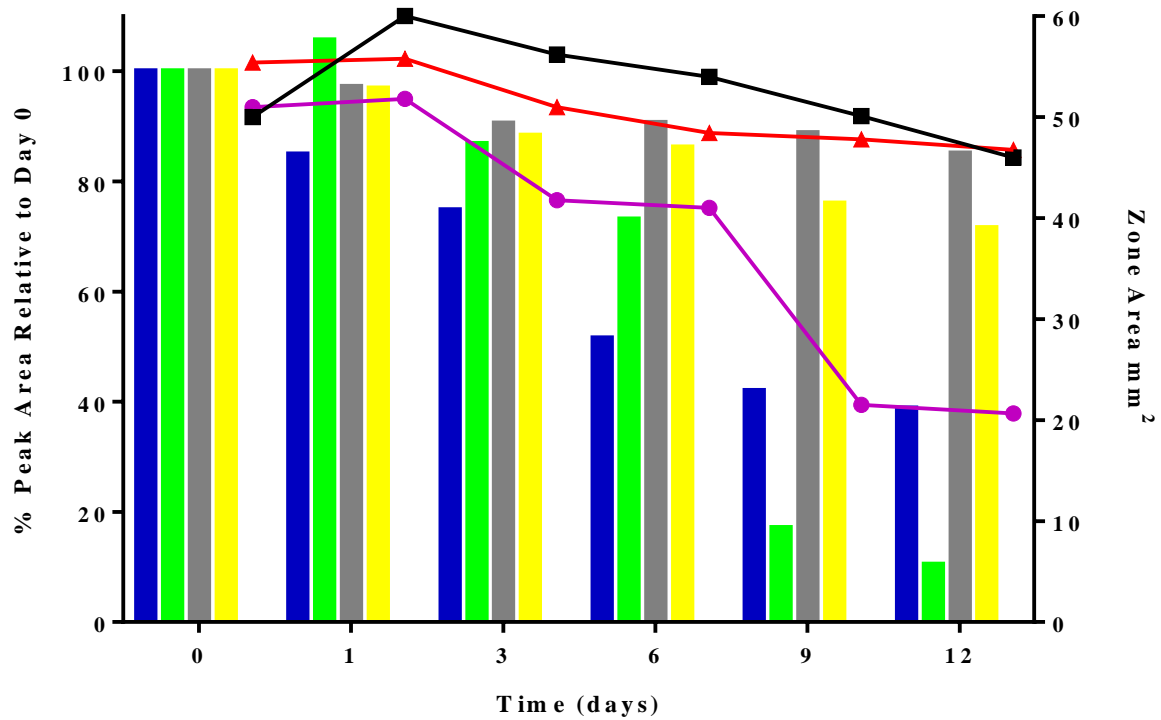
FIG 8 Storage stability of nisin, clausin and AmyA in PBS (pH 7.4). Bars indicate the percentage area of the respective intact lantibiotic HPLC peaks at day *n* relative to day 0. Blue, green, grey and yellow bars represent nisin, clausin AmyAα and AmyAβ, respectively Lines indicate activity on day *n* represented as area of growth inhibition zone in mm^2. Black squares, purple circles and red triangles represent nisin, clausin and AmyA, respectively.

Discussion

Lantibiotics are ubiquitous in nature and have been isolated from a wide range of bacteria and environments. The microbial diversity in the Fynbos soil is very high, increasing the likelihood of finding bacteria (or fungi) that produce antimicrobial substances used for competing in this diverse environment (20, 21). The majority of bacteria associated with soil are Gram-positive bacteria, further increasing the probability of isolating a bacterium that produces antimicrobial substance against other Gram-positive bacteria (19, 20, 39). Soil was isolated near or under plants (mainly *Protea repens*), and these areas are known to be rich in bacterial diversity (20, 21, 39). Therefore, it is not surprising that the two *Bacillus* spp. strains that were isolated both produced potent anti-Gram-positive peptides. These lantibiotic producing *Bacillus* spp. were isolated using traditional techniques that rely not only on the ability to culture the bacteria but also on the production of active antimicrobial compounds. It may be simpler to use genome mining to identify novel lantibiotics, and produce them heterologously (40). However, putative lantibiotics first have to be expressed before activity can be determined. Although heterologous expression for lantibiotics are becoming more efficient, their complex biosynthesis still pose challenges. Traditional techniques provide the advantage of quickly identifying bacteria that actually produce active antimicrobials. Genome mining is also limited by the availability of novel genomes. Therefore, unexploited areas, such as the Fynbos, may contain lantibiotic producing bacteria that have not been sequenced.

Initially antimicrobial activity could be observed on solid media, however, production in liquid broth was either absent or delayed (*B. amyloliquefaciens* AD2 and *Bacillus clausii* AD1, respectively). This was not surprising as some lantibiotics (and other bacteriocins) are better produced on solid media (27, 41, 42). Streptin was also initially only observed on solid media, and only after use of a bi-phasic culture system could activity be observed in liquid broth (41). Using the bi-phasic system the bacteria were grown on solid media for 24 hours followed by addition of liquid broth and a further 24 hour incubation period. Streptin production still essentially relies on the production of streptin on solid media first even with the bi-phasic system (41). Other lantibiotics from streptococci are also only produced on solid- or semi-solid media (43–45). The two-component lantibiotic, haloduracin (HalA) produced by *B. halodurans* C-125 is also only produced on rich solid media (27). The production of lantibiotics, including AmyA, is therefore, most likely controlled by *quorum* sensing in a cell density dependent manner (46). The production of clausin by *B. clausii* is known to be delayed in liquid media

with a correlation between antimicrobial activity and percentage sporulation (26). Both AmyA and clausin could, however, be successfully produced and purified to homogeneity following consecutive solid-phase extraction methods (Fig. 2 and 3). The molecular masses of the purified peptides were compared against those of known bacteriocins. Peptides produced by *B. amyloliquefaciens* AD2 did not correspond to the molecular mass of any known two-component lantibiotics. The peptide produced by *B. clausii* AD1 did correspond to the molecular mass of clausin, which is known to be produced by *B. clausii* (47). Unfortunately, there was little success in obtaining a partial *lanM* sequence for clausin. However, *clausD* could be amplified out of gDNA from *B. clausii* AD1. The *clausD* was identical to one found in *B. clausii* KSM-K16, in which the *clausD* occurs in a lantibiotic gene cluster with a structural gene for clausin (100% amino acid identity to known clausin). Claus D is the LanD putatively responsible for the oxidative decarboxylation reaction in clausin (48). From these results it is proposed that the antimicrobial produced by the *B. clausii* AD1 isolated in this study is indeed clausin. Using the degenerative *lanM* primers a partial *lanM* fragment could be identified in *B. amyloliquefaciens* AD2, which aided subsequent isolation of the complete lantibiotic gene cluster. Initial experiments using Southern- and colony-hybridization revealed a lantibiotic operon. However, further attempts to identify the complete operon using these methods were hampered by a lack of appropriate restriction enzyme sites that were required for successful cloning. Therefore, genome sequencing was utilized to further elucidate the lantibiotic operon. Using genome sequencing and the partial lantibiotic gene cluster as driver, an entire lantibiotic gene cluster could be identified in *B. amyloliquefaciens* AD2 (Fig. 5).

Comparison of the putative AmyA operon with those of other two-component producing lantibiotics revealed that the AmyA operon had the most similarity to another putative operon found in *B. licheniformis* 9945A, also isolated from soil (Fig. 5). The organization of genes in both operons are the same, and along with the high similarity of the individual genes it suggests possible distribution of this gene cluster between these two species. Other lantibiotics, such as nisin, have been associated with transmissible elements such as transposons and conjugative plasmids (28, 49).

The maturation of lantibiotics includes the dehydration of Ser and Thr followed by cyclization and removal of the N-terminal leader peptide. These reactions are mainly performed by three enzymes in two-component lantibiotics from *Bacillus* species (27, 38, 50). The dehydration and cyclization steps are performed by a bi-functional dehydratase/cyclase, LanM, with each peptide requiring its own LanM (29, 51). The LanM enzymes have an N-terminal dehydratase

95

and a C-terminal LanC-like cyclase domain. LanM phosphorylates Ser to Dha and Thr to Dhb followed by cyclization by the C-terminal cyclase, producing Lan/MeLan rings, respectively (1, 52, 53). The LanM proteins in *B. licheniformis* DSM13 and *B. halodurans* C-125 are responsible for the dehydration and cyclization of their respective peptides (27, 38, 50). Disruption of respective *lanM* genes in lichenicidin (LicA) results in loss of activity, further illustrating their essential role in biosynthesis (29). Furthermore, heterologously expressed HalM1 and HalM2 are able to successfully modify HalAα and HalAβ *in vitro*, respectively (50). The LanM enzymes of *B. amyloliquefaciens* AD2 show similarity to those in *B. licheniformis* C-125 and *B. halodurans* DSM13 and therefore are putatively involved in the dehydration and cyclization of the precursor peptides. Following dehydration the peptides are transported out of the cell by the membrane bound LanT which also has an integral protease domain responsible for the removal of the leader peptide (27, 38, 50). The AmyT found in the lantibiotic operon of *B. amyloliquefaciens* AD2 has amino acid identity to those found in *B. licheniformis* DSM13 and *B. halodurans* C-125.

The beta peptides of some two-component lantibiotics such as haloduracin, lichenicidin, plantaricin W and staphylococcin C55 have an additional hexapeptide that needs to be removed after transport (Fig. 4B). The hexapeptide, ADVTPH, in AmyAβ also needs to be removed for the mature peptide to correspond to its observed molecular mass. The supernatant of *B. halodurans* C-125 contains an enzyme that is able to cleave this hexapeptide (50). A *licΔP* mutant in *E. coli,* expressing the entire lichenicidin gene cluster, did not have expression of active lichenicidin (38). Activity could be restored by processing LicAβ *in situ* with sterile supernatant from *E. coli* expressing only *licP* (38). Interestingly, a *licΔT* mutant still produced trace amounts of mature LicAβ suggesting that LicP is still able to process the peptide with the leader attached (38). Both AmyP and LicP also have identity to CylA (30%, 36% and 32% identity for AmyP, LicP DSM13 and 9945A, respectively). The CylA is an extracellular protease responsible for N-terminal trimming of the cytolysin peptides (54). The putative regulators, AmyR1/R2, only have similarity to putative LicR1/R2 in *B. licheniformis* 9945A. However, AmyR1 has identity to several signal transduction histidine kinases and AmyR2 to accessory gene regulators. Together these two may act as a two-component regulatory system similar to that found in other bacteriocins (46). The two-component regulatory system consists of the membrane anchored sensor kinase that recognises a peptide pheromone (potentially the produced lantibiotics) which then transfers the signal to a cytoplasmic response regulator that then regulates expression of either the peptides or other components of the gene cluster (46).

The molecular masses of AmyAα and Amyβ indicate post-translational dehydration of three and eight Ser/Thr, respectively (Fig. 3 and 4). The core-peptide sequence of AmyAα contains three Ser and two Thr that can possibly be dehydrated (Fig. 4A). In order to correspond to the observed molecular mass two of these dehydrations presumably do not take place. Haloduracin-α and LicAα each have a Ser (Ser26 and 30, respectively) that escapes dehydration (28, 38, 50). It is, therefore, proposed that Ser26 of AmyAα may similarly escape dehydration. Due to the identical positions of Cys, Thr and Ser (excluding Ser25) it is also proposed that the only possible Ser/Thr that can also escape dehydration is Ser25. Ten Ser/Thr can possibly be dehydrated in AmyAβ (Fig. 4B). For the molecular mass of mature AmyAβ to correspond to the observed mass two Ser/Thr presumably escapes dehydration. In HalAβ and LicAβ the N-terminal Ser/Thr are all dehydrated, with the exception of Thr10 in LicAβ (28, 38, 50). By analogy it is, therefore, proposed that the two Ser and two Thr in the N-terminal of AmyAβ are dehydrated. The C-terminals of the beta peptides in two-component lantibiotics are highly similar, with exception of BhtA2 and SmbA (Fig. 4B). Relative to AmyAβ, the Ser at position 24 in HalAβ and LicAβ escape dehydration. The Thr at position 20 escapes dehydration in the rest of the compared two-component lantibiotics, with the exception of SmbA (Fig. 4B). Due to the high similarity to HalAβ and LicAβ it is proposed that Thr20 and Ser24 (as oppose to Ser25) in AmyAβ escapes dehydration.

Both HalAα and LicAα have similar ring topologies, with the exception of a disulphide bridge in HalAα instead of a Lan ring (28, 38, 50). It is proposed that the ring structure of AmyAα is similar to that of haloduracin, with one Lan ring (Ser7-Cys17) and two MeLan rings (Thr18-Cys23 and Thr20-Cys27) and possibly also a disulphide bridge between Cys1 and Cys8. As with other two-component lantibiotics. The mersacidin-lipid II binding motif ($\underline{C}$-$\underline{T}$-L$^{/x}$-$\underline{T}^{/S}$-x-E-$\underline{C}$) is also present in AmyAα from position 17 to 23. The beta peptides of haloduracin and lichenicidin both have similar ring topologies (28, 38, 50). It is proposed that AmyAβ also has the same ring topology, with two Lan- (Ser4-Cys8 and Ser19-Cys22) and two MeLan-rings (Thr13-Cys17 and Thr23-Cys26).

Identification and annotation of novel lantibiotic gene clusters provides further information on the biosynthesis of lantibiotics. Using this information lantibiotic production can be optimized, especially with regards to heterologous expression systems. Furthermore, utilizing optimized heterologous expression systems will provide the tools required for the large scale production of lantibiotics.

The two peptides of two-component lantibiotics have to act synergistically in order for them to have antimicrobial activity (28, 38, 50). The alpha peptide binds to lipid II which is followed by the binding of the beta peptide to the alpha peptide-lipid II complex. This interaction then results in pore formation (13, 55, 56). With single peptide lantibiotics, such as nisin, lipid II is also bound via a lipid II-binding motif. After this binding they induce pore formation and inhibit cell wall biosynthesis (57–61). Lantibiotics, including two-component lantibiotics, have activity against a range of Gram-positive bacteria including antibiotic-resistant strains such as MRSA and vancomycin-resistant enterococci (VRE) (13, 29, 62). Similar to other two-component lantibiotics AmyA has activity against clinical isolates of *S. aureus* and enterococci. Activity was, however, not observed against some of the bacteria tested, including *Staphylococcus epidermidis* and one of the enterococci isolates (Table 4). The highest MIC values against *S. aureus* Xen strains were observed with AmyA, with a twofold higher MIC against MRSA strains compared to clausin and nisin which were similar (Table 5). Lacticin 3147 also has higher MIC values against antibiotic-resistant *S. aureus* when compared to nisin, but has lower MIC values against *Enterococcus faecium* (62). Haloduracin on the other hand has mixed results with higher MIC values against some strains but with significantly lower MIC values against others, including VRE (13).

The stability of antimicrobials under various conditions is important if they are to be applied in either the medical or food industry. Resistance to proteolytic cleavage is an important trait, and lantibiotics such as nisin and epidermin show limited stability compared to the more stable globular peptides such as mersacidin. The globular nature of AmyAα was thought to provide protection from proteolytic cleavage, but it is only resistant to pepsin treatment. Resistance against trypsin was at least observed for AmyAβ (Fig. 6). The trypsin cleavage site of AmyAα is in the middle of the first Lan ring which should provide protection from cleavage. Lichenicidin-α is resistant to trypsin cleavage although it has a trypsin cleavage site in-between rings C and D which should provide sufficient protection from cleavage (28, 29). The first Lan ring in AmyAα is, however, made up of nine amino acids, making it a large ring which may not provide sufficient shielding from proteolytic cleavage. This may also explain the resistance observed in AmyAβ, as its trypsin site is in its much smaller C ring. The stability of AmyAβ after 121˚C treatment is also interesting and may imply that the peptide could be fragmented (Fig. 7B). The C-terminal of the beta peptides of two-component lantibiotics are important for interaction with the alpha peptide (38, 50, 63). Therefore, it may be possible for a truncated beta peptide to still be able to bind to the AmyAα-lipid II complex, resulting in activity.

98

Furthermore, AmyAβ digested with pepsin still results in activity when combined with untreated AmyAα. Pepsin possibly cleaves AmyAβ at Phe11, which is not protected by a Lan/MeLan ring, resulting in a truncated C-terminal with rings B-D intact.

Nisin, has shown promise for use in the medical field, however, its instability and low solubility at neutral pH may hamper its use. Nisin is more stable at lower pH with increasing pH also increasing instability (64). Haloduracin-α remains stable for 36 days with more than 90% intact peptide. The beta peptide is less stable with only slightly more than 30% intact peptide after 36 days (13). Up to day 12 the stability of AmyAα and AmyAβ are consistent with previous reports for HalA (13). Similar to previous reports, a decrease in HPLC peak area corrosponding to intact peptide could also be observed for nisin in this study (Fig. 8) (13). Nisin and AmyA stored for 12 days did not undergo drastic activity loss. Similarly nisin was previously found to only start significantly losing activity after 18 days, with HalA being active for longer (13). There was a sudden drop in the peak area of clausin on days 9 and 12. This drop was also observed in the antimicrobial activity. Clausin is an epidermin-like lantibiotic with sequence similarities to nisin. The instability of nisin at neutral pH is believed to be as a result of the unstable nature of Dha5 (64–66). Clausin also has a Dha at position 5 which may explain its instability at neutral pH. Due to limited amount of peptide stability could only be recorded for up to 12 days without replicates. Therefore, further investigation regarding the stability of AmyA is required.

In this study a novel two-component lantibiotic, amyloliquecidin, was characterized from a *Bacillus amyloliquefaciens* strain isolated from Fynbos soil. The operon putatively involved in the biosynthesis of amyloliquecidin was sequenced and found to have the necessary biosynthetic machinery to successfully produce and export the lantibiotic. Amyloliquecidin had similar properties to its other two-component counterparts, with anti-staphylococcal and -enterococcal activity, including activity against MRSA. The heat stability and pepsin degradation of AmyAβ provided interesting results, leading to the hypothesis of a possible truncated beta peptide that is sufficient for synergistic activity. This hypothesis does require further investigation. Furthermore, stability of amyloliquecidin was found to be comparable to that of haloduracin and superior to nisin and clausin. This study provides enough evidence for the continued investigation into the clinical application of amyloliquecidin, especially in the treatment or prevention of *S. aureus* and enterococci infections.

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Chapter 4
Evaluation of the *in vivo* Efficacy of Lantibiotics in the Treatment of *S. aureus*-induced Skin infections in Mice

Chapter Summary

The majority of skin and soft tissue infections (SSTIs) are caused by *Staphylococcus aureus.* Lantibiotics are known to be active against *S. aureus,* including antibiotic-resistant strains. Three lantibiotics namely, nisin, clausin and amyloliquecidin, were evaluated for their effectiveness in treating skin infections caused by *S. aureus* in a murine skin infection model. Lantibiotic treatments significantly reduced *S. aureus* Xen 36 bioluminescence and viable bacterial cell numbers. Lantibiotics were just as effective as the commercially available SSTI treatment, Bactroban (mupirocin). Furthermore, lantibiotics did not have a negative influence on wound closure rates of infected and non-infected wounds, compared to Bactroban- and control-treated wounds. These results further illustrate the potential of lantibiotics as treatment for skin infections caused by *S. aureus*.

Introduction

Skin acts as a protective barrier to the host and is in constant contact with the outside environment. Injury to this critical barrier provides an ideal opportunity for microbial invasion, resulting in infection. Skin and soft tissue infections (SSTIs) are the most common types of infection and have increased dramatically over the past few years, along with increased use of antibiotics for treatment of SSTIs (1, 2). Antimicrobial treatment options are becoming limited and alternatives to traditional antibiotics are needed. Bactroban, which is the recommended treatment for SSTIs caused by *S. aureus,* is ineffective in the treatment of some SSTIs caused by methicillin-resistant *S. aureus* (MRSA) (3, 4). Resistance has also emerged for vancomycin which is considered the "drug of last resort" for some severe MRSA infections (5, 6). Lantibiotics act by binding to bacterial cell wall precursors (i.e. lipid II), resulting in cessation of cell wall biosynthesis and cellular leakage by pore formation (7, 8). Certain lantibiotics have activity against human pathogens, including antibiotic-resistant strains (9–12). Studies have reported on the effectiveness of lantibiotics in the treatment/prevention of infections caused by *S. aureus in vivo* (12–16). Lantibiotics can thus serve as feasible alternatives to antibiotics for the treatment of SSTIs, specifically those caused by *S. aureus.*

Sufficient wound healing is important for successful recovery from infection. However, this is a complex process that requires the completion of highly programmed phases (17–19). These phases are regulated by the immune system, which can be negatively influenced by a variety of factors including stress, diabetes, obesity and nutrition (18). In addition to direct

antimicrobial treatment, antimicrobial peptides also play an important role in modulation of the host immune system (18, 20–23). A group of cationic antimicrobial peptides with immune modulating properties is penol soluble modulins (PSMs). Staphylococci found on the skin, as commensals or pathogens, are able to secrete several compounds that are capable of effecting the innate immune system, including PSMs (18, 22, 23). Secreted PSMs can either play a role in immune defence or pathogenesis, as demonstrated by the ability of pathogenic *S. aureus* PSMs to lure neutrophils and then induce lysis of the infiltrating neutrophils by a subsequently secreting a higher dose of PSMs (22). On the other hand, PSMs from commensal *S. epidermidis* do not induce lysis, but enhance neutrophil bacterial-killing capabilities (21). Lantibiotics are also able to modulate the innate immune system. Nisin has immunomodulatory properties similar to natural host defence peptides, and it is speculated that this trait is shared by all lantibiotics (20). Nisin is able to induce a potent enough immune response to protect against Gram-negative bacterial infections, against which it does not have direct antimicrobial activity (20). Nisin eluting nanofibers also accelerates wound healing (13). It is, therefore, hypothesized, based on preliminary data that lantibiotics may also aid in the wound healing process (13).

Bioluminescent bacteria are used to monitor the real-time progression of infection *in vivo* (3, 13, 14, 24). Bacteria need to express all the genes in the *luxABCDE* operon in order to emit a bioluminescent signal (25). Luciferase is encoded by *luxAB* and catalyses the oxidation reaction of its aldehyde substrate (encoded by *luxCDE*) and $FMNH_2$ to generate a bioluminescent signal (25, 26). This oxidation reaction requires adenosine-5`-tri-phosphate and oxygen. Therefore efficient bioluminescence is only observed in metabolically active cells (27). Bioluminescent bacterial strains are monitored using an *in vivo* imaging system (IVIS) that detects the bioluminescence. The IVIS thus provides a non-invasive way of monitoring the progression of infection and reduces the amount of animals needed to make significant conclusions (3, 13, 14, 24)

The current study aimed to investigate the effectiveness of lantibiotics in the treatment of full thickness wound infections caused by *S. aureus* and also compare them to a commercial anti-bacterial ointment (Bactroban). Progression of infection was monitored using the IVIS and the effect on wound closure rates was also investigated for infected and non-infected wounds.

108

Materials and Methods

Materials

All bacterial growth media were from Biolab Diagnostics (South Africa), unless otherwise stated. Poly vinyl alcohol (PVA, 87-89% hydrolysed, Mw 146000-186000), trifluoroacetic acid (TFA) and XAD-16 beads were from Sigma-Aldrich (Germany). Acetonitrile and isopropanol used for peptide purification were from Merck-Millipore (USA). SepPak C18 columns were from Waters (USA). Nisaplin was from Danisco (Denmark). The bicinchoninic acid (BCA) protein assay was from Pierce Biotechnology (USA). Gauze and micropore type from AlphaPharm (South Africa). Punch biopsies were supplied by Stellenbosch Medical Supplies (South Africa). Isoflurane was from Safe Saline Pharmaceuticals (Isofor; South Africa). Buprenorphine was from Scheiring-Plough Ltd. (Tamgesic; South Africa).

Production and Preparation of Lantibiotics for Use in Mice

Lantibiotics (amyloliquecidin (AmyA), nisin and clausin) were produced as described previously (chapter 3). Briefly, *Bacillus clausii* AD1 was cultured in Mueller Hinton (Sigma-Aldrich) broth for 8-10 days at 30˚C. The cell-free supernatant was obtained by centrifugation ($10,000 \times g$, 25 min at 4˚C) and combined with activated XAD-16 beads. Clausin was eluted from XAD-16 beads as previously described. *Bacillus amyloliquefaciens* AD2 cultured in Luria broth was mixed with sterile activated XAD-16 beads. This suspension was spread onto brain heart infusion (BHI) agar (2% wt/vol agar) and incubated at 37˚C for 96 h. Beads were removed from agar using double distilled water. Elution of AmyA from XAD-16 beads were performed as previously described. Clausin and AmyA XAD-16 purified crude extracts were loaded onto SepPak C18 columns and eluted using an acetonitrile gradient as previously described. Active fractions were frozen at -80˚C, freeze-dried and stored at -20˚C. Nisin was obtained from the commercial nisin preparation, nisaplin. Nisaplin was dissolved in 25% acetonitrile containing TFA (vol/vol/vol) and loaded onto a SepPak C18 column. Nisin was eluted using an acetonitrile gradient as previously described. Active fractions were frozen at -80˚, freeze-dried and stored at -20˚C. Peptides were further purified to homogeneity using high-performance liquid chromatography (HPLC) as previously described. Lantibiotic purity was confirmed by analytical HPLC and electrospray-ionization mass spectrometry. Activity was

confirmed by agar-well diffusion assays as previously described, using *S. aureus* Xen 36 as indicator strain.

Purified lantibiotics were freeze-dried, reconstituted in phosphate buffered saline (PBS, pH 7.4) and peptide concentrations were determined using the BCA protein assay as per manufacturer's instructions. Lantibiotics were prepared to a final concentration of 250 μM in PBS (pH7.4) containing 2.5% PVA (wt/vol) and stored at 4˚C throughout each respective trial. In the case of AmyA the alpha and beta peptides were combined in a 1:1 molar ratio. Lantibiotics were freshly prepared for each trial.

Animals Used

Approval to conduct research on animals was granted by the ethics committee of Stellenbosch University (SU-ACUM14-00009). Adult female nude mice (weighing 20-30g) were used for infection studies and housed in separate cages under controlled environmental conditions (12 hour light/dark cycles, 20-22˚C). Animals were fed sterile standard rodent feed and water. Wound closure was investigated in male nude mice housed under similar conditions. Wound infection studies were conducted in three independent trials and wound closure studies of non-infected wounds were conducted in two independent trials.

Full thickness Wound Generation and Infection with *S. aureus* Xen 36

A full thickness wound model similar to the one described by Heunis et al. (13) was used in this study. Mice were anesthetized with 1.5-2% (vol/vol) isoflurane in O_2, and full thickness excisional wounds were generated using a 6 mm biopsy punch on the dorsal surface of each mouse. Mice received buprenorphine (~0.03 mg/kg) subcutaneously as an analgesic, directly after wound generation and for the first 3 days post-wound generation. A single *S. aureus* Xen 36 colony was used to inoculate BHI broth supplemented with kanamycin (200 μg/ml) and was incubated overnight at 37˚C. The overnight culture was sub-inoculated into fresh media and grown to an OD_{600} of 1.0-1.2 (~2×10^8 CFU/ml). Cell counts were verified by serial dilution and plating onto BHI agar supplemented with kanamycin (200 μg/ml). Bacteria were harvested ($10,000 \times g$, 2 min) and washed twice with sterile PBS (pH 7.4) and resuspended in sterile PBS (pH 7.4) to the original OD_{600}. Wounds were infected with 10 μl *S. aureus* Xen 36 (~2×10^6 CFU/wound) directly after wound generation. Wounds were left for 5 min to dry after which

they were covered with parafilm and gauze. Dressings were kept in place by using micropore surgical tape.

Treatment of and Evaluation of *S. aureus* Xen 36 Wound Infections

Mice (n=9 in each group) were left for 3 h post-wound generation before treatment commenced. For treatment of wounds and bioluminescent imaging mice were anesthetized as previously described. Wounds were treated with 12.5 µl of a 250 µM lantibiotic solution. The lantibiotic solution was dispensed directly onto wounds using a pipette, and spread evenly over the wound surface. The same volume of Bactroban was also dispensed onto wounds. Control mice wounds were treated with 2.5% PVA in PBS (pH 7.4) (CPVA). Mice were left for 5 min after which bioluminescent images were recorded using an *in vivo* imaging system (IVIS 100; Caliper life sciences, Perkin-Elmer). The wounds were subsequently closed by covering with gauze which was held in place using micropore surgical tape. Infection trials were conducted for 7 days. Mice were treated on days 0, 2, 4 and 6, using the same procedure as described previously. Before bioluminescence was recorded wounds were wetted with 50 µl sterile PBS (pH7.4). Bioluminescent images were recorded daily under anaesthesia and images were analysed using the Living Image software (v3.0; Caliper life sciences, Perkin-Elmer). Bioluminescence was quantified by measuring the photons in a region of interest (ROI; 25 × 25 pixels), and was expressed as the $\log_{10}$ of photons per second per cm^2 per steradian ($ps^{-1}cm^{-2}sr^{-1}$). On day 7, mice were euthanized by pentabarbitone overdose (Euthapent, Kyron Laboratories). Wounds were subsequently excised and homogenised in sterile PBS (pH7.4). The homogenate was serially diluted in sterile saline and plated onto BHI agar supplemented with kanamycin (200 µg/ml). Plates were incubated for 24 h at 37˚C and colonies were enumerated to determine CFU/wound.

Digital images were taken of wounds (n=6 per group) to determine the effect of treatment on wound closure. Digital photographs were analysed using the software program ImageJ (version 1.48). Wound size on day n was expressed as a percentage relative to the wound size on day 0 ($D_N/D_0 \times 100$; where D_N is the wound size on day n, and D_0 is the wound size on day 0).

Effect of Lantibiotics on the Wound Closure of Non-Infected Wounds

Full thickness wounds were made as described previously and mice (n=5 per group) were treated with either CPVA, nisin, clausin, AmyA or Bactroban as previously described. Digital

111

images of wounds were taken, and wound closure was determined as previously described. Mice were monitored for 7 days after which they were euthanized by pentabarbitone overdose.

Statistical Analysis

All data were analysed using GraphPad Prism (version 6.05) and statistical differences between groups were determined using two-way analysis of variance (ANOVA) and unpaired t-test. Statistical analysis used are indicated for each data set. A statistical difference was considered when $p < 0.05$. Error is calculated as standard error of mean (SEM).

Results

Efficacy of Lantibiotics in the Treatment of *S. aureus* Xen 36 Wound Infections

All antimicrobial treatments reduced the bioluminescence signal emitted from *S. aureus* Xen 36 throughout the study period and served as an indication of a reduction in bacterial load (Fig. 1 and 2). On day 0 Bactroban and nisin treatments significantly reduced bioluminescence compared to wounds treated with the PVA control (CPVA), clausin and AmyA. From day 1 onwards all treatments reduced bioluminescence significantly compared to CPVA-treated wounds. Bactroban-treated wounds had significantly lower bioluminescence on days 1 and 4, compared to the lantibiotic treatments (Fig. 2). On days 2, 3 and 7, Bactroban-treated wounds only had significantly lower bioluminescence compared to clausin-treated wounds (Fig. 2). Stable bioluminescence was observed for CPVA-treated wounds up to day 6, with a slight decline on day 7.

All of the antimicrobial treatments significantly reduced the viable bacterial cells that could be enumerated from excised wounds, compared to CPVA-treated wounds, with an average 3.1 ($\pm$ 0.2) log drop (Fig. 3). Clausin-, AmyA-, Bactroban- and nisin-treated wounds had individual log drops of 3.03, 3.33, 3.44 and 2.56, respectively, compared to CPVA-treated wounds. There were no significant differences in CFU/wound between wounds treated with lantibiotics or Bactroban. Highest cell counts were observed with CPVA-treated wounds with 5.1×10^7 CFU/wound and a mean of 1.44×10^7 CFU/wound. Clausin and Bactroban treatment resulted in two wounds from which <20 CFU/wound could be detected, respectively. Nisin treatment resulted in one wound from which <10 CFU/wound could be detected. Less than 20 CFU/wound could be detected from three wounds treated with AmyA. Highest cell counts for antimicrobial treatments were recorded for nisin- and clausin-treated wounds, with 6.6×10^5 (mean 1.58×10^5) and 7×10^5 (mean 1.57×10^5) CFU/wound, respectively.

Wound closure rates of infected wounds were minimally affected by all treatments, with a gradual decrease in wound size over the 7 day study period. There were, however, some differences recorded on selected days (Fig. 4). There were statistical differences between Bactroban-treated wounds (107.9 $\pm$4.13%) and that of CPVA- (94.6 $\pm$4.84%) and AmyA-treated wounds (96 $\pm$2.14%) on day 1. Bactroban-treated wounds were also significantly
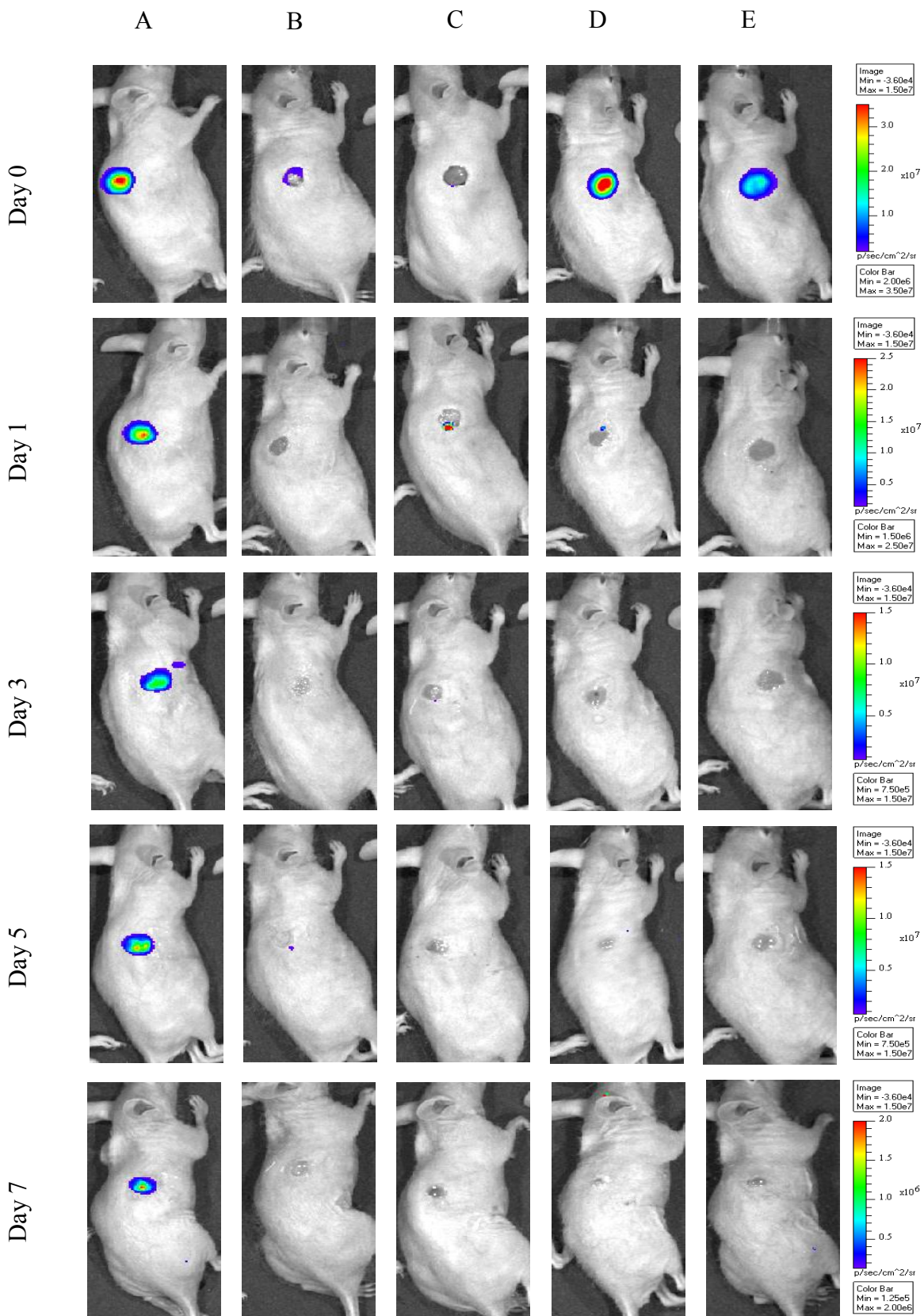
FIG 1 Images to track bioluminescence, used as indication of bacterial load, were taken over a 7 day period using the IVIS and are represented on a colour scale overlaid on top of a greyscale image. **A:** CPVA-; **B:** Bactroban-; **C:** Nisin-; **D:** Clausin- and **E:** AmyA-treated groups. Scale bar represents photons (ps^{-1}cm^{-2}sr^{-1}) for each day.

larger compared to clausin-treated wounds on day 5 (69.6 ±8.00% and 52.7 ±2.69%, respectively). Significant differences were observed between the CPVA-treated wounds (45.2 ±4.59%) and that of clausin- (33.3 ± 1.59%) and AmyA-treated wounds (30.7 ±0.71%) on day 7. Bactroban-treated wounds (47.3 ±6.93) were found to be significantly larger compared to
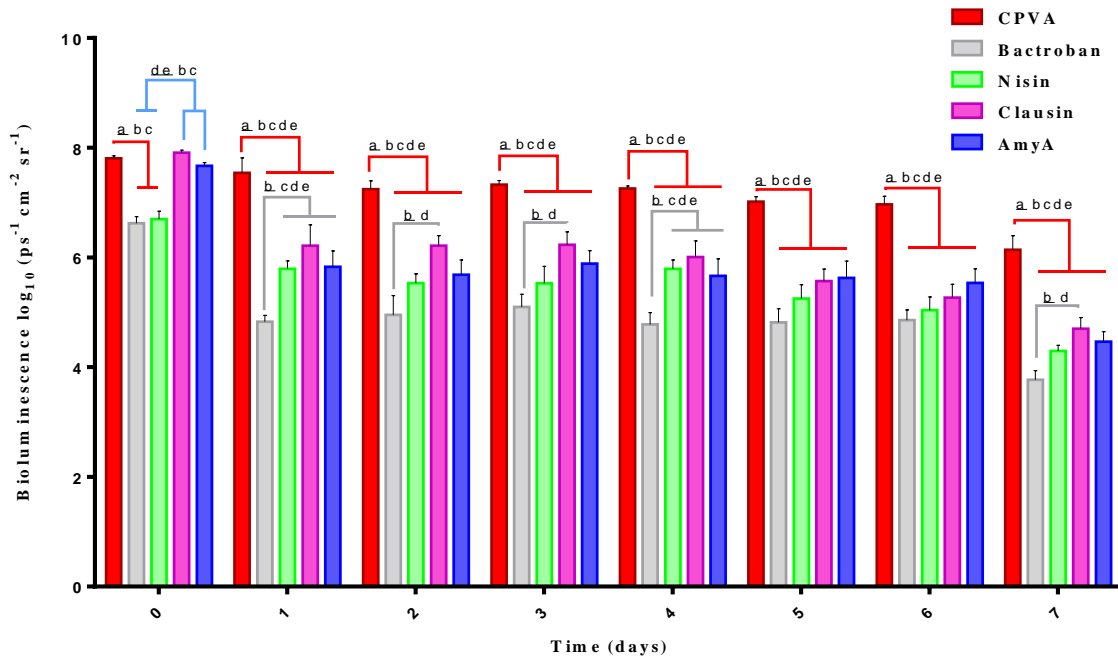
FIG 2 Efficacy of treatments were determined by monitoring bioluminescence of wounds infected with *S. aureus* Xen 36 over a 7 day period. Letters and lines above bars indicate which groups have statistical differences with each other. Statistical analysis was determined by two-way ANOVA.
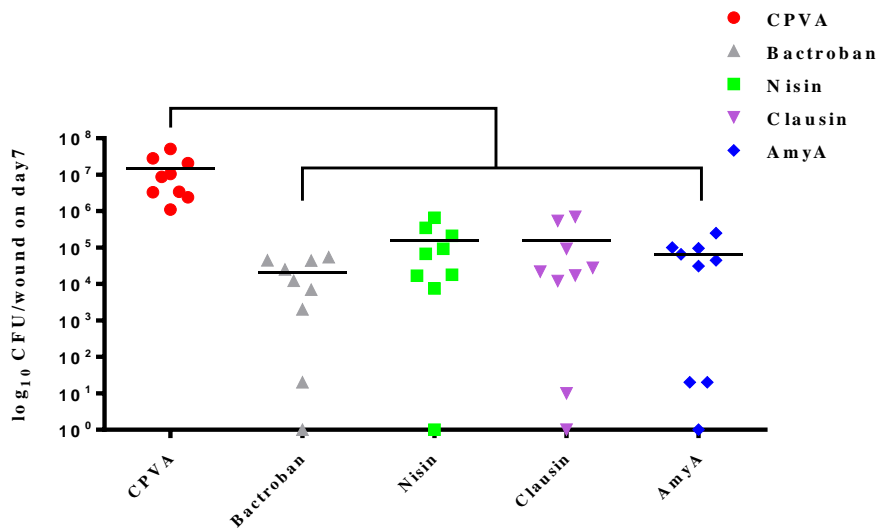
FIG 3 Efficacy of treatments to reduce viable *S. aureus* Xen 36 cells was determined by plating homogonized excised wounds onto BHI agar and determining CFU/wound. CFU/wound is represented on a log_{10} scale. Lines through and above data points represents the mean and statistical differences between groups, respectively. Statistical analysis was conducted using unpaired *t*-test.
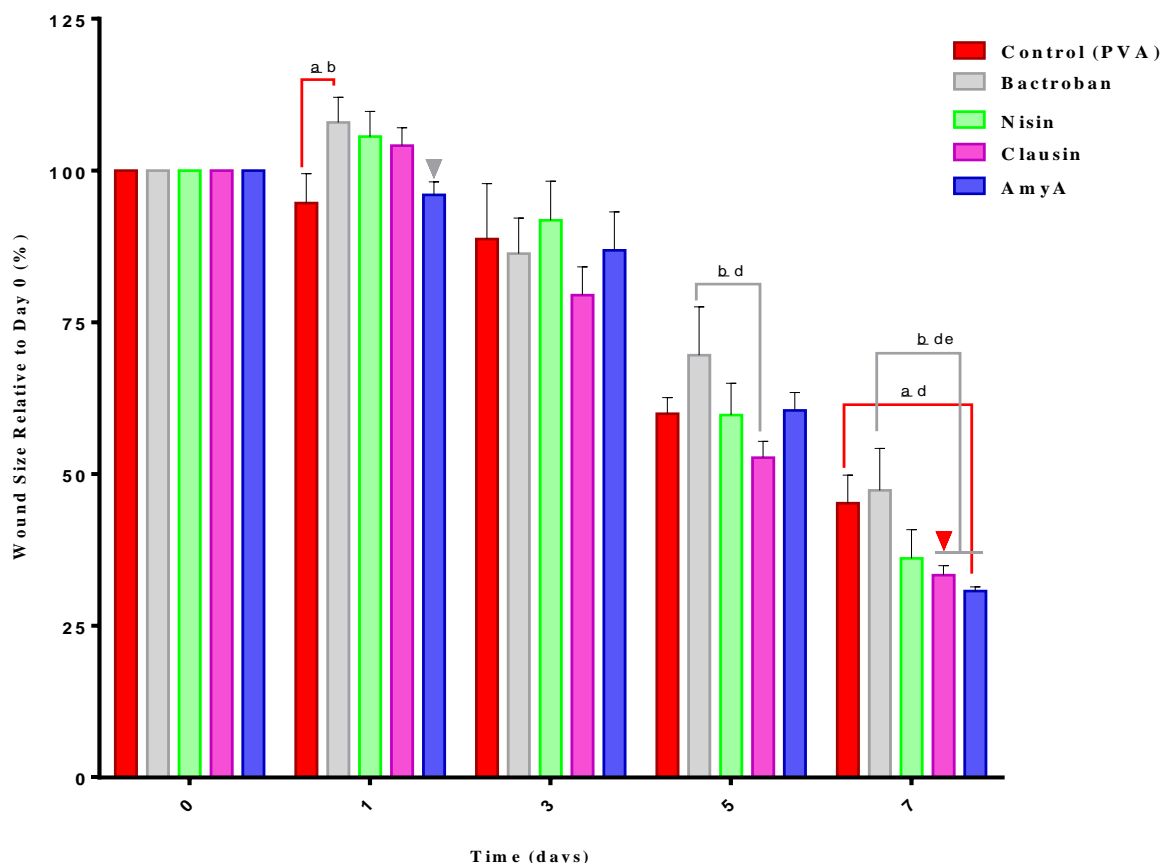
FIG 4 The wound sizes of wounds infected with *S. aureus* Xen 36 were measured for 7 days. Wound sizes on day *n* are expressed as a percentage relative to the wound size on day 0. Letters and lines above bars indicate which groups have statistical differences as determined by two-way ANOVA. The grey arrow indicates statistical difference between bactroban and AmyA-treated wounds, determined by unpaired *t*-test. The red arrow indicates statistical difference between CPVA- and clausin-treated wounds, determined by unpaired *t*-test.

clausin- and AmyA-treated wounds on day 7 (30.7 ±0.71% and 33.3 ± 1.59%, respectively). Although not significant, nisin-treated (36.1% ±4.74) wounds were smaller compared to CPVA- (45.2% ±4.59%) and Bactroban-treated (47.3% ±6.93%) wounds on day 7.

Effect of Lantibiotic Treatment on Wound Closure of Non-Infected Wounds

Wound size steadily decreased over the 7 day study period, with Bactroban-, AmyA- and nisin-treated wounds initially slightly increasing in size (Fig. 5). On day 7 Bactroban-treated wounds (64.3 ±3.04%) were significantly larger than nisin- (44.8 ±3.01%), clausin- (39.9 ±2.20%), AmyA- (44.5 ±4.78%) and CPVA-treated wounds (51.3 ±3.53%). Clausin-treated wounds (39.9 ±2.20%) were significantly smaller than CPVA-treated wounds (51.3 ±3.53%) on day 7. Although not significant nisin- (44.8% ±3.01%) and AmyA-treated wounds (44.5 ±4.78%) were smaller that CPVA treated wounds on day 7 (51.3 ±3.53%).
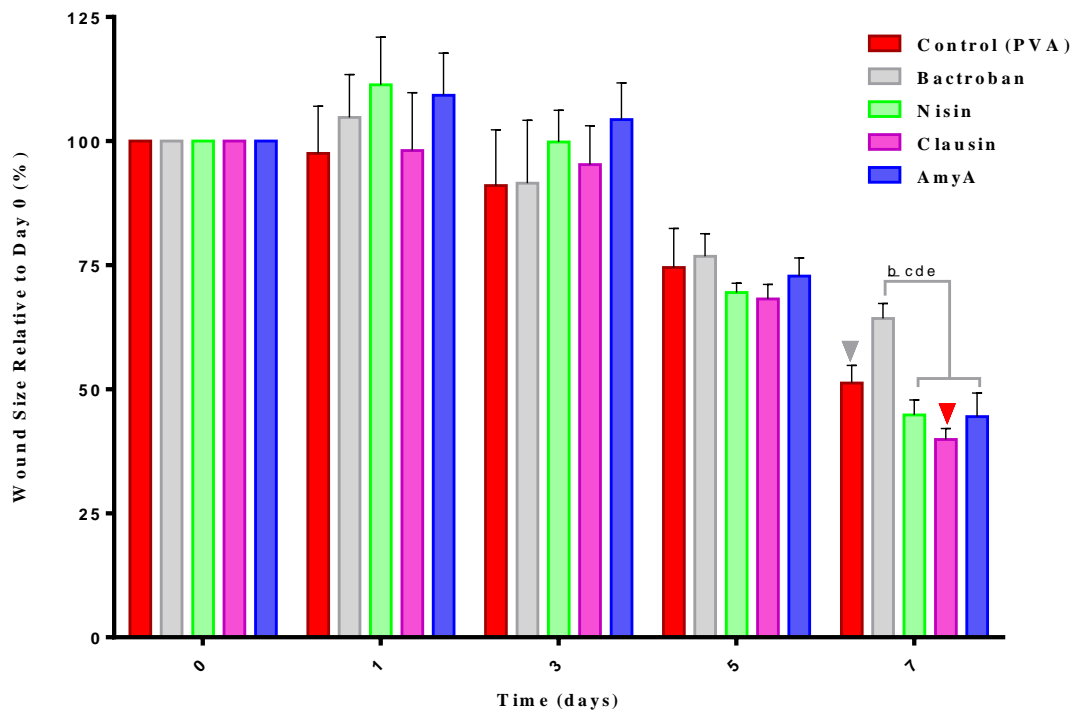
116

FIG 5 The wound sizes of non-infected wounds were measured for 7 days. Wound sizes on day *n* are expressed as a percentage relative to the wound size on day 0. Letters and lines above bars indicate which groups have statistical differences as determined by two-way ANOVA. The grey arrow indicates statistical difference between Bactroban- and CPVA-treated wounds, determined by unpaired *t*-test. The red arrow indicates statistical difference between CPVA- and clausin-treated wounds, determined by unpaired *t*-test.

Discussion

Skin is the largest organ in the human body and protects the host from the outside environment, as well as from infections (28). The microbial community residing on the skin is in symbiosis with the host. However, the same commensal bacteria, along with other non-commensal bacteria, can cause severe skin infections (28). Disruption of the skin can result in dysbiosis and microbial invasion which in turn can lead to infection and delays in wound healing. Therefore, proper treatment and prevention of skin infections is important not only for resolution of infection but also to aid in the healing process. In this study three lantibiotics were evaluated for their effectiveness in the treatment of skin infections caused by *S. aureus*. Their effect on wound closure rates of infected and non-infected wounds was also evaluated.

The mixture of lantibiotics with 2.5% PVA in PBS (pH 7.4) created a more viscous solution that could easily be applied to wounds. Delivery vehicles composed of PVA have been used as for several pharmaceutical drugs as well as in topical formulations (29). Nisin has also been actively released from PDLLA (poly D, L-lactide) and PVA nanofibers were used to control infections caused by *S. aureus* (30).

Bioluminescence has been used in several studies to monitor *in vivo* infections in real-time (3, 13, 14, 24). *S. aureus* Xen 36 bioluminescence was stable throughout the study, although bioluminescence is known to decreases over time. This decrease is most likely due to a decrease in the metabolic activity of the bacteria or loss of the *luxABCDE* operon if carried on a plasmid (3). Bioluminescent *S. aureus* has been successfully used in skin infection models with stable bioluminescence occurring for up to 7 days (3, 13). Four treatments, on days 0, 2, 4 and 6, with the respective antimicrobials were sufficient to control *S. aureus* spread, as determined by bioluminescence (Fig. 1 and 2). Nisin and Bactroban treatment had an immediate effect (within 5 minutes) on bacterial bioluminescence (Fig. 2). Similar results were reported for nisin-eluting nanofibers (NEF), with bioluminescence reported to be significantly less on day zero (13). Nisin and the active ingredient in Bactroban, mupirocin, have two different modes of action. Nisin inhibits cell wall biosynthesis by binding to lipid II thereby disrupting peptidoglycan synthesis (31). Once bound to lipid II it is also able to form pores resulting in cellular leakage (31). Mupirocin inhibits the isoleucyl-tRNA synthetase and has bacteriostatic and bactericidal activity (32–34). Nisin results in rapid cell death while mupirocin has initial bacteriostatic effects followed by bactericidal effects over 24 hours (14, 32, 35). The rapid drop in bioluminescence observed with nisin could be due to the rapid bacteriolytic effect of nisin. The

118

bioluminescent signal emitted by *S. aureus* Xen 36 requires the bacteria to be metabolically active. The decrease observed with mupirocin treatment could, therefore, be due to bacteriostatic effects resulting in *S. aureus* Xen 36 not being able to efficiently produce bioluminescent signal. Mupirocin-resistant *S. aureus* do not have this initial drop in bioluminescence (3). Unlike Bactroban and nisin treatment, clausin and AmyA treatment did not show an initial drop in bioluminescence (Fig. 2). Clausin is an epidermin-like lantibiotic, which are relatively short compared to the nisin-like lantibiotics. Although both epidermin- and nisin-like lantibiotics bind to lipid II, pore formation by epidermin-like lantibiotics is affected by membrane thickness (35). Nisin also results in more rapid leakage than epidermin and gallidermin (epidermin-like lantibiotic) in sensitive strains (35). These observations may explain the delayed reduction in bioluminescence observed in clausin-treated groups. Two-component lantibiotics require two peptides to act synergistically to induce cellular leakage. Pre-incubation of sensitive cells with Haloduracin-α (lipid II binding) followed by Haloduracin-β (responsible for pore formation) results in a more rapid efflux of potassium, compared to simultaneous addition of the two peptides (10). In an *in vivo* situation it can be expected that this reaction may be delayed and could, therefore, account for AmyA not being able to rapidly reduced bioluminescence. Bioluminescence for all the antimicrobial treatments remained significantly less than that of the control (CPVA-treated), with Bactroban having the lowest bioluminescence. This is expected, as Bactroban is a commercially available treatment specifically recommended for SSTIs caused by *S. aureus* (4). However, the lantibiotics compared well with Bactroban, still being able to significantly reduce bioluminescence compared to CPVA-treated wounds (Fig. 1 and 2). Successful treatment of skin and subcutaneous infections caused by *S. aureus* Xen 36 have been reported for NEF as well as nisin-loaded bone cement, respectively (13, 14). In these studies bioluminescence also declined immediately after treatment. Nisin was, however, ineffective in the treatment of *S. aureus* subcutaneous and peritoneal cavity infections (36, 37). The nisin used in these studies were crude samples which could have resulted in inefficient local concentrations of peptide. In the current study lantibiotics were purified to homogeneity and could be used at known concentrations. The results in the current study, therefore, illustrate the importance of using purified antimicrobials in order to accurately determine antimicrobial activity.

Although there is a correlation between bioluminescence and bacterial burden it is important to verify that low bioluminescence is associated with a reduction in viable bacterial cells (3). Bactroban and lantibiotic treatment resulted in decreased viable cells on excised wounds,

confirming that reduced bioluminescence was associated with a reduction in viable bacteria (Fig. 3). Bactroban and lantibiotic treatments performed equally well with regards to reduction in viable bacteria, and are consistent with other reports for treatments of skin infections caused by *S. aureus* (13, 38–40). It is interesting to note that although AmyA has higher minimum inhibitory concetrations against *S. aureus in vitro,* compared to clausin and nisin, it is just as effective as the other lantibiotics *in vivo.* Increasing the contact time with the lantibiotics could increase their effectiveness. This was observed with NEF, which were able to reduce *S. aureus* to 4.3×10^2 CFU/wound compared to the control with 2.2×10^7 CFU/wound (13). The NEF are in constant contact with the wound releasing active peptide over time. In addition to contact time, the formulation used to deliver the lantibiotics can also play a role in their effectiveness. Mupirocin treatment of *S. aureus* and *Streptococcus pyogenes* was affected by the formulation used. A 2% mupirocin oil-water emulsion is more effective in reducing bacterial counts than a 2% mupirocin poly ethylene glycol formulation (38). The importance of drug delivery vehicles on bacterial burden is also reported for ointment formulations of mupirocin and retapamulin (3).

Decreasing the spread of infection should be accompanied by efficient wound healing. Topical treatments satisfying both these requirements would have significant clinical relevance (3). Wound closure rates of infected wounds were similar for all treatments including the control. The only differences were on days 1, 5 and 7 (Fig.4). Subcutaneous treatment with vancomycin, daptomycin and linezolid resulted in smaller lesions on day 7, and in the case of topical treatment, on days 5 and 7 (24). Retapamulin is also effective in wound closure when treating MRSA infections, with significantly smaller lesions occurring from day 1, compared to Bactroban and controls (3). From the results reported in this study it is evident that lantibiotics, in addition to controlling infection, do not negatively influence wound healing. Although Bactroban is able to treat infection it delays wound closure in infected and non-infected wounds, compared to lantibiotic- and CPVA-treated wounds. Similar delayed closure rates following mupirocin treatments have been reported in other comparative studies (41, 42). Non-infected and infected wounds treated with CPVA had similar closure rates, suggesting that in the current model infection does not dramatically influence wound closure (Fig. 4 and 5). Similar results were reported by Kim et al. (43) while studying the dynamics of neutrophil infiltration during wound healing. Mice were either intraperitoneally injected with granulocyte macrophage-colony stimulating factor (GM-CSF) /saline (wounds uninfected) or wounds were infected with *S. aureus*. Wound closure rates were similar for all groups, despite infection with

120

S. aureus and treatment with GM-CSF resulting in increased neutrophil recruitment, compared to the saline injected controls (43). Lantibiotic treatment of infected and non-infected wounds does, however, appear to aid in wound closure (Fig. 4 and 5). Similarly, when non-infected wounds were treated with either NEF or control nanofibers wound closure rates were significantly increased, compared to a gauze-covered (untreated) control wounds. There were, however, no differences in wound closure rates between the control- and NEF-treated wounds (13). Additionally, NEF treated wounds had signs of connective tissue formation, re-epithelialisation, keratinization and no neutrophil infiltration after 7 days (13). Additionally, lantibiotics also affect chemokines involved in the wound healing process. Gallidermin and nisin affect human peripheral blood mononuclear cells, with nisin causing stronger induction of interleukin-8 (IL-8), growth related oncogene-α (Gro-α) and monocyte chemoattractant protein-1 (MCP1; 20). Amongst other roles, IL-8 mediates neutrophil recruitment and is expressed by macrophages and neutrophils, and Gro-α stimulates the movement of neutrophils. MCP-1 participates in different stages of mast cell, monocyte and lymphocyte attraction during wound healing, and can also contribute to epithelial cell movement during angiogenesis (19). Lantibiotics can, therefore, indirectly affect wound healing by stimulating these immune cells. Although there was a significant difference between clausin- and CPVA-treated non-infected wounds, there was no difference between clausin and the other lantibiotic treatments. This suggests that their possible differences in immune modulation do not affect their wound healing capabilities.

There is a high incidence of MRSA associated infections, with emergence of other resistant bacteria also on the increase (44, 45). Furthermore, the ineffectiveness of recommended treatments such as Bactroban against these strains warrants the development of new treatments using alternative antimicrobials (3, 4). Lantibiotics are known for their activity against antibiotic-resistant bacteria, including those involved in skin infections (9, 11, 12, 16, 46). Additionally, using delivery systems such as nanofibers, it is possible to provide a solution to solubility concerns by controlling the release of lantibiotics (13). Therefore, lantibiotics have great potential as topical treatments for infections caused by *S. aureus,* with several lantibiotic treatments already under development for the treatment of infections by Gram-positive bacteria (Novacta Biosystems and Oragenics).

In conclusion, the current study comparatively evaluated the efficacy of three lantibiotics to treat skin infections caused by *S. aureus* using a full-thickness wound model in mice. Lantibiotics are just as effective as Bactroban in reducing bacterial load, as monitored by

121

bioluminescence. A reduction in viable cells from excised wounds confirmed this. Despite having a higher minimum inhibitory concentration *in vitro* the two-component lantibiotic, AmyA, is just as effective as the other lantibiotics *in vivo*. All the tested lantibiotics are superior to Bactroban with respect to wound closure. This is the first comparative *in vivo* study investigating the efficacy of a two-component lantibiotic in the treatment of skin infections. Additionally, it is the first *in vivo* study testing the newly isolated AmyA. The anti-bacterial and wound healing properties of the lantibiotics used in this study further illustrate the potential of their use in treatment of skin infections.

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Chapter 5
General Discussion and Conclusions

General Discussion

Antibiotic resistance is currently one of the most important problems faced by the medical industry (1–8). The wide spread use of antibiotics in the medical and agricultural industries further exacerbates this problem (3, 9). Bacteria can become resistant to antibiotics in a number of ways, including horizontal gene transfer of resistance elements (4). Horizontal gene transfer of antibiotic resistance elements have not only been reported between species but also across genera (10). In contrast to the antibiotic resistance crisis, discovery and development of new antibiotics have decreased over the past few decades (4, 11). Investigation into novel antimicrobials effective against drug-resistant pathogens is therefore essential.

Lantibiotics have long been seen as alternatives to traditional antibiotics and have been isolated from bacteria originating from diverse backgrounds including soil, marine life, food products and human pathogens (12–18). In this study the Fynbos in the Western Cape was chosen as a sampling site to screen for possible lantibiotic producing bacteria. The Fynbos niche was chosen due the diversity of soil bacteria, especially in the rhizosphere of plants. (19, 20). It is an unexploited niche and information regarding antimicrobial producing bacteria is scant. It was predicted that high bacterial diversity would increase the probability of isolating novel antimicrobial producing bacteria. Two antimicrobial producing bacteria, namely *Bacillus clausii* AD1 and *Bacillus amyloliquefaciens* AD2 were isolated from Fynbos soil in this study.

Bacillus amyloliquefaciens AD2 was identified using its *gyraseA* sequence, coding for the DNA gyrase subunit A (21). The *gyraseA* sequence from *B. amyloliquefaciens* AD2 has 92% nucleotide- and 99% amino acid-identity to other *B. amyloliquefaciens* strains, and was characterized as a novel strain. Some lantibiotics, such as streptin and haloduracin, are known to only be produced on rich solid media (13, 15). Similar observations were made for *B. amyloliquefaciens* AD2. Antimicrobial activity could only be observed from the compound produced by *B. amyloliquefaciens* AD2 after purified peak fractions from HPLC were combined. The molecular masses of these peaks did not correspond to any of the known masses of the two-component lantibiotics, indicating that a novel two-component lantibiotic had been isolated. There are two lantibiotics currently known to be produced by *B. amyloliquefaciens*. The most well know is the class II lantibiotic mersacidin, which was only recently shown to be produced by this strain (22). The other is the recently characterized class II mersacidin-like lantibiotic, amylolysin, from *B. amyloliquefaciens* GA1 (23). In this study, a full lantibiotic operon was identified in *B. amyloliquefaciens* AD2 after sequencing the genome, and the

lantibiotic was designated amyloliquecidin (AmyA). Annotation of the operon revealed that, in addition to the two lantibiotic peptide-encoding genes, all the biosynthetic machinery to produce AmyA was present. The genes required for two-component lantibiotic biosynthesis, export and immunity are known to cluster together, consistent with the observations made for *B. amyloliquefaciens* AD2 (15, 24–26). Thus far, the only other two-component lantibiotics characterized from *Bacillus* spp. are lichenicidin (LicA) and haloduracin (HalA). The structural peptides of AmyA were found to be similar to that of LicA and HalA. The Ser, Thr and Cys of AmyAα are in similar positions as that in HalAα. The beta peptide of AmyA has high similarity to both LicAβ and HalAβ. These similarities suggest that the peptides of AmyA have similar ring topologies to the peptides of LicA and HalA.

Bacillus clausii AD1 could be identified using its *16s rDNA* sequence, and was designated *B. clausii* AD1. Clausin, produced by *B. clausii*, is known to only be produced after prolonged incubation in liquid media, and coincides with sporulation (27). Antimicrobial activity in the supernatant of *B. clausii* AD1 could also only be observed after prolonged incubation of the culture. The antimicrobial compound isolated from *B. clausii* AD1 has a molecular mass similar to clausin. The biosynthetic genes associated with lantibiotic production usually cluster together in the same operon (26). The biosynthetic enzyme, LanD, is involved in the oxidative decarboxylation of the C-terminal in epidermin-like lantibiotics (28). Therefore, although the *clausin* structural gene was not elucidated, the presence of *clausD*, along with the similar molecular mass, was sufficient evidence to assume that the peptide produced was similar to, or a variant of, the epidermin-like lantibiotic, clausin (29).

Two-component lantibiotics have activity against a wide range of Gram-positive bacteria including *S. aureus* (30–32). The alpha peptides are classified as class II mersacidin-like lantibiotics and have the mersacidin lipid II-binding motif ($\underline{C}$-$\underline{T}$-L$^{/x}$-$\underline{T}^{/S}$-x-E-$\underline{C}$) (33–35). Two-component lantibiotics act synergistically, with the alpha peptide binding to lipid II to form an alpha peptide-lipid II complex (30, 35). This is followed by binding of the beta peptide to the alpha peptide-lipid II complex resulting in subsequent pore formation (30, 36). The alpha peptide of AmyA also has the mersacidin like-lipid II binding motif, and when combined with the beta has activity against a several Gram-positive bacteria. Activity was observed against clinical isolates of *S. aureus*, enterococci, and methicillin-resistant *S. aureus* (MRSA). Lacticin 3147 is known to be more active against enterococci compared to its activity against *S. aureus* (37). This is consistent with the higher minimum inhibitory concentrations reported for AmyA against *S. aureus*.

129

After incubation with pepsin AmyAβ combined with undigested AmyAα still results in activity. There is, however, a pepsin cleavage site in AmyAβ just before the B-ring and cleavage at this site would result in a C-terminal truncated peptide. The C-terminal rings in beta peptides of two-component lantibiotics have been proposed to be important for interaction with the alpha peptide-lipid II complex (38). In lacticin 3147-β, HalAβ, LicAβ destruction of any of the C-terminal rings results in loss of activity (38–40). However, none of the Ala substitutions in the N-terminal region of lacticin 3147β and LicAβ results in activity loss (38, 40). Destruction of ring A in HalAβ also does not result in activity loss (39). Gardiner et al. (41) reported on a C-terminal truncated lacticin 3147-β after digestion with α-chymotrypsin, with lacticin 3147-α being more susceptible to cleavage. Digestion did not result in complete loss of activity, but a significant reduction was observed. It should be noted that the activity assay was performed by combining peptides that were both digested with α-chymotrypsin. These results suggest that it may be possible for a truncated C-terminal beta peptide to still interact with the alpha peptide-lipid II complex and result in activity. The activity would most likely be as a result of disruption in cell wall biosynthesis as appose to pore formation. Under physiological storage conditions AmyA remained remarkably stable, particularly AmyAα, which is consistent with results for haloduracin (30). The stability of the alpha peptides is proposed to be due to their globular nature and low number of residues that can undergo oxidation (30). The beta peptide is more susceptible to oxidation, similar to nisin, and this is proposed to be due to the high number of thioether bridges (30).

The *in vitro* characterization of AmyA as well as the identifaction and annotation of its operon contributes to the knowledgebase of lantibiotic research. However, from the results in the *in vitro* section of the current study it is evident that there are several areas where future research should focus on:

1. The AmyA operon should be functionally characterized, in order to confirm the putative annotations of genes. Heterologous expression systems have proven useful in assigning function to lantibiotic biosynthetic genes (40, 42–45).

2. The dehydration of Ser and Thr residues must be confirmed in the alpha and beta peptides. Methods have been developed that use mass spectrometry based analysis to elucidate which Ser and Thr are dehydrated and can also be used to determine ring topology (46–48).

3. The hypothesis that a truncated beta peptide could still act synergistically with the alpha peptide, to exert antimicrobial activity, must also be investigated. This can be achieved

by proteolytic cleavage or heterologous expression systems to produce C-terminal truncated beta peptides (49, 50).

In vitro activity of clausin and AmyA against *S. aureus* suggests possible application in the treatment of *S.* aureus-induced skin infections. The lantibiotic treatments compared well with Bactroban in preventing the spread of *S. aureus,* as determined by bioluminescence. Lantibiotics and Bactroban were equally effective in reducing viable bacterial cells, isolated from excised wounds, compared to control polyvinyl alcohol-treated wounds (CPVA; lantibiotic carrier vehicle). Treatment of *S. aureus* wound infections using topical lantibiotic treatment has been reported with nisin nanofibers (NEF) (51). Treatment with NEF resulted in a 5 log drop in viable bacterial cells compared to the control nanofiber group. In the current study the best lantibiotic treatment only resulted in a 3.3 log drop (AmyA). It could be that the NEF provides prolonged contact time between the area of infection and the lantibiotic. These results illustrate how a delivery system could improve effectiveness. Subcutaneous treatment of wounds with lantibiotics has, however, proven to be ineffective (52). However, these results could be due to insufficient peptide concentrations (52). Other studies have reported on the successful treatment of infections caused by of *S. aureus*, where lantibiotic administration routes includes intranasal, subcutaneous, intraperitoneal and intravenous (53–58). However, none of these studies, with the exception of NEF, evaluated the topical efficacy of lantibiotics.

Lantibiotics did not negatively influence wound healing when used to treat infected and non-infected wounds. Delayed wound closure rates were, however, observed for Bactroban-treated wounds. In previous studies Bactroban also resulted in delayed wound closure (59, 60). Non-infected and infected wounds treated with CPVA had similar closure rates. These results suggests that in the current model infection does not significantly influence wound closure rates. These observations are consistent with reports of wounds in mice infected with *S. aureus* or left uninfected (with intraperitoneal granulocyte macrophage colony-stimulating factor/saline injections) having similar closure rates (61). Lantibiotic treatment of non-infected wounds does, however, result in smaller wounds after 7 days, compared to CPVA- and Bactroban-treated wounds. It is possible that the increased wound closure rates reported for lantibiotics can be due to stimulation of innate immune cells. Lantibiotics and other peptides are known to stimulate the immune system (62–65). The cationic peptide IDR-1018 (innate defense regulator), increases wound healing in a porcine *S. aureus* wound model, independent of antimicrobial activity (66). When treated with the pheromone plantaricin A, keratinocytes show increased migration and proliferation as well as differential gene expression of several

growth factors. Furthermore, plantaricin A also induces interleukin-8 expression, which is known to be involved in wound healing (67, 68).

The *in vivo* evaluation of lantibiotics as a topical treatment for infections caused by Gram-positive bacteria have not been extensively investigated. The current study, therefore, adds valuable information regarding the use of lantibiotics as topical treatments. The results of the *in vivo* section, especially regarding wound healing, raises several questions that should be addressed in future research:

1. The immunomodulatory properties of clausin and AmyA should be investigated, along with possible cytotoxicity against human cells. Using ELISA assays the secretion of chemokines from immune cells can be monitored (62). Furthermore, gene expression of cytokines/chemokines can be investigated using real-time PCR/microarrays (69).

2. The effects lantibiotics have on human keratinocytes should be investigated, by making use of *ex vivo* tissue culturing techniques. The effects of lantibiotics on the migration and proliferation of human keratinocytes should be investigated (66, 68, 70). The effect lantibiotics have on the gene expression of growth factors and cytokines/chemokines involved in wound healing can be investigated using real-time PCR/microarrays (68, 69, 71). Furthermore, the secretion profiles of lantibiotic treated keratinocytes can be investigated using ELISA (70).

3. Additional *in vivo* studies should focus on the efficacy of lantibiotics in the treatments of skin infections caused by multiple drug-resistant Gram-positive pathogens. Furthermore, effective delivery systems must be evaluated, such as the polymeric nanofibers already reported for nisin.

Final Conclusion

We have isolated and purified a novel two-component lantibiotic, amyloliquecidin, from *B. amyloliquefaciens* AD2. The entire operon was annotated and contains all the genes required for amyloliquecidin biosynthesis. The novel lantibiotic was active against Gram-positive clinical isolates and MRSA. Amyloliquecidin has remarkable stability at physiological pH, superior to that of nisin and clausin. Lantibiotics are just as effective in controlling the spread of *S. aureus,* as recorded by bioluminescence. Treatment of wounds with lantibiotics and Bactroban are equally effective in reducing viable bacterial cell counts. Furthermore, lantibiotics do not have a negative influence on wound closure in infected and non-infected

wounds. Bactroban, however, results in delayed wound closure. This study adds to the scientific knowledge base with the isolation and characterization of a novel two-component lantibiotic. Additionally, valuable information is gained on the *in vivo* efficacy of lantibiotics from different classes in the treatment of topical *S. aureus*-induced infections.

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