



**Joana Cristina
Pacheco Barbosa**

**Aprofundando o estudo do lantibiótico de dois
péptidos lichenicidina: bioengenharia, toxicidade e
modo de ação.**

**Deepening the study of the two-peptide lantibiotic
lichenicidin: bioengineering, toxicity and mode of
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Em especial, aos meus pais e ao meu irmão, por acreditarem em
mim.
Sempre.**

o júri

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palavras-chave

Lantipéptidos, lantibióticos, lantibióticos de dois-péptidos, *B. licheniformis*, lichenicidina, expressão heteróloga, mutagénese dirigida, péptidos quiméricos, citotoxicidade da lichenicidina, modo de ação de lantibióticos

resumo

Recentemente, têm sido descobertos e caracterizados vários péptidos antimicrobianos eficazes contra estirpes bacterianas clinicamente relevantes, incluindo as multirresistentes. Neste contexto, os lantibióticos apresentam-se como uma possível alternativa aos antibióticos mais tradicionais. Este estudo teve como objetivo clarificar aspetos da biossíntese, estrutura, modo de ação e toxicidade da lichenicidina. A lichenicidina é um lantibiótico constituído por dois péptidos (Bli α e Bli β), produzido por *B. licheniformis* e com atividade contra *Staphylococcus aureus* e *Listeria monocytogenes*. Foi o primeiro lantibiótico a ser produzido totalmente *in vivo* no hospedeiro *Escherichia coli*. Este sistema foi utilizado para expressar variantes de Bli α e Bli β . Os níveis de produção, a bioatividade e a estrutura dessas variantes foram comparadas com os dos péptidos nativos. Identificaram-se algumas variantes com bioatividade ligeiramente melhorada, que deverão ser caracterizadas com maior detalhe no futuro. Verificou-se que o resíduo Glu26 do péptido Bli α é importante para a bioatividade e que não deve ser substituído por outros resíduos, mesmo que estes tenham carga negativa. De uma forma geral, em ambos os péptidos, os aminoácidos Ser e Thr podem ser substituídos entre si, mesmo quando estão envolvidos na formação dos anéis. Relativamente à biossíntese do péptido Bli β , determinou-se que o hexapéptido é uma sequência essencial para a sua proteólise, bem como para a especificidade da sua enzima modificadora. Foi ainda possível utilizar enzimas biosintéticas de Bli β para produzir e secretar outros péptidos (incluindo não lantibióticos) em *E. coli*, o que abriu novas perspectivas para a aplicação biotecnológica destas enzimas. A produção da lichenicidina em *E. coli* foi melhorada pela substituição dos determinantes e reguladores genéticos de *Bacillus* pelos de *E. coli* e pela produção em separado dos péptidos Bli α e Bli β . O método de purificação foi otimizado de forma a garantir a obtenção dos péptidos Bli α e Bli β puros e em níveis elevados. Neste processo, verificou-se que a produção de Bli β é limitada pela etapa de proteólise que ocorre no espaço extracelular. Relativamente à atividade antibacteriana, determinou-se a concentração mínima inibitória (CMI) da lichenicidina e concluiu-se que esta tem atividade contra estirpes de *S. aureus* sensíveis e resistentes à metilicina (MSSA e MRSA), embora a CMI seja superior para as últimas. Separadamente, Bli α e Bli β têm bioatividade, apesar de a atividade de Bli β ser superior à de Bli α , ambas são inferiores à atividade sinérgica dos dois péptidos. Os testes de tempo-dependência revelaram que, à CMI, a lichenicidina inibe completamente o crescimento de MSSA em menos de 3h. A concentrações superiores, a taxa de morte é acelerada, o que aponta para um modo de ação que envolve a lise celular. Em modelos lipídicos de membrana (LUV), Bli β é capaz de induzir vazamento da célula, possivelmente através da formação de poros na membrana. Bli α tem baixa afinidade para a membrana de *S. aureus*, mas liga-se fortemente a LUV contendo lípido II, o que contribui para acelerar o processo de vazamento do conteúdo intracelular. Verificou-se ainda que Bli α e Bli β estabilizam-se mutuamente na interação com as células de *S. aureus*. Por último, confirmou-se que a lichenicidina não é tóxica para células humanas, como eritrócitos e fibroblastos. Este trabalho reuniu informação nova e relevante sobre a lichenicidina, contribuindo assim, para uma melhor avaliação das suas possíveis aplicações biotecnológicas e terapêuticas.

keywords

Lanthipeptides, lantibiotics, two-peptide lantibiotics, *B. licheniformis*, lichenicidin, heterologous expression, site-directed mutagenesis, chimeric peptides, lichenicidin cytotoxicity, lantibiotics mode of action

abstract

Many novel peptides have recently been discovered and characterized that have been shown to be effective against clinically relevant strains, including multi-drug resistant bacteria. Lantibiotics are presented as an alternative to the more traditional antibiotics. The present study aimed to clarify aspects of the biosynthesis, structure, mode of action and toxicity of lichenicidin. Lichenicidin is a two peptide (Bli α and Bli β) lantibiotic produced by *B. licheniformis* with bioactivity against *Staphylococcus aureus* and *Listeria monocytogenes*. It was the first lantibiotic to be produced totally *in vivo* in *Escherichia coli*. This system was used to produce variants of Bli α and Bli β . The production levels, bioactivity and structure of such variants was compared with that of the native peptides. Variants with slightly improved bioactivity were identified and should be further characterized. Bli α Glu26 residue was found to be important for bioactivity and could not be replaced by other residues, even if negatively charged. In general, Ser and Thr can be replaced with each other, even in the positions involved in ring formation. Regarding Bli β biosynthesis, the hexapeptide sequence is essential to the proteolysis step and to ensure the specificity of the modifying enzyme. In addition Bli β biosynthetic enzymes were used to produce and secrete other peptides (including non-lantibiotics) in *E. coli*, opening new perspectives for the biotechnological application of these enzymes. Lichenicidin production in *E. coli* was improved by replacing the original genetic determinants and regulatory regions of *Bacillus* with those from *E. coli* and by producing Bli α and Bli β separately. Lichenicidin purification method was also optimized to ensure high yields of pure peptides. In this process, it was found that Bli β production is limited by the proteolytic step that occurs in the extracellular environment. Regarding antimicrobial activity, lichenicidin minimal inhibitory concentration (MIC) was determined against methicillin-sensitive and methicillin-resistant *S. aureus* strains (MSSA and MRSA), being higher for the latter. Separately, Bli α and Bli β have bioactivity, although Bli β activity is higher than Bli α , both are inferior to their synergistic activity. Time-kill assays showed that, at the MIC, lichenicidin inhibits MSSA in less than 3 h. For higher concentrations, rapid killing occurs, suggesting a mode of action consistent with a pore formation mechanism of action. In lipid membrane models (LUV), Bli β is able to induce leakage. Bli α has low affinity for *S. aureus* membranes, but it strongly binds to lipid II-containing LUV, contributing to accelerate cell lysis. In addition, Bli α and Bli β stabilize each other when binding to *S. aureus* cells. Furthermore, lichenicidin is not toxic against human erythrocytes and fibroblasts. This work gathers new and relevant information on lichenicidin, thus contributing to a better assessment of its possible biotechnological and therapeutic applications.

TABLE OF CONTENTS

Table of Contents	i
List of Figures	v
List of Tables	xiii
List of Abbreviations	xv
Aims and Structure of this Dissertation	xix
Chapter I General Introduction	20
1.1 Historical Overview of Antimicrobials	3
1.2 Lanthipeptides	4
1.3 Lanthipeptides with antimicrobial activity: the Lantibiotics	8
1.4 Biosynthesis of Class I and Class II Lanthipeptides	11
1.5 Mode of Action and Bioactivity Spectrum	13
1.6 Lantibiotic: Resistance and Immunity	18
1.7 Class I Lanthipeptides Produced by <i>Bacillus</i> spp.	22
1.7.1 Subtilin	22
1.7.2 Entianin.	25
1.7.3 Ericin.	26
1.7.4 Sublichenin.	27
1.7.5 Clausin.	28
1.7.6 Thuricin 4A-4 and 4A-4D.	29
1.7.7 Subtilomycin.	30
1.8 Class II Lanthipeptides Produced by <i>Bacillus</i> spp.	31
1.8.1 Mersacidin.	31
1.8.2 Amylolysin.	38
1.8.3 Cerecidin.	40
1.8.4 Pseudomycoicidin	41
1.8.5 Ticins.	42
1.8.6 Haloduracin.	43
1.8.7 Thusin	46
1.8.8 Formicin	47
1.8.9 Bicareucin, a special case.	49
1.9 The case-study of Lichenicidin, a two-peptide lantibiotic produced by <i>B. licheniformis</i>	51
Chapter II Lichenicidin Rational Site-Directed Mutagenesis Library	57
2.1 Introduction	59
2.2 Results and Discussion	61

Table of Contents

2.2.1	<i>Trans</i> complementation and bioactivity assay	61
2.2.2	Substitution of aromatic amino acids	61
2.2.3	Substitution of positively charged amino acids.....	63
2.2.4	Substitution of negatively charged amino acids	64
2.2.5	Substitution of neutral amino acids	65
2.2.6	Substitution of proline residues	65
2.2.7	Intersubstitution of threonines and serines involved in ring formation	66
2.2.8	Intersubstitution of non-dehydrated serines and threonines	67
2.2.9	Inversion and deletion of residues involved in ring formation	68
2.3	Conclusions	69
2.4	Material and Methods	69
2.4.1	Construction of a <i>trans</i> complementation system.....	69
2.4.2	Production of mutants by site directed mutagenesis	70
2.4.3	Peptide detection and analytics	71
2.4.4	Antagonistic deferred bioassay	72
Chapter III Understanding and Exploring the Role of Lichenicidin Bliβ Leader Peptide		73
3.1	Introduction.....	75
3.2	Results and Discussion	76
3.2.1	Is the hexapeptide required for Bli β maturation <i>in vivo</i> ?	76
3.2.2	Impact of hexapeptide's amino acid sequence on Bli β production	79
3.2.3	Can LicP recognize hexapeptides other similar lantibiotics?	80
3.2.4	What is the impact of proteolysis sites for LicP and LicT trimming?	81
3.2.5	Can LicT and LicP process other core peptides?	82
3.3	Conclusions	85
3.4	Material and Methods	85
3.4.1	Construction and analysis of the hexapeptide mutants	85
3.4.2	Construction of the recombinant genes.....	86
3.4.3	Recombinant peptide extraction using Ni-NTA affinity beads.....	87
3.4.4	Analytics of recombinant peptides	87
Chapter IV Improvement of Lichenicidin Production in <i>Escherichia coli</i>		89
4.1	Introduction.....	91
4.2	Results and Discussion	92
4.2.1	Lichenicidin production yields in <i>B. licheniformis</i> I89 and <i>E. coli</i>	92
4.2.2	Separate production of lichenicidin peptides.....	93
4.2.3	Contribution of the immunity and <i>tolC</i> genes for lichenicidin production.....	95

4.2.4	Induction tests of selected strains	96
4.2.5	Effect of LicP proteolysis in Bli β production	97
4.3	Conclusions	98
4.4	Material and Methods	98
4.4.1	Culture media, strains and vectors.....	98
4.4.2	Construction of plasmids	99
4.4.3	Colony deferred antagonistic bioassay	100
4.4.4	Preparation of peptide extracts	100
4.4.5	IPTG induction.....	101
4.4.6	Antagonistic deferred bioassay	101
4.4.7	Mass spectrometry analysis.....	101
4.4.8	Statistical analysis	102
Chapter V Lichenicidin, a promising therapeutic agent: Production, Bioactivity and Toxicity		
		103
5.1	Introduction.....	105
5.2	Results and Discussion	106
5.2.1	Best system for lichenicidin expression and purification.....	106
5.2.2	Antibacterial activity of lichenicidin.....	107
5.2.3	Hemolytic activity of lichenicidin.....	110
5.2.4	Viability of human fibroblasts treated with lichenicidin.....	111
5.3	Conclusions	114
5.4	Material and Methods	115
5.4.1	Lichenicidin expression systems.....	115
5.4.2	Purification of lichenicidin peptides	116
5.4.3	Determination of Minimal Inhibitory Concentration (MIC)	116
5.4.4	Time-kill assay.....	117
5.4.5	Hemolysis assay.....	117
5.4.6	Cell viability assays.....	118
5.4.7	Statistical analysis	119
Chapter VI Insights into the Mode of Action of the Two-Peptide Lantibiotic Lichenicidin.....		
		121
6.1	Introduction.....	123
6.2	Results	124
6.2.1	Zeta-potential measurements	124
6.2.2	Lichenicidin-induced transmembrane voltage changes in <i>S. aureus</i>	125
6.2.3	Lichenicidin interaction with <i>S. aureus</i> imaged by AFM	126

Table of Contents

6.2.4	Interaction studies using LUV	127
6.3	Discussion	129
6.4	Material and Methods	132
6.4.1	Peptide production and purification	132
6.4.2	Bacteria preparation	133
6.4.3	Preparation of lipid vesicles	133
6.4.4	Membrane dipole potential studies with di-8-ANEPPS	133
6.4.5	Zeta-potential measurements	134
6.4.6	Atomic force microscopy imaging of <i>S. aureus</i>	135
6.4.7	Leakage assays with membrane models	135
Chapter VII Synopsis and Future Perspectives		137
7.1	Synopsis	139
7.2	Highlights	147
7.3	Future Perspectives	148
Bibliography		151
Supplementary Information		S1
Chapter II – Supplementary Information		S3
Supplementary Figures		S3
Chapter III – Supplementary Information		S23
Supplementary Figures		S23
Supplementary Tables		S24
Chapter IV – Supplementary Information		S37
Supplementary Figures		S37
Supplementary Tables		S41
Chapter V – Supplementary Information		S43
Supplementary Procedures		S43
Supplementary Figures		S44
Chapter VI – Supplementary Information		S49
Supplementary Tables		S49

LIST OF FIGURES

Figure 1.1. Historical overview of antimicrobial agents discovery. The introduction of the more traditional antibiotics is presented along with the discovery of lantibiotics, from 1928 to the present days. Important landmarks are also highlighted.....	3
Figure 1.2. Posttranslational modifications most commonly found in lanthipeptides. Adapted from [10,35].	6
Figure 1.3. (A) General biosynthesis of lanthipeptides, with indication of the amino acid numbering; a non-representative peptide is shown where * indicates the modified residues Dha or Dhb. The mature peptide has a non-linear structure, which results from bridge formation. (B) Scheme of lanthipeptide synthetases involved in the biosynthesis of the four classes of lanthipeptides. Dark coloured stripes indicate conserved regions.....	7
Figure 1.4. General overview of the dehydration, cyclization and leader peptide removal steps involved in the biosynthesis of class I and II lanthipeptides. The relevant residues for leader peptide removal are represented in maroon. The residues that can undergo dehydration are colored in green and those that are dehydrated are colored in blue. The Lan and MeLan rings are orange.	12
Figure 1.5. Schematic representation of the mode of action of <i>Bacillus</i> spp. lantibiotics, which involves binding of the target molecule, lipid II, preventing the correct cell-wall synthesis. Additionally, pore formation can occur with disruption of the cellular membrane. The mechanism of action of the two-peptide lantibiotics was demonstrated for lactacin 3147 [92].....	14
Figure 1.6. Structures of class I lantibiotic subtilin produced by <i>B. subtilis</i> . The residues that differ in the subtilin-like peptides (entianin, ericin S and sublichenin) are represented in white. The Lan and MeLan rings are orange and dehydrated residues are blue.....	23
Figure 1.7. Schematic representation of the biosynthetic clusters of class I lanthipeptides produced by <i>Bacillus</i> spp. The genes encoding the modification enzymes LanB and LanC are represented in orange and the structural genes in green. The genes of transporters and proteases are blue. The self-protection and regulation genetic determinants are pink and yellow, respectively. The flavoprotein LanD is represented in purple and the genes with unknown function are colorless.....	24

List of Figures

- Figure 1.8. Alignment of the amino acid sequences of the leader and core peptides of all the class I lanthipeptides described for *Bacillus* spp. The colored amino acids represent highly conserved residues.....26
- Figure 1.9. Structure of class I lantibiotic ericin A produced by *B. subtilis*. The Lan and MeLan rings are orange and dehydrated residues are blue. For comparison with ericin S, see Figure 1.6.....27
- Figure 1.10. Structure of class I lantibiotic clausin produced by *B. clausii*. The Lan and MeLan rings are orange and dehydrated residues are blue.29
- Figure 1.11. Structures of class I lantibiotic thuricin 4A-4 produced by *B. cereus*. The Lan and MeLan rings are orange and dehydrated residues are blue. The Obu residue (in pink) refers to 2-oxobutyl.30
- Figure 1.12. Structure of class I lantibiotic subtilomycin produced by *B. subtilis*. The Lan and MeLan rings are orange and dehydrated residues are blue. The Obu residue (in pink) refers to 2-oxobutyl.30
- Figure 1.13. Structure of class II lantibiotic mersacidin produced by *B. methylotrophicus*. The Lan and MeLan rings are orange and dehydrated residues, blue. The conserved Glu motif is represented in purple.....31
- Figure 1.14. Schematic representation of the biosynthetic clusters of class II and lanthipeptides produced by *Bacillus* spp. The genes encoding the modification enzymes LanB and LanC are represented in orange and the structural genes in green. The genes of transporters and proteases are blue. The self-protection and regulation genetic determinants are pink and yellow, respectively. The flavoprotein LanD is represented in purple and the genes with unknown function are colorless.....32
- Figure 1.15. Biosynthesis and regulation mechanism of mersacidin. *mrsA* transcription is regulated by the orphan regulator MrsR1 which lacks a dedicated sensor kinase; still unknown is the nature of the molecule that triggers MrsR1 activation (represented by the triangles with '?') or if mersacidin itself is responsible for triggering the biosynthesis through a mechanism involving also MrsR2/MrsK2 system. The pre-mersacidin is then modified by a single MrsM modifying enzyme, and exported and cleaved by MrsT ABC transporter whose transcription is also activated by MrsR1. Transcription of the immunity genes *mrsFGE* is activated by the two-component system MrsK2/MrsR2, which

recognizes mersacidin from the extracellular environment. No information is available regarding the transcription regulation of MrsK2/MrsR2.	33
Figure 1.16. Alignment of the amino acid sequences of the leader and core peptides of all the class II lanthipeptides described for <i>Bacillus</i> spp. The colored amino acids represent highly conserved residues.....	36
Figure 1.17. Structure of class II lantibiotic amyolysin produced by <i>B. methylotrophicus</i> . Amyolysin A and B correspond to the structure proposed by Arguelles Arias <i>et al</i> [100] and in this work, respectively. The Lan and MeLan rings are orange and dehydrated residues, blue. The conserved Glu motif is represented in purple.	39
Figure 1.18. Structure of class II cerecidin A1 to A6 produced by <i>Bacillus cereus</i> . The residues that differ in cerecidin A7 are represented in white. The Lan and MeLan rings are orange, and dehydrated residues, blue.....	41
Figure 1.19. Proposed structure of class II pseudomycoicidin produced by <i>B. pseudomycooides</i> . The Lan and MeLan rings are orange, dehydrated and non-dehydrated Ser and Thr residues, in blue and green, respectively.....	42
Figure 1.20. Structure of class II ticin produced by <i>B. thuringiensis</i> . The residues that differ in ticins are represented in white. The Lan and MeLan rings are orange, dehydrated Ser and Thr residues, in blue, and non-dehydrated, in green. Ser and Thr residues whose dehydration state could not be determined are represented in turquoise. The conserved Glu motif is represented in purple.	43
Figure 1.21. Structure of class II haloduracin, a two-peptide lantibiotic produced by <i>B. halodurans</i> . The Lan and MeLan rings are orange, dehydrated Ser and Thr residues, in blue, and non-dehydrated, in green. Ser and Thr residues whose dehydration state could not be determined are represented in turquoise. The conserved Glu motif is represented in purple.....	45
Figure 1.22. Structure of class II thusin, a two-peptide lantibiotic produced by <i>B. thuringiensis</i> . The residue that differ in thusin β' is represented in white. The Lan and MeLan rings are marked orange, dehydrated Ser and Thr residues, in blue, and non-dehydrated, in green. Ser and Thr residues whose dehydration state could not be determined are represented in turquoise. The conserved Glu motif is marked in purple. .	47

List of Figures

Figure 1.23. Structure of class II formicin, a two-peptide lantibiotic produced by *B. paralicheniformis*. The Lan and MeLan rings are marked orange, dehydrated Ser and Thr residues, in blue, and non-dehydrated, in green. The conserved Glu motif is marked in purple.48

Figure 1.24. Structure of class II biceuricin, a two-peptide lantibiotic produced by *B. cereus*. The Lan ring is filled in orange and dehydrated Ser and Thr residues, in blue. Dehydrated residues in D-conformation are represented in yellow.....50

Figure 1.25. Structure of class II lichenicidin, a two-peptide lantibiotic produced by *B. licheniformis*. Two different structures are illustrated for Bli α peptide, according to Caetano *et al.*²¹ (A) and Shenkarev *et al.*¹⁰³ (B). The Lan and MeLan rings are orange, dehydrated Ser and Thr residues, in blue and the non-dehydrated, in green. The Obu residue (pink) refers to 2-oxobutyryl. The conserved Glu motif is represented in purple...52

Figure 2.1. Bli α and Bli β amino acid sequences. Summary of the variants produced in this study and respective antagonist effect of crude extracts of the *Escherichia coli* mutant producers against *Kocuria rhizophila*.62

Figure 2.2. Analysis of the *Escherichia coli* extracts against *Kocuria rhizophila*, expressing (a) Bli α and (b) Bli β variants. The ratio between the bioactivity (♦) and relative quantification (x) of mutant and wildtype extracts is presented, considering the wildtype as 1. Mutants with increased bioactivity are represented in green and mutants with decreased bioactivity are represented in red. Grey areas indicate extracts in which the two variants were identified (bioactivity results from the mixture of both). Statistically significant differences of bioactivity ratio are also indicated: * = $p < 0.05$; ** = $p < 0.001$; *** = $p < 0.0001$. In bioactivity ratios, some error bars are not visible as standard deviations are too low to be visualized.....63

Figure 3.1. Lichenicidin gene cluster containing the genes essential for the production of Bli α (green) and for the production of Bli β (blue); LicT is responsible for cleaving the precursor peptides C-terminally of the double-Gly motif (underlined) and for the transport of both peptides out of the cell. LicP cleaves the remaining hexapeptide attached to Bli β in the extracellular space; in grey, are other genes encoding putative regulatory enzymes

and self-immunity genes, not essential for lichenicidin production in *E. coli*. Adapted from [54].76

Figure 3.2. Schematic representation of the mutations inserted into Bli β 's hexapeptide. The native sequence is shown on top: hexapeptide in yellow, core peptide in pink and the remaining leader sequence, in blue. The mutations inserted are represented in green when multiple amino acids were replaced or in red for single amino acid mutations. Deletion of an amino acid is represented by a circle containing a " – ". The cleavage sites of LicT and LicP enzymes are also represented.....77

Figure 3.3. Quantification of peptide (grey bars) and bioactivity against *Kocuria rhizophila* (red diamonds) of Bli β hexapeptide mutants. Native Bli β activity (LicA2) is represented in dark grey/dark red; * indicates statistically significant differences in bioactivity (red) and quantification (black) compared to the control ($p < 0.05$). ^{a)} not detected; ^{b)} quantification not performed by the MRM method, since the first amino acid is different.78

Figure 3.4. Alignment of LicA2 with hexapeptides from other closely related lantibiotics: haloduracin A2, plantaricin W A2, cytolysins and cerecidins. White circles represent amino acids conserved among all the sequences, while the decrease in the conservation level is represented by red circles: light red for higher conservation and dark red for lower. At the bottom, representation of the relative frequency of hexapeptide amino acids (generated using WebLogo [276]).80

Figure 3.5. Representation of constructions made with chimeric genes. In blue, LicA2 leader sequence with double Gly motif highlighted; in yellow the hexapeptide with a Glu residue in position -1; in pink the core peptide is indicated, which is replaced by various core sequences; position 1 was mutated to Thr if required, to maintain the cleavage site. Conditions tested: control (Ctl), LicT and LicP combined activity (licT.P).83

Figure 4.1. Schematic representation of lichenicidin peptides and biosynthetic gene cluster. The essential genes for the biosynthesis of each peptide in *E. coli* are presented: green arrows indicate genes required for Bli α production, blue arrows, for Bli β and the orange arrow for both. Grey arrows represent non-essential genes for lichenicidin production in *E. coli*. Adapted from [54].92

List of Figures

Figure 4.2. Bioactivity (red diamonds) and quantification (bars) of lichenicidin extracts from <i>B. licheniformis</i> and <i>E. coli</i> producing strains. Bli α quantification is shown in pink and Bli β , in blue. * indicates statistical significance ($p < 0.05$).....	93
Figure 4.3. Bioactivity of Bli α (A) and Bli β (B) producers extracts against <i>K. rhizophila</i> . In pink, <i>E. coli</i> BL21Gold strain and in blue, <i>E. coli</i> BL21Star.	94
Figure 4.4. Quantification of Bli α (A) and Bli β (B) peptide in extracts of all the expression systems compared during the present work, using MS. <i>E. coli</i> BL21Gold extracts quantification is presented in pink and BL21Star's, in blue. *indicates statistical significance ($p < 0.05$).....	94
Figure 4.5. Bioactivity of Bli α (A) and Bli β (B) producers extracts containing additional genes, against <i>K. rhizophila</i> . In pink, <i>E. coli</i> BL21Gold strain and in blue, <i>E. coli</i> BL21Star. *indicates statistical significance ($p < 0.05$).....	95
Figure 4.6. Quantification of Bli α (A) and Bli β (B) produced by Gold pA1M1T and HP β , respectively without IPTG induction (purple) and with 0.1mM (pink) or 1 mM (blue) IPTG added to the growing media.....	96
Figure 4.7. Relative concentration of LicA2 with the hexapeptide attached (blue) comparison with concentration of Bli β (pink), produced by Gold HP β , over time.....	98
Figure 5.1. Quantification of Bli α (A) and Bli β (B) present in extracts using the respective <i>E. coli</i> expression systems constructed. Blue represents the expression in <i>E. coli</i> Gold and red the expression in <i>E. coli</i> Star. For standard calibration curves, see Figure S4.4. Error bars represent the standard deviation of three independent replicates; when these are not visible, standard deviation is too low to be noticed.....	107
Figure 5.2. Lichenicidin killing time curve. <i>S. aureus</i> ATCC 29213 growth curve is shown in black full circles. Three lichenicidin concentrations were tested, corresponding to the MIC value (5 μ M; red full circles), 2-fold MIC (10 μ M; empty triangles) and 5-fold MIC (25 μ M; empty squares). All the conditions were followed for 24 h, although only the first 10h are shown. Error bars represent standard deviation of three independent replicates.	109
Figure 5.3. Percentage of hemolysis caused by different lichenicidin concentrations after 1 h (white) and 24 h (black) of incubation. Error bars represent standard deviation of three independent replicates; when these are not visible, standard deviation is too low to be	

noticed. Triton X-100 was used as positive hemolytic control. The MIC value is indicated in the xx axis. The 10% hemolysis threshold, above which a compound is considered hemolytic, is depicted as a red dashed line. *** shows a statistically significant ($p < 0.001$) increase in hemolysis for that specific sample in comparison to all the other conditions tested. Controls with ampicillin, which was used as non-hemolytic agent, and methanol are shown in Figure S5.1110

Figure 5.4. Percentage of viability of HFF-1 human fibroblasts after 2 h (white bars) and 24 h (black bars) of incubation with different lichenicidin concentrations, using XTT cell proliferation assay. Control samples containing the same percentage of methanol that was used to dissolve lichenicidin can be found in Figure S5.2. Error bars represent standard deviation of three independent replicates; when these are not visible, standard deviation is too low to be observed.....111

Figure 5.5. Flow cytometry analysis of HFF-1 fibroblasts treated with increasing lichenicidin concentrations and respective gating strategy. Controls, including those with methanol, are shown in Figure S5.3.....113

Figure 5.6. Viability assay by flow cytometry of HFF-1 human fibroblasts after 2h of incubation with different lichenicidin concentrations. Unstained cells are represented in grey; live cells, marked with calcein, are shown in green; dead cells, marked with ethidium homodimer-1, are shown in red; double stained cells are represented in purple. Controls with the same percentages of methanol are shown in Figure S5.4. Error bars represent standard deviation of three independent replicates; when these are not visible, standard deviation is too low to be noticed.114

Figure 6.1. Zeta potential (ζ) changes of *S. aureus* induced after incubation with different concentrations of lichenicidin (red dots). ζ changes induced by higher concentration of Bli α (white dot) and Bli β (black dot) alone are also presented for comparison. Error bars represent the standard deviation of three independent replicates.125

Figure 6.2. Membrane dipolar potential of *S. aureus* in the presence of different concentrations of lichenicidin (red circles), Bli α (white circles) and Bli β (black circles). Binding profiles of lichenicidin was assessed by calculating the excitation ratio R (I455/I525), normalizing for the value obtained in the absence of peptide. Experimental

results fitted using equation (1). Error bars represent the standard deviation of three independent replicates.....	125
Figure 6.3. Effect of lichenicidin on <i>S. aureus</i> , imaged by AFM: (A) control, <i>S. aureus</i> in the absence of lichenicidin; (B) incubation for 1 h with 5 μ M lichenicidin (MIC); (C) incubation for 5 h with MIC; and (D) incubation for 24 h with 5 x MIC (25 μ M).	126
Figure 6.4. Zeta potential (ζ) of the lipid vesicles (A); percentages of leakage induced by lichenicidin (B) and by the sequential addition of Bli α followed by Bli β (C) and Bli β followed by Bli α (D). Error bars represent the standard deviation of three independent replicates; when these are not visible, standard deviation is too low to be noticed.	127
Figure 6.5. Lichenicidin affinity to LUV: dissociation constants (A) of lichenicidin (red), Bli α (white) and Bli β (black dots) calculated for the interaction with DOPC 100% (B), DOPC:LipII 99.9:0.1% (C) and DOPC:DPPG:CL:LipII 70:20:9.9:0.1% (D). The MIC value is represented by the vertical red line.....	128
Figure 6.6. Proposed mode of action of lichenicidin. (A) target membrane containing lipid II; (B) possible mode of action of Bli α by sequestering lipid II and destabilizing the membrane without pore formation; (C) mode of action of Bli β with transient pore formation without using lipid II as anchoring molecule; (D) dual mode of action of lichenicidin: Bli α recognizes and binds lipid II, recruiting Bli β that then inserts within the target membrane, forming a stable pore.	132

LIST OF TABLES

Table 1.1. List of class I and class II lanthipeptides produced by <i>Bacillus</i> spp. and their respective molecular masses. The information not available is represented with n.a..	10
Table 1.2. Minimal inhibitory concentrations of <i>Bacillus</i> spp. lantibiotics determined against different indicator strains. It should be noted that the test conditions and compound purity could differ among the different assays reported.....	15
Table 3.1. List of genes used in this study and their respective masses.....	82
Table 3.2. Peptides and their derivatives identified in the soluble cellular fractions and in the supernatant of <i>E. coli</i> , in the presence (+LicTP) or absence of LicTP (-LicTP). ND indicates that none of the peptides was detected.	84
Table 5.1. MIC of lichenicidin, Bli α and Bli β against selected Gram-positive target strains.	108

LIST OF ABBREVIATIONS

ABC	– ATP-binding cassette
Abu	– α -aminobutyric acid
AFM	– atomic force microscopy
ATCC	– American Type Culture Collection
ATP	– adenosine triphosphate
AviCys	– S-[(Z)-2-aminovinyl]-D-cysteine
AviMeCys	– aminovinyl methylcysteine
Dha	– 2,3-didehydroalanine
Dhb	– (Z)-2,3-didehydrobutyrine
CL	– cardiolipin
dNTP	– deoxynucleoside triphosphate
DMEM	– Dulbecco's modified Eagle medium
DOPC	– 1,2-dioleoyl-sn-glycero-3-phosphocholine
DPPG	– 1,2-dipalmitoyl-sn-glycero-3-phospho-(1'-rac-glycerol)
DSM	– Deutsche Sammlung von Mikroorganismen
EDTA	– ethylenediamine tetra-acetic acid
EUCAST	– European Committee on Antimicrobial Susceptibility Testing
FAO	– Food and Agriculture Organization (United Nations)
FDA	– Food and Drug Administration (United States of America)
GRAS	– Generally recognized as safe
HPLC	– high performance liquid chromatography
HTH	– helix-turn-helix
LA	– Luria-Bertani agar medium
Lac	– lactyl
Lab	– labionin
Lan	– lanthionine
LanA	– generic designation for precursor peptides in lanthipeptide biosynthesis
LanB	– generic designation for lanthipeptide dehydratases
LanC	– generic designation for lanthipeptide cyclases
LanCL	– LanC-like protein
LanD	– generic designation for lanthipeptide oxidative decarboxylases
LanE	– generic designation for component of the ABC transport complex involved in lanthipeptide self- immunity

List of Abbreviations

LanF – generic designation for component of the ABC transport complex involved in lanthipeptide self- immunity

LanG – generic designation for component of the ABC transport complex involved in lanthipeptide self- immunity

LanI – generic designation for lanthipeptide immunity proteins

LanJ – generic designation for lanthipeptide dehydrogenases

LanK – generic designation for lanthipeptide regulatory histidine kinases

LanKC – generic designation of class III bifunctional enzymes catalysing both dehydration and cyclization reactions

LanL – generic designation of class IV bifunctional enzymes catalysing both dehydration and cyclization reactions

LanM – generic designation of class II bifunctional enzymes catalysing both dehydration and cyclization reactions

LanO – generic designation for lanthipeptide oxidoreductases that convert Pyr to Lac

LanP – generic designation of proteases that remove lanthipeptide leader peptides

LanR – generic designation for lanthipeptide response regulator protein

LanT – generic designation of ABC transporters that excrete lanthipeptides after biosynthesis

LB – Luria-Bertani broth

LUV – large unilamellar vesicles

MALDI-ToF – matrix-assisted laser desorption/ionization-time of flight

MeLan – methyllanthionine

MeLab – methyllabionin

MIC – minimum inhibitory concentration

MRM – multiple reaction monitoring

MRSA – methicillin-resistant *Staphylococcus aureus*

MSSA – methicillin-sensitive *Staphylococcus aureus*

MS – mass spectrometry

MW – molecular weight

NMR – nuclear magnetic resonance

NRPS – nonribosomal peptide synthetase

NSR – nisin resistance protein

Obu – 2-oxobutyryl

ORF – open reading frame

PEG – polyethylene glycol

Pi – phosphate
PKS – polyketide synthase
PTM – post-translational modification
Pyr – 2-oxopropionyl (pyruvyl)
RBS – ribosomal binding site
RiPP – ribosomally synthesized and post-translationally modified peptides
SAR – structure-activity relationship
SDM – site-directed mutagenesis
SDS – sodium dodecyl sulfate
TSA – tryptic soy agar medium
TSB – tryptic soy broth
VISA – vancomycin intermediate-resistant *Staphylococcus aureus*
VRE – vancomycin-resistant Enterococcus
VRSA – vancomycin-resistant *Staphylococcus aureus*
VSE – vancomycin-sensitive Enterococcus
WHO – World Health Organization

AIMS AND STRUCTURE OF THIS DISSERTATION

The present work aims to contribute to a deeper knowledge of lantibiotics, specifically the two-peptide lantibiotics. Lichenicidin, produced by *Bacillus licheniformis* I89, was used as a case-study. The genus *Bacillus* has been considered a prolific source of natural products, including new antimicrobials of clinical interest.

The following aspects were addressed:

- the structure-activity relationship of the amino acids of lichenicidin;
- the flexibility of lichenicidin biosynthetic machinery;
- lichenicidin expression in Gram-negative host, *Escherichia coli*;
- lichenicidin bioactivity, toxicity and mode of action.

The thesis is organized into seven chapters which are structured as follows:

Chapter I, a general introduction is made which provides an overview on lanthipeptides, their biosynthesis and classification, and general aspects of lantibiotics. A comprehensive review of the current state-of-the-art of lantibiotics produced by *Bacillus* spp. is included.

In the following chapters, the strategies employed and the main results obtained throughout the work are described.

Chapter II: a rationally designed mutant library of lichenicidin was made to assess the impact of the substitution of important residues on the bioactivity and structure of the peptide, aiming at obtaining mutants with improved activity.

Chapter III: the role of Bli β peptide leader sequence was elucidated and the importance of the hexapeptide amino acids residues was evaluated. In addition, the ability of this leader sequence to direct the activity of lichenicidin proteolytic enzymes to process other (non-)lantibiotic peptides was also investigated.

Chapter IV: improvement of lichenicidin expression in the heterologous host *Escherichia coli* was attempted, which included the development of optimized vectors and the optimization of culture conditions.

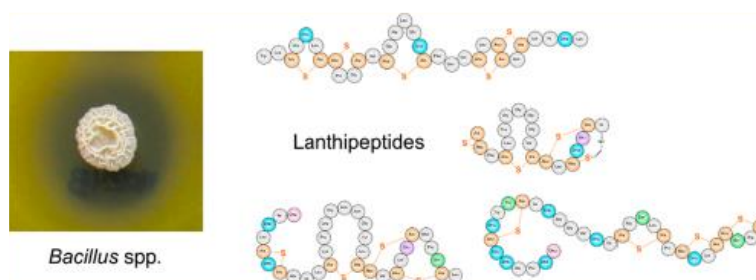
Chapter V: using the best conditions and an optimized procedure, lichenicidin peptides were purified. Next, the lichenicidin bioactivity spectrum and toxicity to human cells were evaluated.

Chapter VI: applying biophysical approaches, purified lichenicidin was used to evaluate the mode of action of two-peptide lantibiotics against *Staphylococcus aureus* and the interaction with model membranes containing lipid II.

Finally, **Chapter VII** gives a synopsis of all the work done, highlighting the most relevant aspects and presenting future perspectives.

CHAPTER I

GENERAL INTRODUCTION



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1.1 HISTORICAL OVERVIEW OF ANTIMICROBIALS

The discovery of antibiotics has long been considered one of the most significant discoveries of the twentieth century. Over the last decades, several antibacterials have been discovered and extensively applied to treat infections (Figure 1.1). Although resistance development is a normal evolutionary process for bacteria, it is accelerated by the selective pressure due to widespread use of antibacterial drugs. As Alexander Fleming predicted in 1945, the development of each drug was accompanied by detection of resistance to it. This has become a serious problem, particularly regarding the development of multi-drug resistant bacteria and the report of infections without effective therapeutic options [1,2]. This phenomenon is enhanced by the misuse and overuse of antibiotics for both medical and veterinary applications, as well as the reduced investment in antimicrobial research combined with high development costs, including clinical trials, and the low success rates in identifying new compounds [1,3].

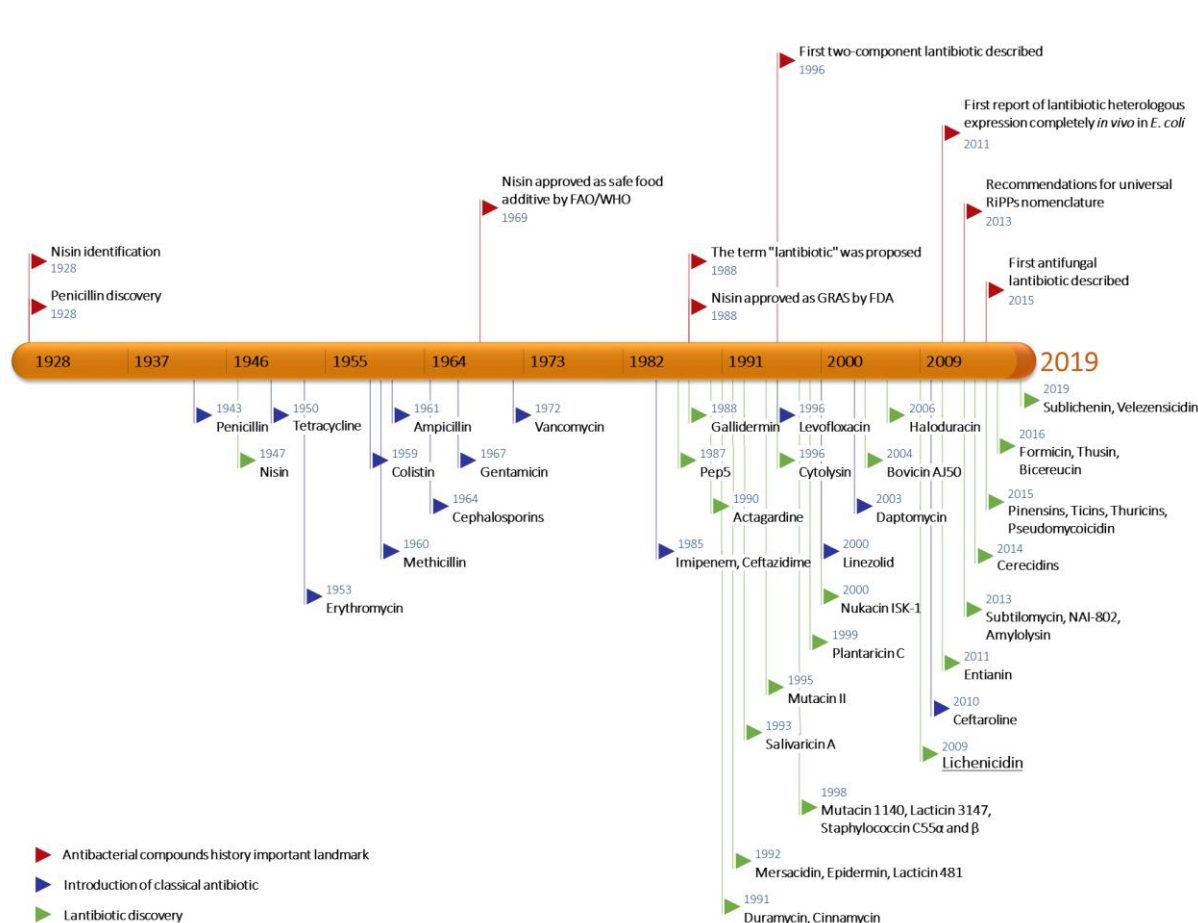


Figure 1.1. Historical overview of antimicrobial agents discovery. The introduction of the more traditional antibiotics is presented along with the discovery of lantibiotics, from 1928 to the present days. Important landmarks are also highlighted.

Since the “golden era” of antibiotic discovery, between the 1950s and the 1960s, few new classes were introduced to the market (Figure 1.1). Since the 1980s, the total number of approved antibiotics has fallen significantly, slightly increasing only in 2011 and 2016 [1]. Alternative strategies to overcome this worldwide problem are being investigated, including phage therapy and modulation of the immune system, in parallel with attempts to modify and improve the currently used bioactive molecules as well as the search for novel antimicrobials, with improved bioactivities and new modes of action [3].

Bioactive natural peptides produced by microorganisms have their origin in two different biosynthetic pathways: *i*) non-ribosomal biosynthesis (NRPS), which included the more traditional antibiotics, and *ii*) ribosomally synthesized and post-translationally modified peptides (RiPP). Recently, RiPP have been drawing attention due to their characteristics, namely, bioactivity levels and targets, proteolytic degradation stability, low toxicity toward mammals and low immunogenicity, and lower resistance development to date [3,4]. Among these are the lanthipeptides – lanthionine containing peptides – a promising class of natural compounds produced primarily by Gram-positive bacteria. Lanthipeptides with antibiotic properties, the so called lantibiotics, are the main focus of the present thesis. The term lantibiotic was first proposed by Kellner and co-workers in 1988 when reporting the discovery of a natural lanthionine containing peptide, which was named gallidermin [5]. However, even before that, the first and most widely used lantibiotic, nisin, was approved by FAO and WHO as a safe food additive in 1969, although at the time it was not classified as a lantibiotic (Figure 1.1; [6]). Interestingly, the first observation of nisin’s bioactivity was reported in the same year that penicillin was described by Alexander Fleming, 1928 [7,8]. After that, there was a gap of around 20 years, when no new lantibiotics were reported in the literature. Since the 1990s, several lantibiotics have been described and this class of antimicrobial compounds has gained some attention from the academia and the pharmaceutical industry, as evidenced by the number of studies on newly discovered peptides (Figure 1.1). Lantibiotics and their characteristics, biosynthesis, classification and mode of action will be described in more detail in the next sections, along with some model examples.

1.2 LANTHIPEPTIDES

Lanthipeptide biosynthetic gene clusters can be found in the genome of various genera of Firmicutes, Actinobacteria, Proteobacteria, Bacteroidetes and Cyanobacteria [9,10]. Genes encoding lanthipeptide biosynthetic enzymes homologues are also be found in some Archaea and higher eukaryotes, including mammals [11–13], although until recently, lanthipeptides could only be detected and isolated from bacteria. Several biological activities

have been reported for these peptides: antimicrobial (lantibiotics), morphogenetic [14,15], antiviral [16], antifungal [17], antiallodynic [18] or antinociceptive [19] and also immunomodulatory [20]. In addition to nisin, that has been used for more than 50 as a food preservative, other lanthipeptides are currently under clinical tests for various therapeutic applications, including: *i)* a semisynthetic actagardine analogue (NVB302, Novacta Biosystems Limited, phase I clinical trial to treat *Clostridium difficile* infections); *ii)* microbisporicin (NAI-107, NAICON and Sentinella Pharmaceuticals INC, targeting nosocomial infections caused by multiresistant bacteria); *iii)* synthetic mutacin 1140 (Mu1140-S, Organics, in preclinical development to treat Gram-positive infections); *iv)* duramycin (completed phase II of clinical trials to be used against cystic fibrosis), *v)* labyrinthopeptin A2 (clinical trials for treatment of several virus infections) and *vi)* the synthetic lanthipeptide MOR107 (Lanthio Pharma B. V./MorphoSys, completed phase I clinical trials as an agonist of the angiotensin II type 2 receptor) [3,21–24].

Lanthipeptides are characterized by the presence of lanthionine and/or lanthionine related amino acids, resulting from post-translational modifications of peptide substrates [10]. A lanthionine is an amino acid in which two alanine residues are linked by a thioether group connecting their β -carbons and when incorporated into a peptide chain via both the amino and acid groups results in a thioether crosslink. These linkages are introduced by a two-step modification that involves: *i)* the dehydration of Ser and Thr residues to 2,3-didehydroalanine (Dha) and 2,3-didehydrobutyrine (Dhb), respectively, and *ii)* the addition of a Cys onto the dehydrated amino acid followed by protonation, originating a lanthionine (Lan) from Ser or a methylanthionine (MeLan) from Thr (Figure 1.2) [4,10]. This structure can undergo additional modifications, resulting in a higher structural diversity as for example when the previously formed structure attacks another dehydrated amino acid to form labionin (Lab) or methylabionin (MeLab) (Figure 1.2) [10,25]. Several other post-translational modifications have been reported so far. Together with Dha and Dhb residues, these modifications are associated with higher resistance to proteolytic activity or increasing peptide bioactivity (e.g. lactacin 3147 [26]), and include (Figure 1.2): *i)* reduction of a C-terminal pyruvic group (epilancin 15X [27]); *ii)* oxidation of the thioether bond (actagardine [28]); *iii)* hydroxylation of proline and aspartate (microbisporicin [29], cinnamycin [30]); *iv)* halogenation of tryptophan (microbisporicin [29]); *v)* C-terminal decarboxylation (mersacidin [31]); *vi)* covalent linkage of lysine and alanine (microbisporicin [29]); *vii)* N-terminal acetylation (paenibacillin [32]); *viii)* disulfide bridge formation (bovicin HJ50 [33], haloduracin α -peptide [34]); and *ix)* tryptophan N-glycosylation (NAI-112 [19]).

General Introduction

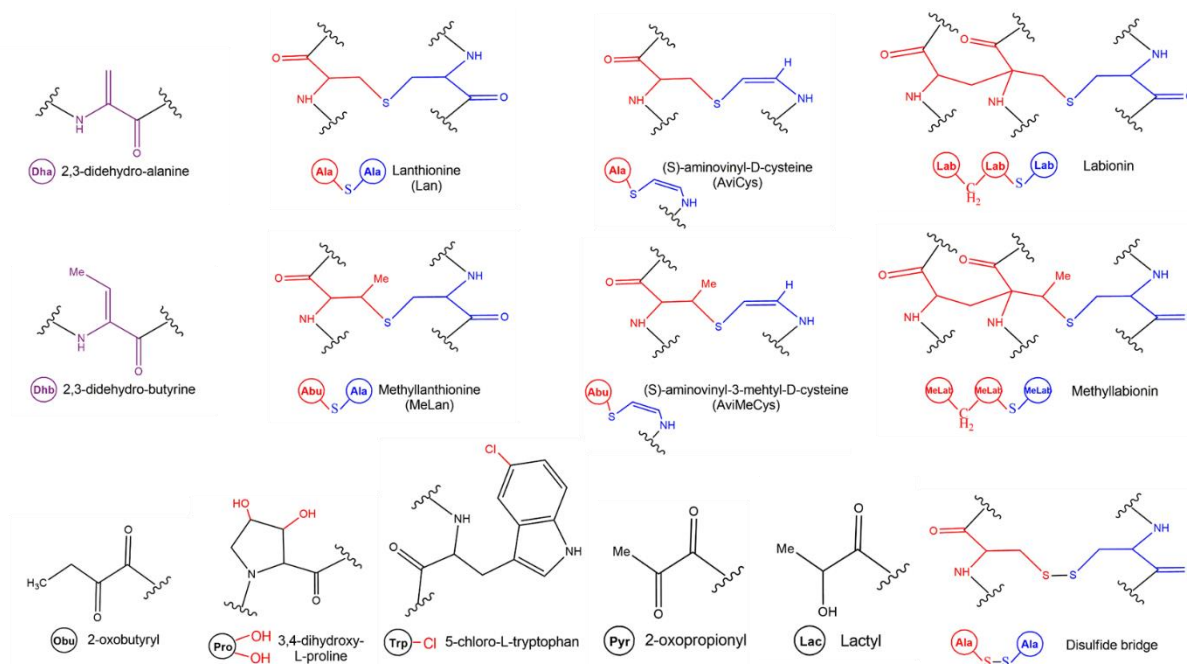


Figure 1.2. Posttranslational modifications most commonly found in lanthipeptides. Adapted from [10,35].

Lanthipeptide biosynthesis involves the ribosomal synthesis of a precursor peptide, comprising an N-terminal leader sequence and a C-terminal core sequence (Figure 1.3), where the post-translational modifications are installed during the biosynthetic process [10]. The mechanism and the enzymes involved in the installation of the characteristic thioether cross-links and other post-translational modifications is one of the criteria used to divide lanthipeptides into four different classes [10,25]. Class I lanthipeptides are modified by two independent enzymes: a LanB dehydratase, responsible for dehydrating serine and threonine residues, and a LanC cyclase, that performs the thioether cyclization (Figure 1.3B). For all the other classes, a single multifunctional enzyme catalyses both reactions, although they have different domains. As so, class II peptides are modified by a bifunctional lanthipeptide synthetase, LanM, containing an N-terminal dehydratase and a C-terminal LanC-like cyclase domains. Class III and IV are modified by trifunctional enzymes: class III modification enzymes bear an N-terminal lyase domain, a central kinase and a putative C-terminal cyclase domains, which lack the zinc ligands characteristic of the other classes; in the recently described class IV, LanL contains the N-terminal lyase and the kinase domains, such as those of class III, and a C-terminal LanC-like cyclase domain (Figure 1.3B) [25,35].

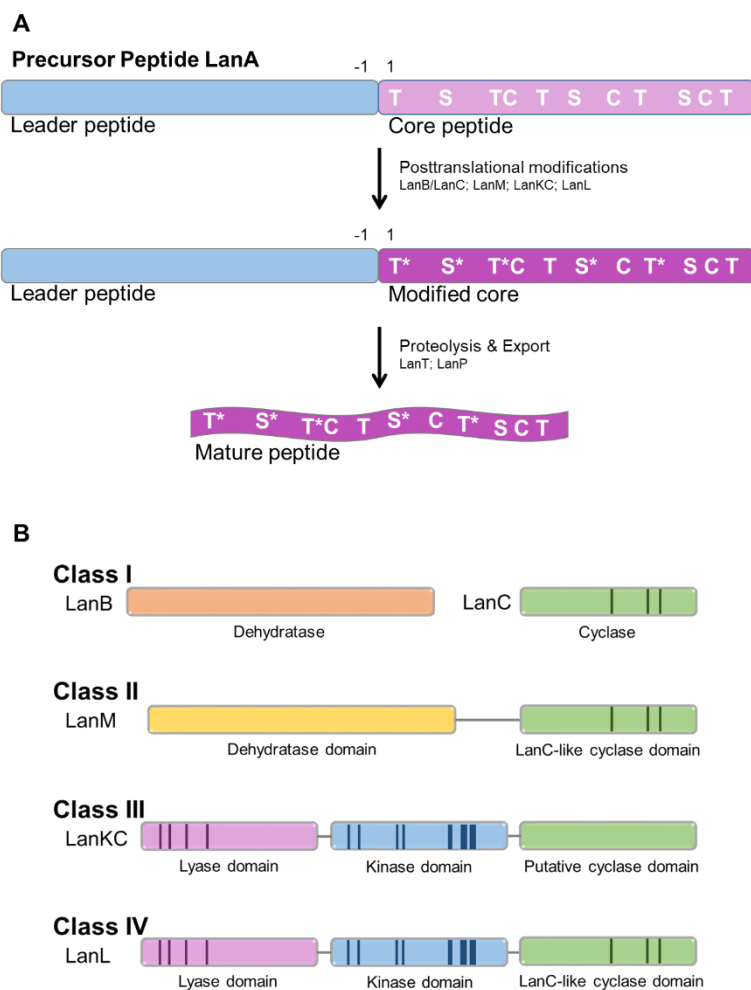


Figure 1.3. (A) General biosynthesis of lanthipeptides, with indication of the amino acid numbering; a non-representative peptide is shown where * indicates the modified residues Dha or Dhb. The mature peptide has a non-linear structure, which results from bridge formation. (B) Scheme of lanthipeptide synthetases involved in the biosynthesis of the four classes of lanthipeptides. Dark coloured stripes indicate conserved regions.

1.3 LANTHIPEPTIDES WITH ANTIMICROBIAL ACTIVITY: THE LANTIBIOTICS

The need for new antimicrobials for clinical, veterinary and food applications is a reality [36]. Outbreaks of many hospital-acquired infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *S. aureus* (VRSA) and vancomycin-resistant enterococci (VRE) [37] are difficult to treat due to the resistance developed by these microorganisms to the drugs currently available. Foodborne infections caused by pathogenic agents, including *Escherichia coli*, *Listeria monocytogenes* and *Salmonella typhimurium* are also of great concern. As such, the scientific and medical community has emphasized that research leading to new antimicrobials is a priority [36–38]. Several approaches can be employed to enlarge the number of available antimicrobials, including the design of new peptides by synthetic biology, genome mining for novel metabolites and traditional screening methods using organisms from diverse environments [39].

Lanthipeptides with antimicrobial activity, the so-called lantibiotics, are a class of compounds that has been gaining relevance and may be a promising alternative to the currently used antibiotics and can also be employed as food preservatives [35,40], as is the case of nisin that has been used as a food additive since the 1950s [41]. Potential applications include human and veterinary medicine as well as in the biochemical, pharmaceutical, agricultural and food industries [23]. A number of features that make lantibiotics ideal candidates for applications in these areas are: *i*) they are active against important pathogenic strains including MRSA and VRE, *ii*) they have high levels of efficacy against relevant pathogens (similar or even higher than the currently used antibiotics) [23,42,43] and *iii*) they present low toxicity toward mammals [37] and have low immunogenicity [23]. These interesting properties are bringing lantibiotics to the spotlight of clinical research and testing [42]. For instance, the results that have been obtained with nisin and microbisporicin to treat *Clostridium difficile* and *S. aureus* infections, respectively, have encouraged their application in medical fields [42]. Nisin, produced by *Lactococcus lactis*, was the first and, so far, the only lantibiotic commercialized both as a biological food preservative and in veterinary medicine [23]. Microbisporicin (also known as NAI-107) is produced by the actinomycetes *Microbispora corallina* and is considered the most potent lantibiotic, with high potential in therapy of nosocomial infections [23,44–46], as shown by *in vivo* models involving several multi-drug resistant Gram-positive pathogens including MRSA [47]. There are pending patents for the use of actagardine (produced by *Actinoplanes liguriae* ATCC 31048) to treat halitosis [42], and mutacin 1140 (produced by *Streptococcus mutans*) to treat dental caries and streptococcal throat infections [48]. The lantibiotic

duramycin, produced by *Streptomyces cinnamomeus* DSM 40646, is in phase II clinical trials for the treatment of cystic fibrosis [42,48].

In general, lantibiotics are poorly absorbed orally, transdermally or via the pulmonary system, and the majority of applications under investigation focus on topical or gastrointestinal use [48]. Another advantage of lantibiotics may be their low propensity to resistance development [23], although *in vitro* studies on the mode of action of nisin suggest that repeated exposure to the lantibiotic can induce resistance [49,50].

Lanthipeptides are ribosomally synthesized and are thus more amenable than non-ribosomally synthesized compounds to the application of molecular biology techniques, including mutagenesis and other bioengineering approaches.[51] The application of these methodologies allows the generation of variants that may result in compounds with improved bioactivity, stability and/or broader spectrum of activity. These methodologies are also powerful for structure-activity relationship (SAR) studies investigating the relevance of each constituting amino acid on the biosynthetic process of the lanthipeptide and on bioactivity [23,51]. Successful examples of modifications performed were already reported for nisin [38], mersacidin [52,53] and lichenicidin [54]. Solid-phase synthesis and *in vitro* mutasynthesis are also useful strategies for the synthesis of modified and novel analogues of lantibiotics, which may be valuable tools to better understand lantibiotics properties [55,56].

The genus *Bacillus* is a prolific source of bioactive compounds, including ribosomal and non-ribosomal antibacterials [57]. In the following sections, we gather general information on lantibiotics, including their biosynthesis (section 1.4), mode of action (section 1.5) and mechanisms of immunity and resistance (section 1.6). In all of them, special attention will be given to the lantibiotics produced by *Bacillus* spp. The subsequent sections (sections 1.7, 1.8 and 1.9) will present the information gathered specifically on lanthipeptides produced by this genus, including (Table 1.1): subtilin, entianin, ericins, clausin, subtilomycin, thuricins, sublichenin, mersacidin, amylolysin, cerecidins, pseudomycoicidin, ticins, haloduracin, thusin, formicin, bicereucin, a particular case, and lichenicidin, the lantibiotic investigated in this study. Special emphasis is given to gene cluster organization, biosynthesis, peptide structure, spectrum of activity and bioengineering. Subtilosin A [58,59] and sublancin [59,60], initially considered lanthipeptides, will not be discussed, since they were reclassified as sactibiotic and glycocin peptides, respectively [35,61,62].

Table 1.1. List of class I and class II lanthipeptides produced by *Bacillus* spp. and their respective molecular masses. The information not available is represented with n.a..

Lantibiotic	Producer(s)	Producer source	Molecular Mass (Da)	Ref.
Class I				
Subtilin			3320	
N-succinylated subtilin	<i>B. subtilis</i> ATCC 6633	n.a.	3421	[63]
Entianin			3303,9	
N-succinylated entianin	<i>B. subtilis</i> subsp <i>spizizenii</i> DSM 15029 ^T	Tunisian desert	3447.6	[64]
Ericin A ¹⁾	<i>B. subtilis</i> A1/3 ¹⁾ and 2)	Greenhouse tomato culture	2987.7	
Ericin S ²⁾	<i>B. velezensis</i> RC 218 ²⁾	Wheat anthers	3342.8	[65]
Clausin	<i>B. clausii</i> O/C	Enterogermina®	2107.5	[66]
Subtilomycin	<i>B. subtilis</i> MMA7	Marine sponge <i>Haliclona simulans</i> , Ireland	3235	[57]
Thuricin 4A-4	<i>B. thuringiensis</i> T01001	Mediterranean flour moth, <i>Ephestia kuhniella</i>	2786.3	[67]
Thuricin 4A-4D			2886.3	[67]
Sublichenin			3348	
N-succinylated sublichenin	<i>B. licheniformis</i> MCC 2512 ^T	Rhizobial soil of <i>Hedysarum coronarium</i>	3448	[68]
Class II				
Mersacidin	<i>Bacillus</i> HIL Y-85,54728, <i>B. amyloliquefaciens</i> Y2	Soil from Mulund (salt pan), India	1825	[69]
Amylolysin	<i>B. amyloliquefaciens</i> GA1	Strawberry fruits, Italy	3318	[70]
Cerecidin	<i>B. cereus</i> As 1.1846	Spoiled soybean milk	A1 to A6: 1988.17 A7: 1958.03	[71]
Pseudomycoicidin	<i>B. pseudomycooides</i> DSM 12442	Soil, Ghana	2786	[72]
Ticins	<i>B. thuringiensis</i> BMB 3201	Soil, China	A1: 4062.9 A3: 4048.9 A4: 4063.0	[73]
Haloduracin	<i>B. halodurans</i> C-125	Soil, Japan	α : 3043.3 β : 2330.0	[34]
Thusin	<i>B. thuringiensis</i> BGSC 4BT1	Red soil, China	α : 3928.9 β : 2908.5 β' : 2922.5	[74]
Formicin	<i>B. paralicheniformis</i> APC 176	Mackerel (<i>Scomber scombrus</i>) intestinal tract, coast of Ireland	α : 3254.3 β : 2472.1	[75]
Bicereucin	<i>B. cereus</i> SJ1	Chromium-contaminated wastewater a metal factory	α : 3486 β : 4100	[76]
Lichenicidin	<i>B. licheniformis</i> ATCC 14580, DSM13, 189 ^{a)} , VK21, WIT562/564/566 ^{b)}	^{a)} Hot spring, Portugal; ^{b)} Marine seaweed	α : 3250 β : 3020	[77]

Due to the increasing number of available genomes, as well as the emergence of better platforms for genome mining of secondary metabolites encoding genes, such as antiSMASH 2.0 [78], BAGEL4 [79], RiPPquest [80] or RiPPMiner [81], more lanthipeptides will almost certainly be discovered. Currently, none of the lantibiotics produced by *Bacillus* spp. have entered clinical trials. Even so, the lanthipeptides produced by *Bacillus* spp. are effective against clinically relevant pathogens, such as MRSA and VRE. Furthermore, these peptides

should be investigated as food preservatives. Despite the advances already made, more studies are needed regarding the application of these peptides in both food and for medical use. These studies should focus on a more comprehensive characterization of the activity of all the lantibiotics produced and the potency determined against the wide range of bacteria which impact the food industry and animal and human health.

The Genbank accession numbers of the lantibiotics produced by *Bacillus*: Subtilin: U09819; Entianin: HQ871873; Ericin: AF233755; Clausin: AP006627; Subtilomycin: JX912247; Thuricin: KP133062; Sublichenin (partial sequence): MF399478; Mersacidin: AJ250862.2; Amylolysin: KC415250; Cerecidin: KJ000001; Pseudomycoicidin: NZ_CM000745 and NZ_ACMX01000000; Ticins: KR869822; Haloduracin: BA000004.3 genome, gene cluster identified from bh0445 to bh0455; Thusin: KT454399; Formicin: LXP000000000 genome; Velezensicidin: RBZX01000000 genome assembly; Bicereucin: ADFM00000000.1 genome; Lichenicidin: AE017333.1 genome, gene cluster identified from BLi04116 to BLi04128.

1.4 BIOSYNTHESIS OF CLASS I AND CLASS II LANTHIPEPTIDES

Lanthipeptides are a group of post-translationally modified peptides characterized by the presence of lanthionine (Lan) or methyllanthionine (MeLan) bridges. Currently, they are classified into four classes [35], but the lantibiotics described to date belong only to classes I and II. Also, only gene clusters of these two classes were identified in *Bacillus* spp. strains.

Their biosynthesis involves the formation of a precursor peptide LanA, which is converted to an active compound after several post-translational modifications. LanA can be physically divided into an N-terminal leader peptide and a C-terminal core peptide (Figure 1.4). Cys residues are never found within the leader sequence, whereas core peptides always possess several Ser, Thr and Cys amino acid residues that are required for the formation of Lan and MeLan thioether rings. In this process, Ser and Thr residues may be dehydrated to 2,3-didehydroalanine (Dha) and (*Z*)-2,3-didehydrobutyrine (Dhb), respectively. Lan and MeLan are subsequently formed by intramolecular Michael addition of Cys thiols to Dha or Dhb, respectively. Typically, class I and class II lanthipeptides contain three to six Lan/MeLan and several Dha and Dhb units; other structural modifications include S-aminovinyl-D-cysteine (AviCys; e.g., clausin produced by *Bacillus* spp., and epidermin and gallidermin produced by *Staphylococcus* spp. strains) [25,82], S-aminovinyl-3-methyl-D-cysteine (AviMeCys; e.g., mersacidin, produced by *Bacillus* spp.) [25], hydroxylation (in cinnamycin and duramycins) and epimerization (in lacticin 3147) reactions [23,83]. The structural gene encoding the LanA precursor peptide is usually clustered in an operon together with genes encoding the respective modification enzymes [23]. The main difference

between class I and II lanthipeptides lies in the enzymes involved in maturation and transport. Accordingly, in class I lanthipeptides, LanB and LanC enzymes catalyze dehydration and cyclization, respectively, whereas in class II lanthipeptides, both reactions are carried out by the bifunctional synthetase LanM (Figure 1.4) [23]. The leader peptide seems to be important for secretion of the compound from the producer cell [25,84], albeit its removal is required for full activity [25,85,86]. In class I lanthipeptides, transport is accomplished by a generic ABC transporter, LanT, and the leader peptide is cleaved by a LanP protease. In class II lanthipeptides, both processes are mediated by a single bifunctional enzyme – LanT – comprising an ABC transporter, a transmembrane protein and a proteolytic domain (Figure 1.4). Generally, this protein cleaves at a GG/GA/GS-motif, which is present in all class II leader peptides. In addition to the genes encoding proteins involved in processing of lantibiotics, the biosynthetic clusters of class I and class II lanthipeptides contain the genetic determinants for self-protection of the producer (*lanFGEHI*) and peptide synthesis regulation (*lanRK*). Within the class II lanthipeptides there is a subclass known as two-peptide lantibiotics (also referred to as two-component peptides) [35]. In this case, the biosynthetic cluster comprises two structural genes which encode the α - and β -peptides. Distinct LanM proteins modify each peptide, but the same LanT protein removes the leader peptides and their transport from the cell [87]. Haloduracin and lichenicidin are examples of lanthipeptides produced by *Bacillus* spp. belonging to this group. More detailed information regarding the general biosynthesis of class I and class II lanthipeptides is available in published reviews [23,25,35,88].

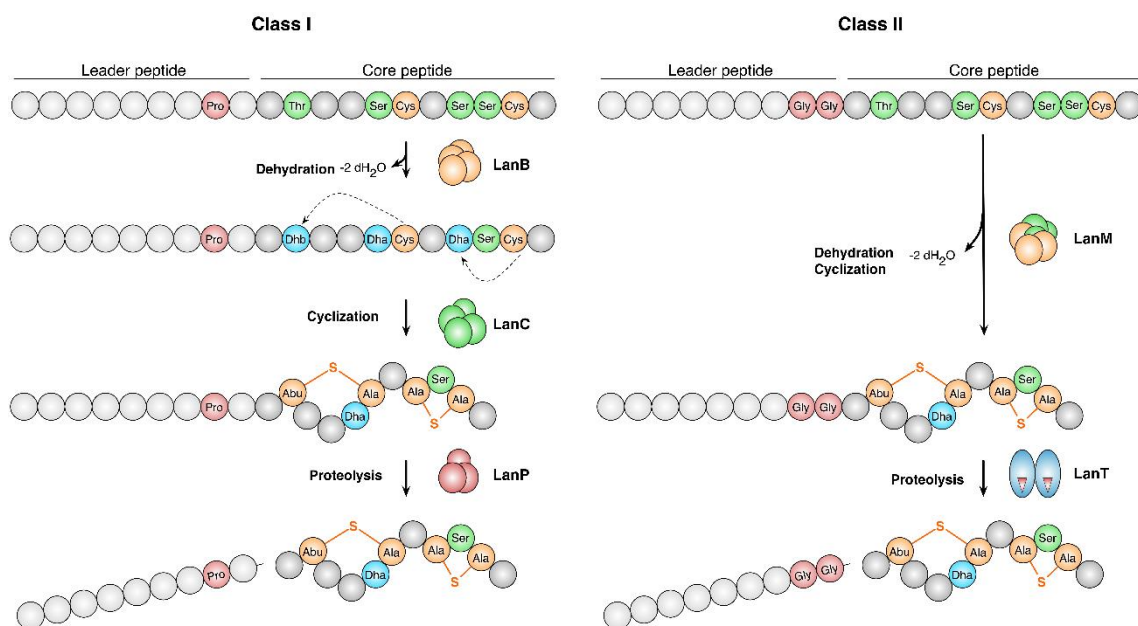


Figure 1.4. General overview of the dehydration, cyclization and leader peptide removal steps involved in the biosynthesis of class I and II lanthipeptides. The relevant residues for leader peptide removal are represented in maroon. The residues that can undergo dehydration are colored in green and those that are dehydrated are colored in blue. The Lan and MeLan rings are orange.

1.5 MODE OF ACTION AND BIOACTIVITY SPECTRUM

Despite the numerous studies conducted to unravel the mode of action of class I and class II lanthipeptides, further investigation is still needed to fully clarify how lantibiotics interact with the target cells at the molecular level. Such investigations could contribute to a better understanding of the biosynthesis and functional role of post-translational modifications. The majority of class I and class II lanthipeptides are lantibiotics, typically with activity against Gram-positive bacteria. In Gram-negative bacteria, the presence of the outer membrane constitutes a natural barrier to these compounds [89]. However, it was shown that some strains of *E. coli*, *Helicobacter pylori* and *Neisseriae* can be affected if high lantibiotic concentrations are applied [23]. A large number of the lantibiotics described so far target lipid II, which is a precursor of the peptidoglycan, found in the outer layer of the bacterial membrane. Lipid II is the target binding molecule of several lantibiotics, mediating two different modes of action (Figure 1.5): *i*) causing physical sequestration of lipid II molecules, thus inhibiting peptidoglycan synthesis by preventing the catalysis of important steps of the cell wall assembly, such as the polymerization of lipid II and the crosslinking of the glycan chains; and *ii*) serving as docking molecule to induce the formation of defined and stable pores that cause membrane damage and depolarization, leading to intracellular content leakage and ultimately cell death [23,37,90–92].

Mersacidin, actagardine, and cinnamycin block cell wall synthesis (Figure 1.5). In the case of nisin, the same peptide performs both activities to induce bacterial death. In addition to these, the class of two-peptide lantibiotics, such as lacticin 3147, haloduracin and lichenicidin, requires the presence of two mature peptides acting synergistically to achieve full activity [93]. The general mechanism of action of the two-peptide lantibiotics has been proposed based on the mode of action of lacticin 3147 and haloduracin (Figure 1.5). In these cases, each peptide performs one of the functions described above, so that the combined activity of the two peptides resembles that of nisin [91,92,94,95]. Briefly, the α -peptide binds to the peptidoglycan precursor lipid II in a 2:1 stoichiometry, thus inhibiting cell wall biosynthesis [94,95]. This complex then recruits the β -peptide that inserts within the membrane leading to pore formation. The synergistic activity between Lan α and Lan β occurs at equimolar concentrations, with the formation of a 2:2:1 Lan α :Lan β :lipid II complex [91,94].

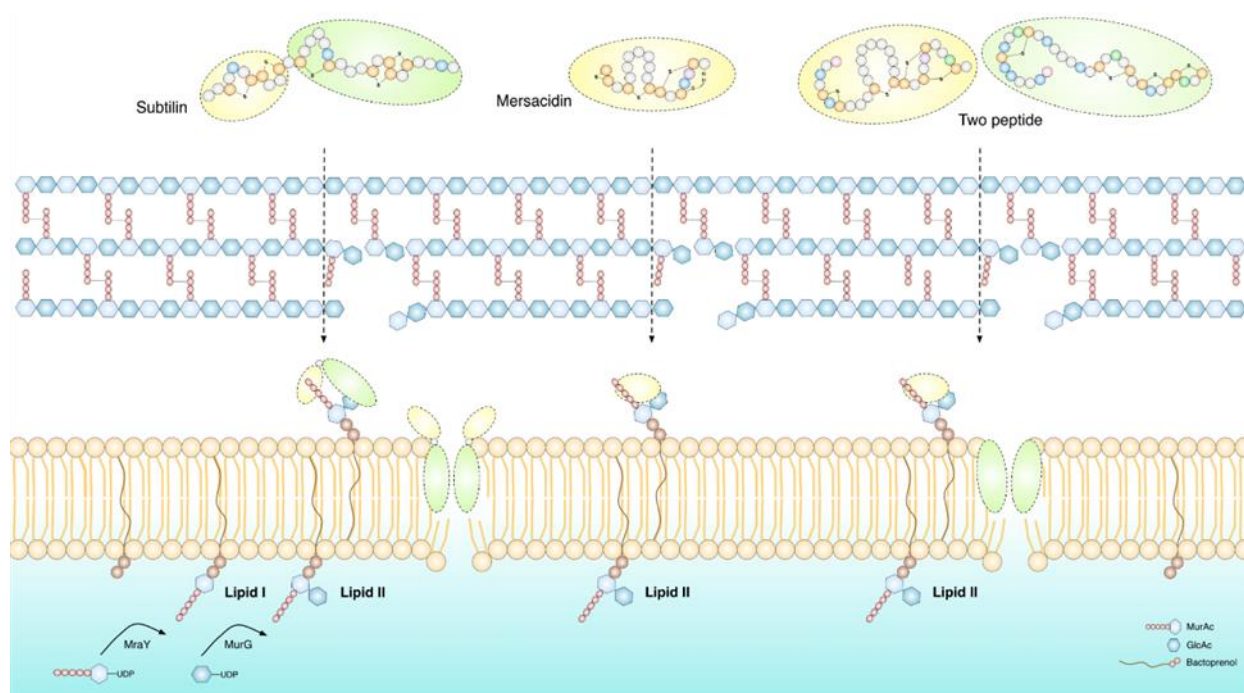


Figure 1.5. Schematic representation of the mode of action of *Bacillus* spp. antibiotics, which involves binding of the target molecule, lipid II, preventing the correct cell-wall synthesis. Additionally, pore formation can occur with disruption of the cellular membrane. The mechanism of action of the two-peptide antibiotics was demonstrated for lactacin 3147 [92].

Among the antibiotics produced by *Bacillus* spp., only the mode of action of subtilin, clausin, mersacidin and haloduracin have been investigated in detail and will be later discussed (sections 1.7.1, 1.7.5, 1.8.1 and 1.8.6, respectively). Regarding the minimal inhibitory concentrations (MIC) and the spectrum of activity of antibiotics produced by *Bacillus* spp., subtilin seems to be the most potent antibiotic produced by *Bacillus* spp. strains, with a MIC of 0.05 µg/mL against *Micrococcus luteus* NDCO8166 (Table 1.2) [63,96]. As shown in Table 1.2, the efficacy of antibiotics seems to be strain-specific. Some examples are: *i*) MIC of mersacidin against *S. aureus* SG511 is 35 times higher than against *S. aureus* 137/93A, *ii*) MIC of the same antibiotic against *Enterococcus faecium* ATCC 29212, is 2 times higher than against *E. faecium* ATCC 19579 and *iii*) haloduracin, which is approximately 8 times more active against *L. lactis* HP (MIC of 0.4 µg/mL) than against *L. lactis* ATCC 11454 (MIC of 3.4 µg/mL). However, the methodologies employed to determine the MIC for these compounds are not standardized, particularly with respect to: *i*) the initial inoculum of the indicator strain, *ii*) the culture medium to perform the tests, *iii*) the species and strains tested and *iv*) the compounds to be used for quality control and normalization. Thus, it remains unclear whether the discrepancy in MIC values is due to the strain used, to the structural differences of the peptides (or their purity) or even an outcome of the different methodologies used for MIC determination. This shows the need to adopt a

standardized procedure for MIC determination, and the importance of standard positive controls for comparison. Some possible solutions to these problems are: *i)* MIC determination of the pure peptides according to the guidelines proposed by EUCAST, *ii)* pure compounds already available in the market (such as nisin) should be established as quality control and included in each MIC determination test and *iii)* a minimum of indicator strains to be tested should be defined *à priori*, and all of these strains must be available in culture collections. The adoption/implementation of such simple measures will also allow the normalization of results between research laboratories.

Therefore, a comparison between the activities of the different lantibiotics is sometimes difficult. For example, despite the structural similarity of subtilin and entianin, the MIC of subtilin against *M. luteus* NDCO8166 is 0.05 µg/mL [63,96], whereas the MIC of entianin against *M. luteus* ATCC 9341 is 4 to 8 µg/mL [64] (Table 1.2).

Table 1.2. Minimal inhibitory concentrations of *Bacillus* spp. lantibiotics determined against different indicator strains. It should be noted that the test conditions and compound purity could differ among the different assays reported.

Lantibiotic	Indicator Strains	MIC	Ref.
<i>Class I</i>			
Subtilin; S-Subtilin ^{a)}	<i>Lactococcus lactis</i> MG1614	1 µg/mL; 19 µg/mL	[63,96]
	<i>Micrococcus luteus</i> NDCO8166	0.05 µg/mL; 0.33 µg/mL	
Entianin (unsuccinylated)	<i>Enterococcus faecalis</i> ATCC 29212	16 µg/mL	4842.8 nM
	<i>Enterococcus faecalis</i> ATCC 51299	8 – 16 µg/mL	2320.4 - 4842.8 nM
	<i>Micrococcus luteus</i> ATCC 9341	4 – 8 µg/mL	1160.2 - 2320.4 nM
	<i>Staphylococcus aureus</i> ATCC 29213	4 – 8 µg/mL	1160.2 - 2320.4 nM
	<i>Staphylococcus aureus</i> ATCC 43300 (MRSA)	8 µg/mL	2320.4 nM
Ericins	MIC values not available; Obs.: Bioactivity profile similar to subtilin		[97]
Clausin	MIC values not available; Obs.: Active against Gram-positive bacteria (e.g. <i>Clostridium difficile</i> , <i>Clostridium perfringens</i> , <i>Enterococcus faecium</i> , <i>Lactococcus lactis</i> , <i>Listeria monocytogenes</i> , <i>Micrococcus</i> spp. and <i>Staphylococcus aureus</i>)		[98]
Subtilomycin	MIC values not available; Obs.: Active against Gram-positive (e.g. <i>Listeria monocytogenes</i> , <i>Clostridium sporogenes</i> , MRSA) and some Gram-negative bacteria (e.g. <i>Aeromonas hydrophila</i> , <i>Vibrio anguillarum</i> , <i>Alteromonas</i> sp. and <i>Pseudomonas aeruginosa</i>)		[57]
Thuricin 4A-4; Thuricin 4A-4D ^{a)}	<i>Bacillus cereus</i> UW85	21.7 µg/mL; 62.3 µg/mL	7800 nM; 21600 nM
	<i>Bacillus firmus</i> BCRC11944	0.91 µg/mL; 3.4 µg/mL	326 nM; 1350 nM
	<i>Bacillus subtilis</i> 168	14.5 µg/mL; 31.2 µg/mL	5200 nM; 10800 nM
	<i>Bacillus subtilis</i> Bsn5	14.5 µg/mL; 31.2 µg/mL	5200 nM; 10800 nM
	<i>Bacillus thuringiensis</i> BMB171	3.6 µg/mL; 15.0 µg/mL	1300 nM; 5200 nM
	<i>Bacillus thuringiensis</i> YBT1518	3.6 µg/mL; 7.5 µg/mL	1300 nM; 2600 nM
	<i>Bacillus pumilus</i> SCG I	1.8 µg/mL; 250.0 µg/mL	650 nM; 86600 nM
	<i>Enterococcus faecalis</i> Bom3	7.2 µg/mL; 15.0 µg/mL	2600 nM; 5200 nM
	<i>Staphylococcus sciuri</i> Bom1	5.4 µg/mL; 124.7 µg/mL	1950 nM; 43200 nM
	<i>Microbacterium</i> Pri2	14.5 µg/mL; 15.0 µg/mL	5200 nM; 5200 nM

Lantibiotic	Indicator Strains	MIC	Ref.
Sublunchenin	<i>Pediococcus lolii</i> MCC 2972	1.5 µg/mL	450 nM
	<i>Enterococcus durans</i> B20G1	0.5 µg/mL	150 nM
	<i>Enterococcus faecalis</i> MF3	0.5 µg/mL	150 nM
	<i>Enterococcus faecalis</i> MM2	0.25 µg/mL	75 nM
	<i>Enterococcus faecalis</i> CHL1	0.5 µg/mL	150 nM
	<i>Enterococcus faecalis</i> CHL3	0.25 µg/mL	75 nM
	<i>Enterococcus faecalis</i> CHL	0.5 µg/mL	150 nM
	<i>Enterococcus faecalis</i> MCC 30630.5 µg/mL	150 nM	
	<i>Enterococcus faecalis</i> MCC 27730.5 µg/mL	150 nM	
	<i>Enterococcus faecium</i> MCC 2763	0.75 µg/mL	225 nM
	<i>Enterococcus avium</i> CS32+	1.5 µg/mL	450 nM
	<i>Enterococcus cecorum</i> 1-40a	1.5 µg/mL	450 nM
	<i>Lactobacillus plantarum</i> MCC 2774	0.75 µg/mL	225 nM
	<i>Kocuria rhizophila</i> ATCC 9431	0.5 µg/mL	150 nM
	<i>Listeria monocytogenes</i> Scott A	2.0 µg/mL	600 nM
	<i>Staphylococcus aureus</i> MTCC 96	5.0 µg/mL	1500 nM
	<i>Staphylococcus aureus</i> ATCC 43300 (MRSA)	7.0 µg/mL	2100 nM
	<i>Escherichia coli</i> ETEC	>8.0 µg/mL	>2400 nM
	<i>Klebsiella pneumoniae</i> ATCC 10031	10.0 µg/mL	3000 nM
Class II			
Mersacidin; MersacidinP3W ^{a)}	<i>Enterococcus faecium</i> ATCC 19579	32 µg/mL; 16 µg/mL	17534.2 nM; 8583.6 nM
	<i>Enterococcus faecium</i> 7131121 (VRE)	64 µg/mL; 32 µg/mL	35068.5 nM; 17167.3 nM
	<i>Enterococcus faecalis</i> ATCC 29212	64 µg/mL; 32 µg/mL	35068.5 nM; 17167.3 nM
	<i>Micrococcus luteus</i> ATCC 4698	1.2 µg/mL; 0.5 µg/mL	657.5 nM; 268.2 nM
	<i>Staphylococcus epidermidis</i> NCTC 11047	16.32 µg/mL; 8 µg/mL	8942.5 nM; 4291.8 nM
	<i>Staphylococcus aureus</i> SH1000	32 µg/mL; 16 µg/mL	17534.2 nM; 8583.6 nM
	<i>Staphylococcus aureus</i> R33 (MRSA)	32 µg/mL; 8 µg/mL	17534.2 nM; 4291.8 nM
	<i>Staphylococcus aureus</i> SG511	1 µg/mL; nd	547.9 nM; nd
	<i>Staphylococcus aureus</i> 137/93A	35 µg/mL; nd	19178.1 nM; nd
	<i>Staphylococcus aureus</i> 137/93G	30 µg/mL; nd	16438.4 nM; nd
Amylolysin	<i>Streptococcus pneumoniae</i> BAA-255	2 µg/mL; 2.4 µg/mL	1095.9 nM; 1287.5 nM
	<i>Bacillus cereus</i> RFB125 ^c	0.7 µg/mL	200 nM
	<i>Bacillus megaterium</i> RFB124	1.3 µg/mL	400 nM
	<i>Bacillus subtilis</i> ATCC 6633	4.6 µg/mL	1400 nM
	<i>Enterococcus faecalis</i> ATCC 29212	4.6 µg/mL	1400 nM
	<i>Enterococcus faecium</i> RFB128	0.3 µg/mL	100 nM
	<i>Listeria innocua</i> ATCC 33090	2.3 µg/mL	700 nM
	<i>Listeria monocytogenes</i> LMG 21263	1.7 µg/mL	500 nM
	<i>Listeria monocytogenes</i> LMG	1.3 µg/mL	400 nM

Lantibiotic	Indicator Strains	MIC	Ref.
	23905		
	<i>Micrococcus luteus</i> ATCC 9341	2.3 µg/mL	700 nM
	<i>Staphylococcus aureus</i> ATCC 25923	9.3 µg/mL	2800 nM
	<i>Staphylococcus aureus</i> ATCC 43300 (MRSA)	1.3 µg/mL	400 nM
	<i>Staphylococcus aureus</i> RFB127	4.6 µg/mL	1400 nM
	<i>Staphylococcus epidermis</i> ATCC 1228	9.3 µg/mL	2800 nM
	<i>Streptococcus agalactiae</i> RFB141	9.3 µg/mL	2800 nM
Cerecidins	MIC values not available; Obs.: Active against <i>Staphylococcus aureus</i> 1-1, <i>Enterococcus faecalis</i> V583 and some <i>Bacillus</i> species ^{b)}		[71]
Pseudomycoicidin	MIC values not available; Obs.: Active against <i>Micrococcus luteus</i> ATCC 1856, <i>Staphylococcus aureus</i> (including MRSA strains), <i>Staphylococcus simulans</i> , <i>Lactococcus lactis</i> NCTC 497, <i>Streptococcus</i> G 3645 and several <i>Bacillus</i> species		[72]
Ticins (A4)	<i>Bacillus amyloliquefaciens</i> X1	4.0 µg/mL	980 nM
	<i>Bacillus cereus</i> ATCC 14579	16.0 µg/mL	3910 nM
	<i>Bacillus cereus</i> ATCC 49064	2.0 µg/mL	490 nM
	<i>Bacillus cereus</i> VD 048	2.0 µg/mL	490 nM
	<i>Bacillus firmus</i> DS-1	8.0 µg/mL	1950 nM
	<i>Bacillus subtilis</i> 168	32.0 µg/mL	7810 nM
	<i>Bacillus subtilis</i> Bsn5	8.0 µg/mL	1950 nM
	<i>Bacillus thuringiensis</i> BMB171	3.0 µg/mL	975 nM
	<i>Bacillus thuringiensis</i> YBT1518	2.0 µg/mL	490 nM
	<i>Bacillus pumilus</i> SCG I	12.0 µg/mL	3010 nM
	<i>Listeria monocytogenes</i> LM201	8.0 µg/mL	1950 nM
	<i>Listeria monocytogenes</i> LM605	4.0 µg/mL	980 nM
	<i>Paenibacillus</i> strain X2	8.0 µg/mL	1950 nM
	<i>Staphylococcus aureus</i> CMCC 26003	64.0 µg/mL	15630 nM
	<i>Staphylococcus aureus</i> ATCC 43300	128.0 µg/mL	31250 nM
	<i>Staphylococcus sciuri</i> Bom1	16.0 µg/mL	3910 nM
	<i>Microbacterium</i> strain Pri2	16.0 µg/mL	3910 nM
	<i>Enterococcus faecalis</i> ATCC 29212	2.0 µg/mL	490 nM
Haloduracin*	<i>Bacillus subtilis</i> ATCC 6633	2.5 µg/mL	469 nM
	<i>Enterococcus faecium</i> C33105	4.2 µg/mL	781 nM
	<i>Lactococcus lactis</i> ATCC 11454	3.4 µg/mL	625 nM
	<i>Lactococcus lactis</i> HP (ATCC 11602)	0.4 µg/mL	73.4 nM
	<i>Lactococcus lactis</i> 481 (CNRZ 481)	1 µg/mL	195 nM
	<i>Micrococcus luteus</i> ATCC 4698	6.7 µg/mL	1250 nM
	<i>Staphylococcus aureus</i> ATCC 12600	8.4 µg/mL	1560 nM
	<i>Staphylococcus aureus</i> C5 (MRSA)	25.2 µg/mL	4690 nM
Thusin*	<i>Staphylococcus epidermidis</i> 15X	1.7 µg/mL	313 nM
	<i>Staphylococcus mutans</i> ATCC 25175	13.4 µg/mL	2500 nM
	<i>Bacillus amyloliquefaciens</i> X1	11.3 µg/mL	1560 nM
	<i>Bacillus cereus</i> ATCC 14579	5.65 µg/mL	780 nM
	<i>Bacillus subtilis</i> Bsn5	5.65 µg/mL	780 nM
	<i>Bacillus thuringiensis</i> BMB171	2.8 µg/mL	390 nM

Lantibiotic	Indicator Strains	MIC	Ref.
	<i>Bacillus pumilus</i> SCG I	2.8 µg/mL	390 nM
	<i>Listeria monocytogenes</i> LM201	5.65 µg/mL	780 nM
	<i>Listeria monocytogenes</i> LM605	5.65 µg/mL	780 nM
	<i>Staphylococcus aureus</i> CMCC 26003	11.3 µg/mL	1560 nM
	<i>Staphylococcus aureus</i> ATCC 43300	11.3 µg/mL	1560 nM
	<i>Staphylococcus aureus</i> MRSA	11.3 µg/mL	1560 nM
	<i>Staphylococcus sciuri</i> Bom1	5.65 µg/mL	780 nM
	<i>Enterococcus faecalis</i> ATCC 29212	22.6 µg/mL	3130 nM
	<i>Streptococcus pneumoniae</i> ATCC 49619	11.3 µg/mL	1560 nM
Formicin*	MIC values not available; Obs.: Highly active against several <i>Lactobacillus</i> species, <i>Micrococcus luteus</i> and <i>Streptococcus mutans</i> ; also active against <i>Enterococcus</i> , <i>Clostridium</i> and <i>Listeria</i> species, and <i>Staphylococcus aureus</i>		[75]
Bicereucin ^{c)}	<i>Bacillus subtilis</i> ATCC 6633	3.5+2.0 µg/mL	1000+500 nM
	<i>Micrococcus luteus</i> ATCC 4698	1.75+1.0 µg/mL	500+250 nM
	<i>Lactococcus lactis</i> 481 CNRZ 481	17.5+10.0 µg/mL	5000+2500 nM
	<i>Staphylococcus epidermidis</i> 15x	43.75+25.0 µg/mL	12500+6250 nM
	<i>Streptococcus mutans</i> ATCC 25175	70.0+40.0 µg/mL	20000+10000 nM
	<i>Staphylococcus aureus</i> Rosenbach ATCC BAA-1717 (MRSA)	43.75+25.0 µg/mL	12500+6250 nM
Lichenicidin*	<i>Enterococcus faecium</i> ATCC 700221 (VRE)	8,75+5.0 µg/mL	2500+1250 nM
	<i>Bacillus megaterium</i> VKM41	3 µg/mL	480 nM
	<i>Bacillus subtilis</i> L1	6 µg/mL	960 nM
	<i>Micrococcus luteus</i> B1314	1 µg/mL	170 nM
	<i>Staphylococcus aureus</i> 209p	6 µg/mL	960 nM

* Considering 1:1

a) The MIC values for both compounds are included and separated by the “;” symbol.

b) IC₅₀ (µg/mL) values are available for some indicator strains: *Micrococcus flavus* NCIB8166 – 0.5 for CerA1, 0,125 for CerA7; *Bacillus cereus* As 1.352 – 4 for CerA1, 1 for CerA7; *Enterococcus faecalis* V583 – 8 for CerA1, 2 for CerA7; *Staphylococcus aureus* 1-1 – 8 for CerA1, 4 for CerA7 [71].

c) Considering bicereucin peptides ratio 2:1 Bsja:Bsjβ

1.6 LANTIBIOTIC: RESISTANCE AND IMMUNITY

One of the advantages to support more intensive application of lantibiotics is that significant levels of resistance to nisin, the most widely used lantibiotic in the food industry to date, have not spontaneously developed. [23]. Thus, it is assumed that, due to their particular mode of action, lantibiotics are less likely to induce the development of resistance. However, a widespread use of these compounds for clinical and veterinary applications might lead to the selection of resistant strains. More importantly, several mechanisms of innate resistance have been already described, for a vast number of bacteria [93]. First of

all, the natural composition of the membrane of several bacterial cells works as a barrier to lantibiotics activity. This is the case of most Gram-negative cells that are naturally resistant to the majority of the lantibiotics described so far, due to the presence of an outer membrane that prevents the entry of the lantibiotics [102]. Also, the lipid composition influences the interaction of these (usually cationic) peptides with the cell membrane, i.e. a higher percentage of negatively charged lipids on the surface facilitates the interaction with the positively charged peptide. On the contrary, decreased amounts of anionic lipids and the presence of zwitterionic lipids increases the levels of resistance [93]. D-alanylation of the cell wall teichoic acids and lysinylation of a class of membrane phospholipids (phosphatidylglycerol) contribute to the addition of positive charges to the cell surface and that was reported to occur in several pathogens, including in *S. aureus* [103,104]. Regarding lipid II, which is the anchor molecule for the activity of various lantibiotics, it seems that resistance levels are independent of its amount in the target cells, as this molecule is essential for the correct assembly and functionality of the cell membrane [105].

Some resistance mechanisms are specific for one lantibiotic or lantibiotic-type, but most of them are related to intrinsic responses against environmental stressors [93]. Among these, several two-component systems have been reported. They allow the cells to recognize and respond to specific environmental conditions, inducing a cascade of reactions that might result in one or a combination of the following: *i*) activation of efflux pumps that actively impair lantibiotic activity; *ii*) alteration of the thickness, fluidity or charged state of the cell membrane surface; *iii*) internalization of the lantibiotic target that becomes inaccessible for binding; and *iv*) chelation of the lantibiotic's target or the lantibiotic itself, impairing its docking. Some of these two-component systems sense the presence of the lantibiotic itself while others recognize the cellular damage being induced, regardless of the causing agent. Additionally, for some cell wall inhibitors, it was reported that inducing the overexpression of the regulatory genes does not elicit higher expression of the effector genes, rather the presence of the inhibitor itself is required to induce a response [93]. General resistance mechanisms also include the production of biofilms that become more resistant than the planktonic cells. While some lantibiotics are able to prevent the formation of biofilms if previously applied to the planktonic cells, its application to established biofilms is not as efficient, requiring higher peptide concentrations and depending on the age of the biofilm [93].

With respect to nisin, the first lantibiotic applied industrially, two different proteins that are able to impair its activity were identified: the nisin resistance protein (NSR) and the nisinase. NSR is a 35 kDa protein that cleaves nisin, originating a truncated peptide with significantly reduced bactericidal activity due to its reduced ability to form pores in the target membrane [106]. The NSR protein gene has been identified in plasmids of various bacterial species,

which may indicate possible dissemination by horizontal gene transfer [106,107]. Nisinase is an enzyme that can inactivate both nisin and subtilin [108]. This specific enzyme is a dehydropeptide reductase that specifically reduces the C-terminal lysine on nisin [109]. The efficacy of the general resistance mechanisms is dependent not only on the lantibiotic mode of action but also on the target strain, the initial number of bacterial cells and their growth phase.

Self-immunity is the natural process by which producing strains prevent cell death due to the action of their own lantibiotic. These self-immunity mechanisms are usually highly specific [110]. Because of this, cross immunity is not frequently observed, meaning that a certain lantibiotic producer might be susceptible to other lantibiotics, even if they are closely-related [110]. However, although extremely rare, the occurrence of cross immunity was already identified [111]. Self-immunity genes are part of the lantibiotic biosynthetic cluster and they often possess their own regulatory determinants. The immunity proteins described so far belong to one of the following categories: *i)* LanI, an individual immunity protein; *ii)* LanFEG, an ABC transporter; and *iii)* LanH, that has different functionalities and is not required for the most lantibiotics. Some lantibiotic producers possess only one type of these proteins (subtilomycin and thuricin – *lanI*; mersacidin – *mrsFGE*), others two (subtilin and lacticin 3147) and others have the three types (lichenicidin). The haloduracin gene cluster, in particular, possesses two groups of *halFGE* genes [110].

LanI proteins have low homology between them, as they are thought to directly interact with their related lantibiotics [110,112]. They are usually involved in sequestering the lantibiotic molecules causing aggregation, thus impairing lantibiotic-membrane interaction [107]. This mechanism was proposed for LctI, lacticin 3147 immunity protein. However, this is a two-peptide lantibiotic, which raised questions regarding the interaction between LctI and both lacticin peptides: is the protein able to aggregate both peptides? If not, with which peptide does it interact? Is the immunity protein responsible for impairing the interaction between the two peptides? Recent studies suggest that, in this case, the immunity protein does not bind to lacticin peptides, rather it shields lipid II, the lacticin target on the cell membrane, preventing lacticin bactericidal activity [110,113].

The ABC transporter LanFEG actively transports the lantibiotic molecules that are able to cross the cytoplasmic membrane into the cell of its producer. They are the product of the expression of three genes (except for lacticin 3147, which have only two genes reported): *i)* *lanF* corresponding to an ATPase domain that binds and hydrolyses ATP, facilitating the export process; and *ii)* *lanE* and, in most of the cases, *lanG*, that encode hydrophobic transmembrane domains. LanG is thought to be able to interact with the pore forming domain of the lantibiotic itself in some cases. This is consistent with the fact that LanG

proteins from different producers share less similarities among them, when compared to LanF and LanE proteins [110].

When present, LanH usually plays a role on the correct assembly of the ABC transporter LanFEG. This type of auxiliary proteins is often associated with systems that require the immediate release of substrates into the extracellular medium. NukH, involved in nukacin ISK-1 self-immunity, was reported to present a unique activity: it binds and inactivate nukacin, that can not be recovered in its active form, unlike in similar assays involving other lantibiotics [110].

When more than one system is present in a lantibiotic producer, their specific contribution to its immunity does not follow a clear pattern. For example, NisI has higher impact on nisin self-immunity than NisFEG while subtilin SpaFEG seems to provide higher immunity than SpaI. This last scenario was also observed for nukacin immunity proteins NukFEG and NukH. However, the effect of both Nuk systems seems to be cooperative rather than simply additive, implying some kind of interaction between them [110]. This is also the case of lacticin 3147 immunity genes LtnI and LtnFEG cooperative activity [111]. The expression of the self-immunity genes occurs constitutively in the most of the producing strains, as well as the respective structural gene *lanA*. The basal expression levels of the immunity genes provide immediate protection for the cell when first contact with the lantibiotic occurs. When production of high amounts of lantibiotic is activated, usually by auto-induction, both *lanA* and the self-immunity genes promoters are activated and intensely expressed. Epidermin regulation is unique as it seems to involve a transcriptional activator that elicits the expression of both the structural gene and the respective self-immunity genes [114]. Another unusual lantibiotic is Pep5, as its self-immunity gene, *pepI*, is translated into a single mRNA along with *pepA* structural gene [115]. Also noteworthy, is the regulation of the two-peptide lantibiotic cytolysin, including the self-immunity protein CylI, which is through a quorum sensing mechanism, where the trigger molecule is only one of the peptides, CylILs, in its active form [116].

Concerning all this, it is now clear why cross immunity is not common among lantibiotic producers. However, studies have demonstrated that the insertion of lantibiotic immunity genes into a susceptible strain is, most of the times, effective to provide immunity to that specific lantibiotic. For example, staphylococcin C55 producers present cross immunity to the closely related lacticin 3147; in addition, homologues of lacticin immunity genes were identified in other non-producer strains, which were shown to provide some level of resistance against this lantibiotic on the heterologous producer [111].

The hypothesis that this might also occur in nature, through horizontal gene transfer, can not be excluded, which accompanied by the selective pressure exerted by the use of these compounds may lead to the prevalence of lantibiotic resistance phenotypes. In fact, genes

conferring resistance to nisin, mersacidin, lactacin 481, among others, which resemble their self-immunity genes, were already reported in some human pathogenic bacteria, namely, *S. aureus* and *C. difficile*. In general, these resistance genes encode ABC transporters and are regulated by dedicated two-component systems, consisting of a histidine kinase and a response regulator responsible for sensing the presence of the lantibiotics and inducing gene transcription [117].

Even so, resistance levels reported to date are still low compared to commonly used antibiotics. Thus, highly resistant strains, especially to nisin, are being artificially developed to predict possible paths underlying this process [105,118,119]. At the same time, peptides that can withstand the resistance mechanisms already described are being developed [120,121]. This might provide tools to prevent possible outbreaks as the one observed nowadays for the antibiotics currently in use.

1.7 CLASS I LANTHIPEPTIDES PRODUCED BY *BACILLUS* SPP.

1.7.1 Subtilin.

Subtilin (Figure 1.6) is a cationic lantibiotic produced by *B. subtilis* ATCC 6633. It has structural and antibacterial spectrum similarities with nisin. Subtilin was the first lantibiotic produced by a *Bacillus* strain to be described [122]. Its chemical structure was unveiled in 1973 by Gross and Kiltz [123], prior to the characterization of its structural gene *spaS* [124]. This gene encodes a 56-residue peptide, consisting of a 24-residue leader peptide and a 32-residue core peptide. The other essential genes for subtilin biosynthesis are adjacent to the *spaS* and are organized in an operon-like structure (Figure 1.7) [63,125]. This operon includes genes encoding proteins for post-translational modification (*spaBC*), transport (*spaT*) and self-protection (*spaIFEG*) [125,126]. Regarding the latter, it was initially proposed that immunity of the producer strain was encoded only by *spaIFG*. However, another open reading frame (ORF) was identified within the putative *spaF* gene, with similarity to the *lanE* counterparts [126]. Surprisingly, and in contrast with other class I lantibiotics already identified, the subtilin gene cluster does not encode a *lanP* protease, suggesting that intrinsic *B. subtilis* protease(s) are able to remove the subtilin leader peptide. One possibility is that Vpr protease, one of the five *B. subtilis* extracellular serine proteases, is responsible for this task, since the deletion of its encoding gene resulted in the absence of active subtilin [127].

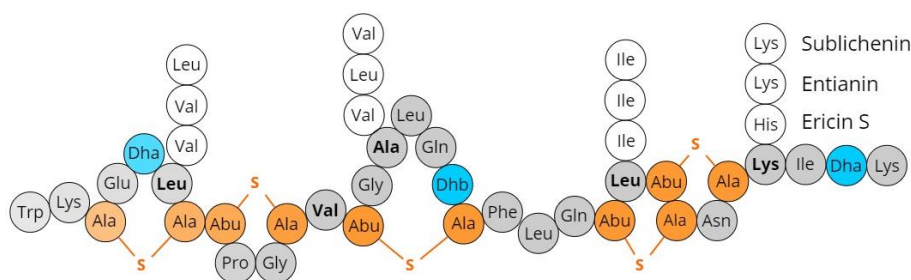


Figure 1.6. Structures of class I lantibiotic subtilin produced by *B. subtilis*. The residues that differ in the subtilin-like peptides (entianin, ericin S and sublichenin) are represented in white. The Lan and MeLan rings are orange and dehydrated residues are blue.

Subtilin biosynthesis is regulated by a two-component system, comprising a histidine kinase and a response regulator (*spaRK*), both encoded in the *spa* gene cluster. A detailed description of the regulatory system for subtilin production was discussed by Hyungjae and co-workers [125]. Briefly, the peptide acts as a quorum sensing molecule that induces the expression of its own structural gene and the whole biosynthetic cluster, including the immunity genes [125,128]. When extracellular subtilin concentration reaches a certain threshold, it activates SpaK which autophosphorylates at a conserved histidine residue. The phosphoryl group is then transferred to a conserved asparagine residue within the regulatory DNA binding SpaR protein, which becomes activated and is able to bind to the DNA motif, thus initiating the transcription of the *spa* gene cluster [125,128,129]. The regulation of this operon is also dependent on other factors such as a sigma factor and the transition state regulator AbrB [88,125]. Detailed information regarding subtilin biosynthesis and self-immunity regulation was explored in previous publications [88,130].

During the characterization of subtilin (M= 3320 Da), an additional peptide with antibacterial activity was identified [63,131]. This peptide is a subtilin variant containing a succinylated N-terminus, currently designated succinylated subtilin (S-subtilin; M= 3421 Da) [63]. It was suggested that this post-translational modification may be part of a self-protection mechanism, since S-subtilin is considerably less active than subtilin (Table 1.2) [63]. As with nisin, subtilin shows antimicrobial activity at a nanomolar range (Table 1.2) against a broad spectrum of Gram-positive bacteria, including other *Bacillus* spp. strains, *Lactococcus* spp. and *M. luteus*. The molecular mechanism of target recognition by subtilin was investigated using a carboxyfluorescein leakage assay and solid-state nuclear magnetic resonance spectroscopy (NMR) [96]. It was found that subtilin is able to affect the integrity of membranes but only in the presence of lipid II. Thus, the ability of subtilin to induce pores is dependent on the formation of a complex involving its N-terminal Trp residue and lipid II (Figure 1.5). Additionally, subtilin forms stable complexes with pyrophosphate-containing intermediates, suggesting its role as a cell-wall biosynthesis inhibitor [96]. Formation of such

pores lead to the dissipation of transmembrane electrostatic potential and loss of low-molecular-mass metabolites that are vital for the targeted cell [96,132].

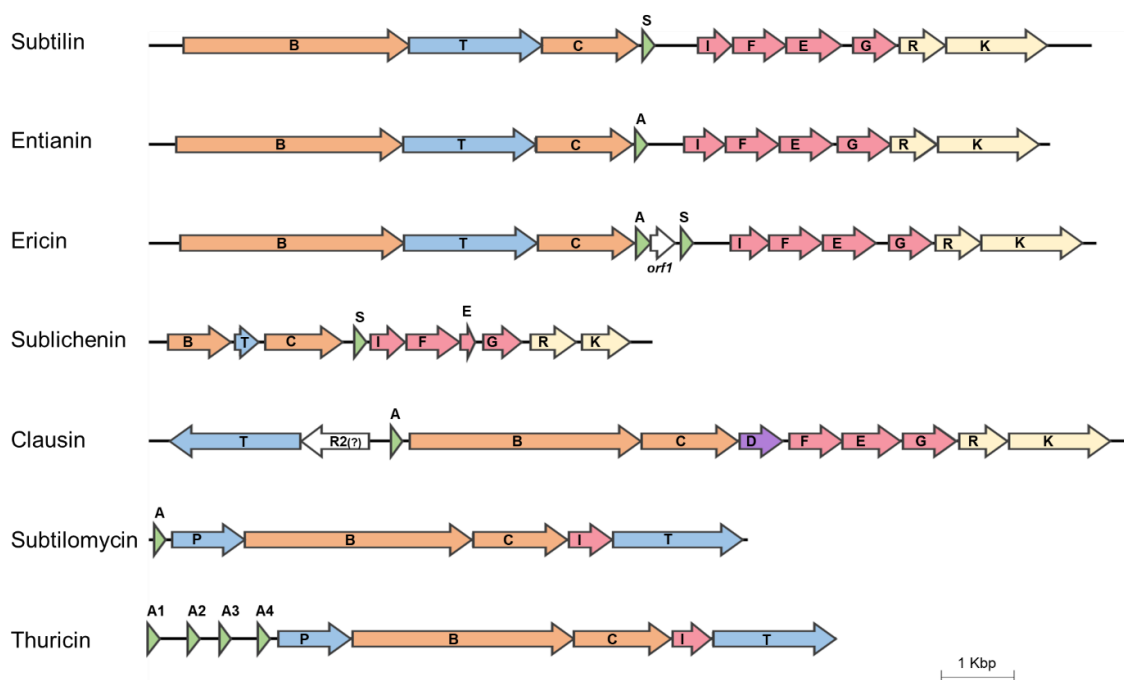


Figure 1.7. Schematic representation of the biosynthetic clusters of class I lanthipeptides produced by *Bacillus* spp. The genes encoding the modification enzymes LanB and LanC are represented in orange and the structural genes in green. The genes of transporters and proteases are blue. The self-protection and regulation genetic determinants are pink and yellow, respectively. The flavoprotein LanD is represented in purple and the genes with unknown function are colorless.

Heinzmann and co-workers [133] engineered *B. subtilis* ATCC 6633 to improve subtilin production yields. The strategy involved the increase of producer's self-protection by the insertion of additional copies of the *spaIFEG* genes, and deletion of *abrB* gene from the producer-strain genome. *Abr* is a general transition state regulator of *B. subtilis* that is also involved in the suppression of subtilin biosynthesis and expression of the immunity genes [128,134]. Both approaches were successful, but subtilin production yields were six-fold higher in the absence of *abrB* gene [133]. However, $\Delta abrB$ mutant strain predominantly produced S-subtilin, which explains the decreased antimicrobial activity observed [133].

One of the first reports on the fermentative production of subtilin used a polyethylene glycol (PEG)/potassium phosphate (KPi) aqueous two-phase systems (ATPS). The application of these systems up- and downstream of the fermentation processes allowed the production and separation of biomolecules from the producing microorganisms in a continuous fashion, according to the hydrophobicity of such molecules. Fermentation was carried out at 37°C with shaking at 130 rpm using a biphasic medium with different

concentrations and proportions of PEG/KPi, and PEG6000 (20%)/KPi (5.5%) was defined as optimum for the two-phase production. Compared with single-phase fermentations, the ATPS revealed higher yields of subtilin production per unit cell (4.9 U/mg against 4.0 U/mg of the two single-phase media) and also better stability of subtilin over time. It was suggested that the physical separation between cells (in the bottom phase) and peptide (in the top phase) could improve the stability of the peptide [135]. More recently, a large variety of parameters were investigated to optimize the production of this lanthipeptide, including varying the time of cultivation, temperature, pH and carbon and nitrogen sources. The best results were obtained at 28°C, using peptone as the nitrogen source and corn flour as the carbon source, and with the pH of the medium adjusted to 8.0. It was observed that the higher amounts of subtilin were present after 96 h, whereas no compound was detectable after 192 h fermentation [136].

1.7.2 Entianin.

Entianin is a subtilin-like lantibiotic produced by *B. subtilis* subsp *spizizenii* DSM 15029^T. The lantibiotic is produced in both succinylated (S-entianin; M= 3,446.6 Da) and unsuccinylated isoforms (M= 3,346.6 Da) [64] and it was discovered during an autoinduction bioassay developed to identify subtilin [64,137]. Only three amino acids differ between entianin and subtilin: Leu6Val, Ala15Leu and Leu24Ile (subtilin → entianin) as shown in Figure 1.6. Entianin shows a high similarity to ericin S, and they both differ from subtilin in the same amino acid positions, except for position 29, in which both entianin and subtilin contain a Lys while ericin S contains an His (Figure 1.6) [138]. The *ent* gene cluster (Figure 1.7) has a high degree of homology (93%) with the subtilin biosynthetic cluster of *B. subtilis* ATCC 6633 [64].

The unsuccinylated entianin is active against several bacterial species, including MRSA and VRE. In addition, it completely inhibited the growth of *B. subtilis* strains that do not produce subtilin-like peptides. As with subtilin, succinylation clearly decreases the antibacterial activity, and S-entianin exhibits a 40-fold decreased bioactivity when compared with its unsuccinylated form. However, this N-terminal modification does not affect the recognition of entianin by the regulation-sensing system (*entRK*), since S-entianin reaches 70% of the autoinduction level of its unsuccinylated form [64]. Due to all the similarities identified, the mode of action of entianin is regarded as the same as subtilin. This was further supported by results showing that Spal has a sequence similarity of 95% with EntI and is able to mediate immunity against entianin [138]. The same was observed with ericin (described below): Eril shows 77% homology with Spal and it is still able to mediate a certain degree of immunity against entianin [138].

1.7.3 Ericin.

B. subtilis A1/3 produces a large number of peptides with antibacterial activity [97]. Among these are the two related lantibiotics, ericin S and ericin A (Figure 1.6). Both peptides share a high degree similarity with subtilin. The genes that constitute the ericin gene cluster also present high sequence identity with those of subtilin (between 71 and 96% homology) [97]. More recently, ericin gene cluster was also identified in *B. velezensis* RC 218 and the production of ericin S was confirmed [139].

The ericin gene cluster possesses only one *lanB* gene (*eriB*) and one *lanC* gene (*eriC*), but two structural genes, *eriA* and *eriS* (Figure 1.7). Thus, the same post-translational modification enzymes modify both EriA and EriS precursor peptides. This was clearly demonstrated after disruption of *lanB* gene, which resulted in no detectable production of ericin A or ericin S peptides. This system contrasts with that of the two-peptide lantibiotics, which require distinct modification enzymes for each peptide (for examples, see the sections on haloduracin and lichenicidin below). The leader peptides of ericin A and ericin S have identical amino acid sequences (Figure 1.8) and it is not surprising that all the modification enzymes (EriB, EriT and EriC) were able to use EriA and EriS as substrates. Due to the high similarity (around 85%) between EriC and SpaC, a functional hybrid consisting of SpaB-EriC-SpaT was shown to be able to modify subtilin [140]. A distinct feature of the ericin cluster is the presence of an ORF (*orf1*) between *eriA* and *eriS* genes containing a fragment of the *eriC* sequence and whose function remains unknown. It was proposed that at least one duplication took place within this gene cluster creating another copy of a subtilin-like gene and *orf1* [97].

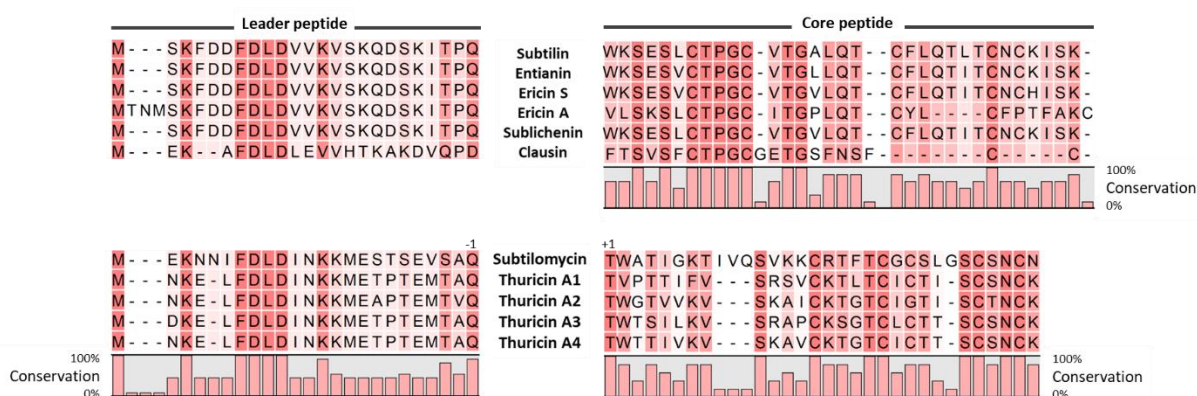
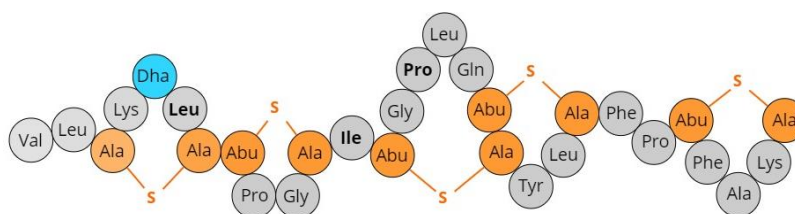


Figure 1.8. Alignment of the amino acid sequences of the leader and core peptides of all the class I lanthipeptides described for *Bacillus* spp. The colored amino acids represent highly conserved residues.

The amino acid composition of ericin S (M= 3342.8 Da) is 92% identical to that of subtilin and differs by only four amino acid residues: Leu6Val, Ala15Leu, Leu24Ile and Lys29His



Ericin A (M= 2987.7 Da) is smaller than ericin S and shares only 75% sequence homology with subtilin. When compared with subtilin sequence, ericin A has 13 amino acid changes and a three residue truncation at its C-terminus. Furthermore, the lanthionine-bridging pattern differed from that of subtilin and ericin S (Figure 1.9). Despite the fact that its physical properties are similar to ericin S, its bioactivity is clearly reduced (approximately 100-fold less active than ericin S), even when tested against strains highly sensitive to ericin S. So far, a synergistic action between ericin A and ericin S was not observed suggesting that ericin does not present characteristics of a two-peptide lantibiotic [97].

Sublichenin is a subtilin-like lantibiotic isolated from *Bacillus licheniformis* MCC 2512^T, a probiotic bacterium from the rhizobial soil of the medicinal herb *Hedysarum coronarium* [141]. Sublichenin and entianin gene clusters share high similarity, with some differences that resemble that of subtilin and ericin gene clusters (Figure 1.7) [68]. The structural gene *lanS* has 95% sequence similarity with subtilin and entianin, and has only a difference in two residues with these lantibiotics, and a similarity of 94% with ericin S (Figure 1.6). Like subtilin and entianin, sublichenin is produced in both succinylated (S-sublichenin; M= 3448 Da) and unsuccinylated forms (M= 3348 Da). The succinylated form appears to be less active than its unsuccinylated counterpart and seem unable to self-induce its own production. Crude

extracts of sublichenin show bioactivity against a large number of bacteria, including MRSA and some Gram-negative pathogens, although with lower efficacy [68]. The sublichenin structural gene was heterologously expressed in *B. subtilis* B15029p (containing the whole entianin gene cluster except the structural gene *etnS*) but only the unsuccinylated form was detected. As succinylation is considered a self-protection mechanism, this indicates that the host may be tolerant to the most active form of sublichenin. Additionally, succinylation seems to be dependent of host's enzymatic machinery, as the heterologous host could not produce the succinylated form, despite that the whole sublichenin gene cluster is present [68].

1.7.5 Clausin.

Clausin is a 2107.5 Da lantibiotic produced by *Bacillus clausii* O/C strain [142]. It is one of the constituents of the probiotic Enterogermina® used for the treatment of diarrhea and also in the prevention of infectious diseases [143]. Its structure was confirmed by NMR [142], showing similarities to subtilin-like lantibiotics, with its N-terminus presenting a high homology with these peptides (Figure 1.10). In its C-terminus, clausin possesses an AviCys residue that is formed by oxidative decarboxylation [142]. Besides being found in the strain O/C, the biosynthetic gene cluster of clausin is also present in the genome of *B. clausii* KSM-K16 and encodes all the essential elements of class I lanthipeptides (*claA*, *claB*, *claC* and *claT*), the self-protection (*claFEG*) and the regulatory genes (*claRK*) (Figure 1.7). Additionally, between *claC* and *claF*, a gene is found encoding a protein belonging to the HFCD (homo-oligomeric flavin-containing Cys decarboxylases) family of flavoproteins (*claD*), which catalyzes the formation of the AviCys residue (Figure 1.10). Lastly, upstream of the *claA* structural gene, an uncharacterized ORF which encodes a 224 amino acid protein is present. The first 70 amino acids have homology with the helix-turn-helix XRE-family like proteins from the group of HipB transcriptional regulators. Therefore, this protein may be part of a second regulatory system, as described for mersacidin, and the gene is herein referred to as *claR2* (Figure 1.7). Clausin possesses *in vitro* activity against Gram-positive bacteria, including *C. difficile*, *Clostridium perfringens*, *E. faecium*, *L. lactis*, *L. monocytogenes*, *Micrococcus* spp. and *S. aureus* [142]. However, clausin MIC values against these bacteria have not been reported. It was established that clausin interacts, with similar affinities, with the intermediates of peptidoglycan biosynthesis lipid I and II. Additionally, it was found that the clausin:lipid I/II complex should occur at the micelle/water interface in a 2:1 stoichiometry, indicating that the active form of clausin is most probably a dimer. Furthermore, this lantibiotic seems to interact with the lipid intermediate C₅₅-PP-GlcNAc, suggesting its interference in the biosynthesis of other cell wall polymers, such as teichoic acids [144]. A patent covering the clausin's biological activity, preparation and application

has been published [142]. Details of the patent describe that the method for its preparation requires growth of *B. clausii* O/C strain for three days in Mueller-Hinton medium at 37°C, followed by extraction and purification of clausin from the cell-free supernatant.

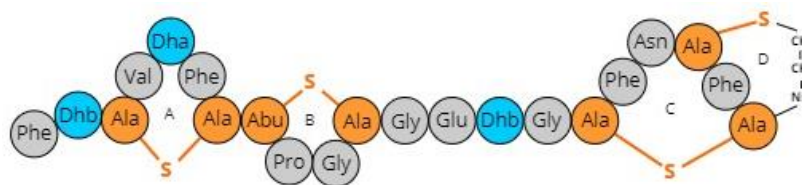


Figure 1.10. Structure of class I lantibiotic clausin produced by *B. clausii*. The Lan and MeLan rings are orange and dehydrated residues are blue.

1.7.6 Thuricin 4A-4 and 4A-4D.

Thuricin 4A-4 was first identified by *in silico* screening that targeted 223 strains of the *Bacillus cereus* group (2014) [67]. It is worth mentioning that thuricins 4A do not belong to the same family as thuricin CD, which is a narrow-spectrum bacteriocin composed by two post-translationally-modified sactibiotic peptides [145]. Its biosynthetic cluster was identified in 10 *B. cereus* and *Bacillus thuringiensis* strains, however the number of structural genes present in each of those clusters differed from one to five [67]. The cluster of *B. thuringiensis* T01001 strain (Figure 1.7) was confirmed with Sanger sequencing and it is formed by four structural genes (*thiA1-thiA4*), followed by the biosynthetic machinery-encoding genes *thiP*, *thiB* and *thiC*. As with the subtilomycin cluster, only one self-immunity gene is present (*thiI*), which is located upstream of the class I transporter *thiT*. Using the T01001 strain, it was found that all four *thiA* genes were transcribed during exponential growth phase, although the cDNA amount of *thiA4* gene was much higher than that of the other three genes [67]. The leader peptide of the four Thi peptides is identical, with just one amino acid difference in ThiA2 and ThiA3 (Figure 1.8). However, a higher variation is found in their core peptides. Nevertheless, the similarities between ThiA1, ThiA3 and ThiA4 suggest that their structure should be similar and includes the formation of 4 Lan/MeLan rings (Figure 1.8 and Figure 1.11). The ThiA2 possesses only 3 Cys residues which implies the formation of a maximum of 3 thioether rings (Figure 1.8). The mature peptide encoded by the *thiA4* gene, with a molecular weight of 2786.3 Da, was successfully identified in the fermentation supernatant and designated as thuricin 4A-4 (Figure 1.11). In the same supernatant, a second antibacterial peptide with a molecular weight of 2886.3 Da was detected. This MW did not match any of the predicted masses of the four mature thuricins 4A (from 4A-1 to 4A-4). After MS/MS analysis, it was predicted that the 2886.3 peptide, referred to as thuricin 4A-4D, is a derivative of thuricin 4A-4 wherein the side chain of Lys7 or Lys10 reacted to an unknown

118 Da substance (Figure 1.11). The complete *thi* gene cluster was heterologously expressed in *B. thuringiensis* BMB171 and both thuricin 4A-4 and thuricin 4A-4D were detected in the supernatant of the transformed strain (designated BMB1661). Both lantibiotics were recovered from 16 h fermentations performed at 28°C in Luria-Bertani medium broth. Thuricin 4A-4 and thuricin 4A-4D have activity against Gram-positive bacteria, including *B. cereus*, *Bacillus firmus*, *B. subtilis*, *Bacillus pumilus*, *Enterococcus faecalis*, *Microbacterium*, *Paenibacillus*, *S. aureus*, and *S. sciuri* (Table 1.2). The activity of thuricin 4A-4 is greater than that of thuricin 4A-4D, for all of these indicator strains tested [67].

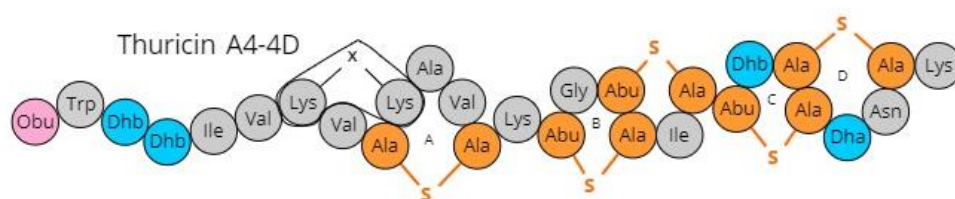


Figure 1.11. Structures of class I lantibiotic thuricin 4A-4 produced by *B. cereus*. The Lan and MeLan rings are orange and dehydrated residues are blue. The Obu residue (in pink) refers to 2-oxobutyryl.

1.7.7 Subtilomycin.

One of the most recently described class I lanthipeptides produced by *Bacillus* spp. is subtilomycin (M= 3235 Da; Figure 1.12). It is produced by *B. subtilis* MMA, a strain isolated from the marine sponge *Haliclona simulans* [57]. The structural gene encoding this lantibiotic is found in several other *B. subtilis* strains isolated from different shallow and deep-sea marine sponges [57]. However, it seems to be absent in *B. subtilis* strains isolated from other environments [57].

The subtilomycin gene cluster (Figure 1.7) contains 6 ORF comprising the structural gene (*subA*), two genes encoding the modification proteins SubB and SubC (LanB-like dehydratase and LanC-like cyclase, respectively), a gene that encodes a putative extracellular serine protease (*subP*) and an ABC transporter (*subT*). Another ORF was identified with no significant homology to other sequences in the database. This gene was designated *subI*, since it was proposed it could mediate the host protection to subtilomycin. Thus, self-protection seems to be conferred by a single dedicated immunity protein, excluding the involvement of ABC transporter proteins, in a similar way to Pep5 [57,146].

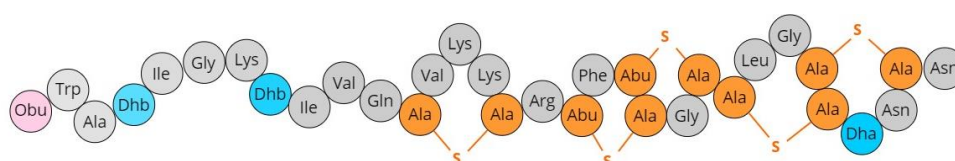


Figure 1.12. Structure of class I lantibiotic subtilomycin produced by *B. subtilis*. The Lan and MeLan rings are orange and dehydrated residues are blue. The Obu residue (in pink) refers to 2-oxobutyryl.

Subtilomycin displays inhibitory activity against a variety of Gram-positive bacteria, including pathogens associated with food and others of clinical relevance such as *L. monocytogenes*, *Clostridium sporogenes*, MRSA and heterogeneous vancomycin intermediate-level resistant *S. aureus*. It is also active against the Gram-negative bacteria *Aeromonas hydrophila*, *Vibrio anguillarum*, *Alteromonas* sp. and *Pseudomonas aeruginosa*. This activity requires a much higher concentration of peptide (around 100-fold more) is required. These results were obtained by antagonistic activity testing and no MIC values were reported [57]. Subtilomycin was purified from cell-free supernatants of the *B. subtilis* MMA7 strain after growth in Marine Broth medium for 12h at 30°C.

1.8 CLASS II LANTHIPEPTIDES PRODUCED BY *BACILLUS* SPP.

1.8.1 Mersacidin.

Mersacidin (Figure 1.13) was firstly isolated from *Bacillus* sp. HIL Y-85,54728 by Fehlbauer and Kogler in 1992 [69]. More recently, DNA comparison studies and biochemical tests showed that *Bacillus* sp. HIL Y-85,54728 is a member of the *B. methylotrophicus* (former *amyoliquefaciens*) species [147].

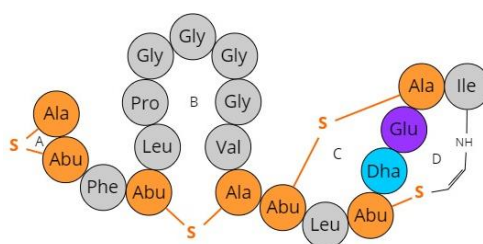


Figure 1.13. Structure of class II lantibiotic mersacidin produced by *B. methylotrophicus*. The Lan and MeLan rings are orange and dehydrated residues, blue. The conserved Glu motif is represented in purple.

The gene cluster of mersacidin (Figure 1.14) contains 10 open reading frames. In addition to the structural gene *mrsA*, it possesses genes encoding the enzymes required for post-translational modifications of the peptide (*mrsD* and *mrsM*), a gene that encodes a bifunctional protein with a transporter and protease domain (*mrsT*), three immunity genes (*mrsF*, *mrsG*, *mrsE*) and genes involved in the regulation of the biosynthesis (*mrsK2*, *mrsR2* and *mrsR1*) [148]. This cluster was also identified in *B. methylotrophicus* Y2 strain [149] and YAU B9601-Y2 strain (closely-related to Y2) [150]. An incomplete cluster, comprising the immunity genes *mrsF*, *mrsG* and *mrsE*, and the transcriptional regulators *mrsK2* and *mrsR2*, was found in *B. methylotrophicus* FZB42 [147] as well as in JS25R, NAU-B3, CC178 and UCMB5036 genomes [150].

General Introduction

Unlike the mersacidin's original producer *B. methylotrophicus* HIL Y-85,54728, the FZB42 strain is naturally competent. This biological feature allowed the insertion of the remaining components of the gene cluster (*mrsA*, *mrsM* and *mrsT*) into FZB42 strain. In this way, a new mersacidin producer was obtained, which is more amenable to transformation facilitating the genetic manipulation of mersacidin gene cluster [147].

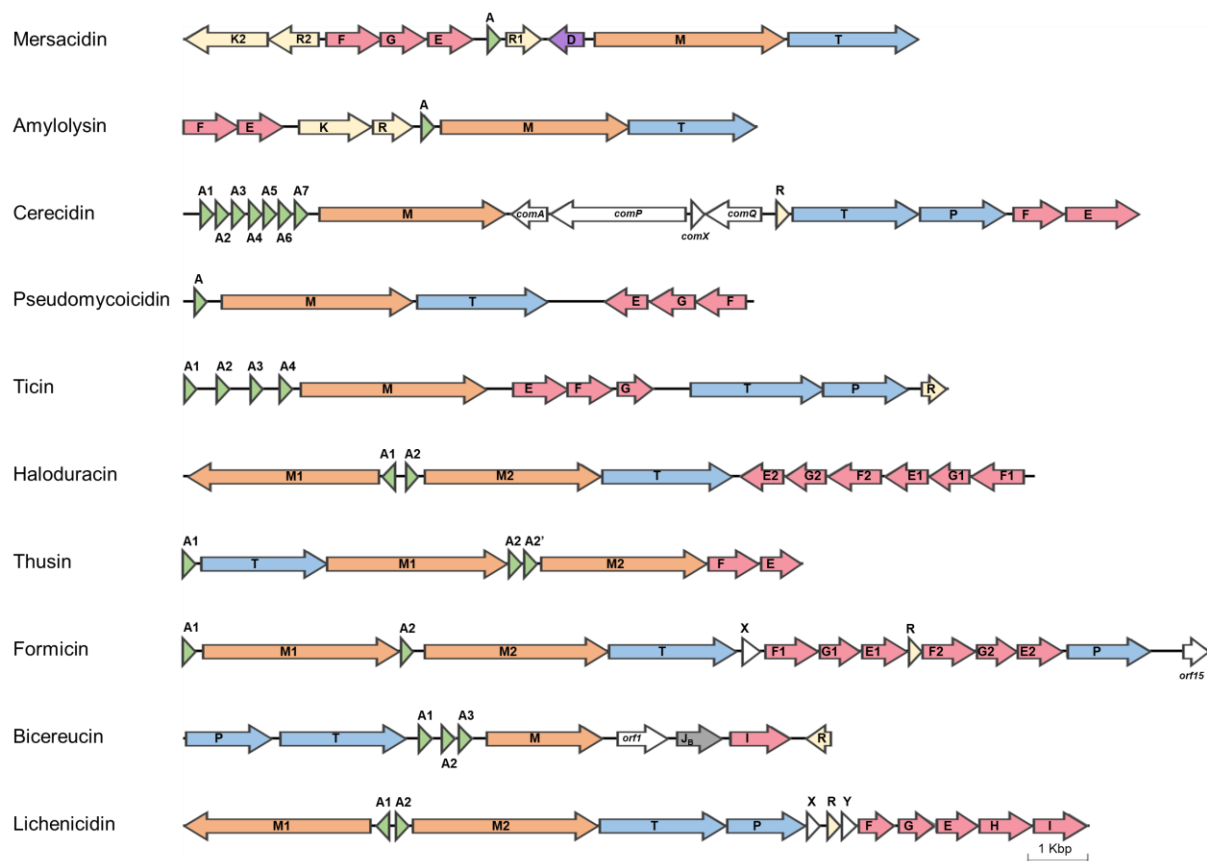


Figure 1.14. Schematic representation of the biosynthetic clusters of class II and lanthipeptides produced by *Bacillus* spp. The genes encoding the modification enzymes LanB and LanC are represented in orange and the structural genes in green. The genes of transporters and proteases are blue. The self-protection and regulation genetic determinants are pink and yellow, respectively. The flavoprotein LanD is represented in purple and the genes with unknown function are colorless.

Mersacidin induces its own biosynthesis since its addition to an exponential phase culture of the *Bacillus* sp. HIL Y-85,54728 strain (before the onset of mersacidin biosynthesis), resulted in increased *mrsA* transcription [151]. In this study relatively high concentrations of mersacidin (2 µg/mL) were employed in comparison to a similar study with nisin (0.01 µg/mL) [151,152]. The *mrs* biosynthetic cluster includes two regulatory proteins (MrsR1 and MrsR2) and histidine kinase (MrsK2). The MrsR1 protein seems to play an important role in the regulation of *mrsA* transcription as the deletion of *mrsR1* led to the abolishment of mersacidin production (Figure 1.15) [151]. There is no dedicated histidine kinase to MrsR1 in the *mrs* cluster and it is unknown if phosphorylation of MrsR1 is required for its functionality

[153]; possibly this is not the case since MrsR1 lacks the Lys residue that mediates the conformational change upon phosphorylation [148]. The two-component system MrsR2/MrsK2 seems to be related to the regulation of the producer's self-protection mechanism (Figure 1.15). This was supported by results revealing that transcription of *mrsFGE* genes was affected in a $\Delta mrsR2/K2$ strain and also that the immunity to mersacidin in this knockout mutant decreased to 37% that of the wild type [153]. It was shown that this mutant was still able to produce the lantibiotic, although the mersacidin auto-induction system was inhibited [153].

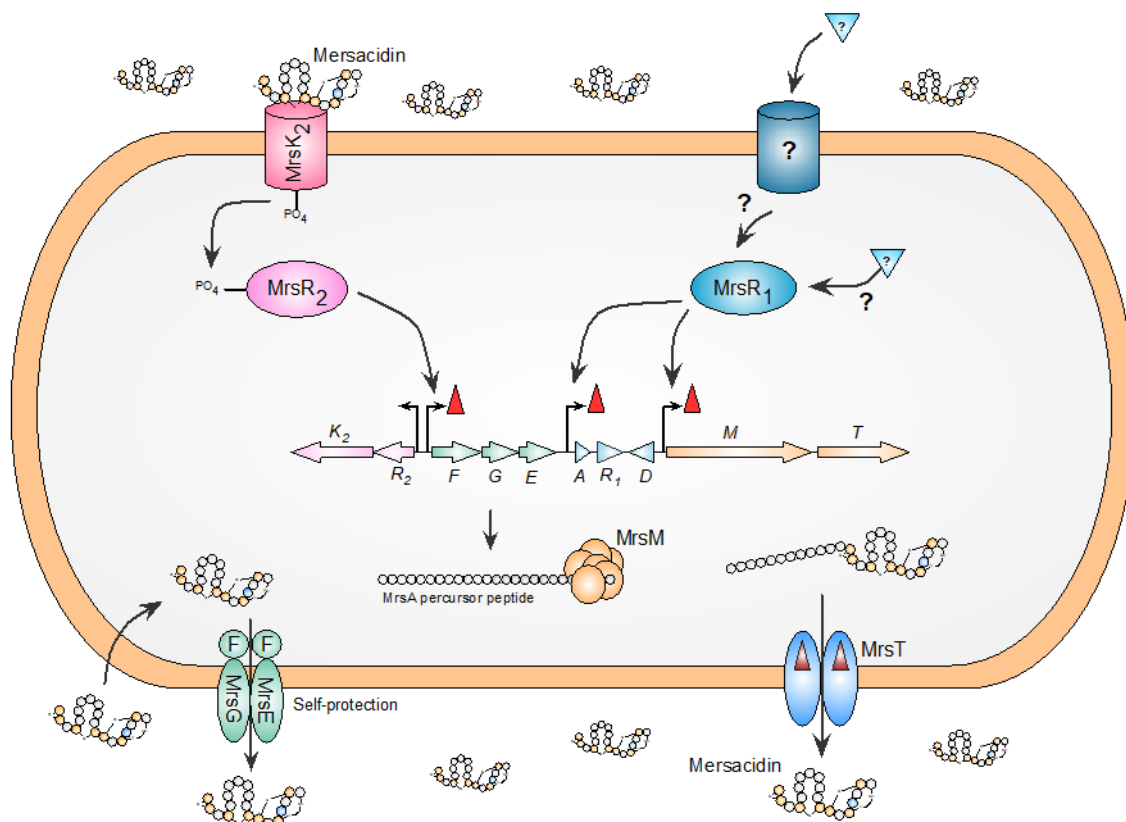


Figure 1.15. Biosynthesis and regulation mechanism of mersacidin. *mrsA* transcription is regulated by the orphan regulator MrsR1 which lacks a dedicated sensor kinase; still unknown is the nature of the molecule that triggers MrsR1 activation (represented by the triangles with '?') or if mersacidin itself is responsible for triggering the biosynthesis through a mechanism involving also MrsR2/MrsK2 system. The pre-mersacidin is then modified by a single MrsM modifying enzyme, and exported and cleaved by MrsT ABC transporter whose transcription is also activated by MrsR1. Transcription of the immunity genes *mrsFGE* is activated by the two-component system MrsK2/MrsR2, which recognizes mersacidin from the extracellular environment. No information is available regarding the transcription regulation of MrsK2/MrsR2.

Mersacidin is relatively small (M= 1825 Da; Table 1.1) and it was the first lantibiotic structure determined by crystallography [154]. It is a neutral molecule with overall hydrophobic properties, containing MeLan but not Lan in its structure, which is uncommon among *Bacillus* spp. lantibiotics (Figure 1.13) [155,156]. Mersacidin contains an AviMeCys

residue at its COOH-terminal, being the only class II lanthipeptide produced by *Bacillus* spp. described so far with this type of post-translational modification. The biosynthesis of AviMeCys is catalyzed by the MrsD protein, which is also a member of the HFCD flavoproteins family aforementioned ClaD [31]. It was experimentally demonstrated that MrsD functionality is dependent on flavin adenine dinucleotide (FAD) instead of flavin mononucleotide (FMN) [157]. The analysis of the MrsA precursor peptide (Figure 1.16) reveals the presence of 6 amino acids between the GA-motif (site of leader peptide removal by MrsT) and the first Cys residue of the mature peptide. This sequence is found in other class II lanthipeptides and it is commonly referred to as the hexapeptide (Figure 1.16). It is assumed that the removal of the hexapeptide is essential to obtain the mature and active mersacidin, however the protease catalyzing this reaction is unknown.

Mersacidin has activity against Gram-positive bacteria such as *S. aureus* (including methicillin-resistant strains), Streptococci (*S. faecalis* and *pyogenes*) and *B. subtilis*, but is inactive against Gram-negative bacteria and fungi [155,156]. *In vivo* studies with infected mice showed that mersacidin salt form has bioactivity comparable to that of mersacidin [156]. In fact, the ED₅₀ against *Streptococcus pyogenes* A77 infections, was approximately 5-fold reduced when using the salt form [69]. Surprisingly, mersacidin activity was better in *in vivo* than in *in vitro* studies [69,155], which might indicate that its application may be more effective than anticipated. Unlike subtilin, mersacidin does not affect the energy-transducing cytoplasmic membrane of target cells (Figure 1.5). In the presence of the latter it was demonstrated that the membrane potential is retained, and the uptake and presence of low-molecular-mass metabolites was not affected [155]. The same study showed that mersacidin blocks the incorporation of glucose and D-alanine into cellular macromolecules, interfering with the biosynthesis of the cell wall at the transglycosylation level [158]. This occurs due to a strong association between mersacidin and the lipid II, which forms a stable complex even in the presence of 1% SDS [99]. The Glu17 residue of mersacidin is probably involved in the binding of mersacidin to lipid II, since its replacement by Ala resulted in a completely inactive peptide [90,159]. In contrast to glycopeptide antibiotics (e.g., vancomycin that also binds to lipid II), mersacidin does not bind to the C-terminal D-alanyl-D-alanine moiety of lipid II, indicating a different mode of action from the currently used antibiotics [99,160]. In fact, mersacidin can distinguish between lipid I and lipid II, indicating that GlcNAc, the only difference between them, is possibly part of the recognition motif [159].

Results from NMR studies showed that structural changes of mersacidin after binding lipid II affected the exposure of its charged residues, suggesting that electrostatic interactions play an important role in the binding process despite the hydrophobic nature of mersacidin [159]. Hsu and co-workers also demonstrated a certain flexibility of the hinge region Ala12-Abu13 (Figure 1.13), which changes its conformation in the presence of lipid II,

exposing its only charged amino acid Glu17. It is believed that this ring structure, also present in other class II lantibiotics, might be essential for the interaction of mersacidin with lipid II. The bioactivity seems to be ion-dependent, as the presence of the calcium ions enhanced its activity *in vivo* [159,161]. Since both lipid II and the Glu17 side chain are mostly negatively charged, calcium ions may provide a bridge between mersacidin and lipid II [159]. The distinct mode of action of mersacidin is also highlighted by the fact that it induces a strong cell wall stress response in *S. aureus* at very low concentrations when compared with other antibiotics such as nisin, bacitracin or vancomycin [160].

General Introduction

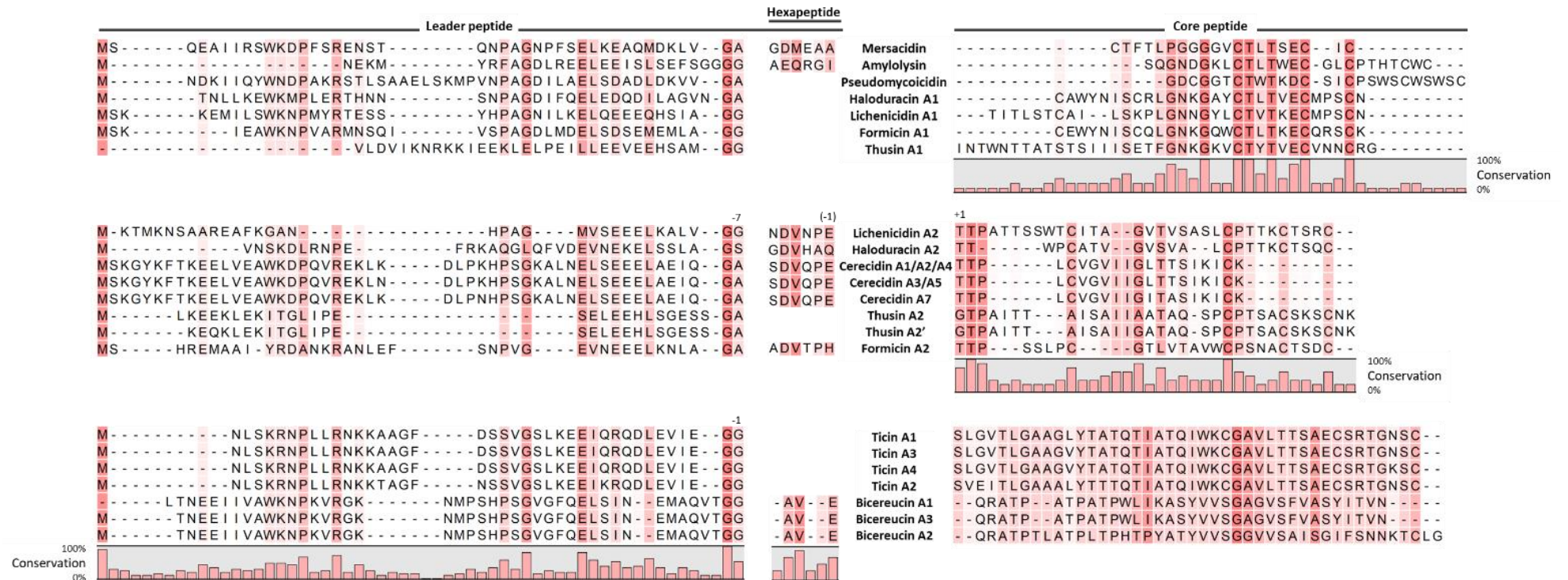


Figure 1.16. Alignment of the amino acid sequences of the leader and core peptides of all the class II lanthipeptides described for *Bacillus* spp. The colored amino acids represent highly conserved residues.

To better understand structure-function relationship of mersacidin, Szekat and co-workers developed an expression system for site-directed mutagenesis (SDM) in which a temperature-sensitive vector was used to replace the wild type gene by a mutated one, directly into the chromosome [53]. However, this procedure was difficult and entailed several steps and a large number of mutants. Hence, Appleyard *et al.* developed an improved system, which allowed the construction of a saturation mutagenesis library of mersacidin residues. This system involved the *trans* complementation of a $\Delta mrsA$ -knockout mersacidin producer strain with a shuttle plasmid able to express *mrsA* and its variants [52]. From the 228 mersacidin mutant strains produced in this study, 80 produced good levels of mature mersacidin variants; the remaining presented lower production yields. These results revealed the flexibility of the biosynthetic machinery once the enzymes responsible for the post-translational modifications were able to cope with variants of the wild-type peptide, although with reduced efficiency in some cases [52,56]. Valuable information regarding the lantibiotic structure, which seems to be strictly controlled, was also assessed. For example, no deletions were tolerated within the B-ring (Figure 1.13), suggesting that a minimum ring-size is required for mersacidin bioactivity. In contrast, insertions were accepted, depending on the number and type of amino acids introduced. For example, insertion of polar and nonpolar residues were well tolerated, while neither proline nor aromatic amino acids were accepted, except tryptophan that was successfully introduced between positions 4 and 5 (Figure 1.13) [52]. A decrease in the production levels was observed in variants with insertions close to the MeLan bridges. The addition of residues in both A-ring and hinge region (between B- and C-rings) of mersacidin (Figure 1.13) were not tolerated. As already mentioned, mersacidin contains only MeLan bridges, and mutants with Lan bridges were not produced [52,56]. It was also found that substitutions involving Cys residues were not accepted. Most of the mutated peptides were inactive or showed decreased activity when compared to the natural mersacidin. A variant where the Phe3 residue was replaced by Trp showed improved activity against Gram-positive pathogens, including *S. aureus* (MSSA and MRSA), *E. faecium* (VSE and VRE) and *E. faecalis* (Table 1.2) [52]. Two patents describe the improvement of mersacidin production and the generation of mersacidin variants [162,163].

The first report on mersacidin fermentation was published in 1992 [156]. In this study, mersacidin production was achieved in a synthetic minimal medium containing sucrose, KH_2PO_4 , Na_2HPO_4 , $(\text{NH}_4)_2\text{SO}_4$, Na-glutamate, MgSO_4 , MnSO_4 , $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$, ZnSO_4 and FeSO_4 , at pH 7.2 [156]. The fermentation proceeded at 28°C with aeration and mersacidin

was detected after 48 h following the active growth phase [156]. These results were quite surprising since the production of other lantibiotics, e.g., pep5, epidermin and gallidermin, could not be obtained in defined medium and always required complex media [156]. Some years later, a new synthetic medium containing glucose and maleate buffer was used for the production of mersacidin. In this medium, mersacidin was present in the culture supernatant after 14 h of incubation. The same medium at twice the concentration has been used in recent studies of mersacidin production [164]. Such medium has been often referred to as mersacidin production medium or 2X BPM [52,147].

1.8.2 Amylolysin.

Amylolysin was described by Halimi *et al.* in 2010 [70]. This lantibiotic is produced by *B. methylotrophicus* (former *B. amyloliquefaciens*) GA1 isolated from strawberries. Structurally it is closely related to mersacidin-like peptides. Its biosynthetic cluster (Figure 1.14) possesses the structural gene (*amIY*) and all the essential elements for peptide modification and transport (*amIM* and *amIT*), regulation (*amIK* and *amIR*) and self-protection (*amIF* and *amIE*) [100]. Interestingly, amylolysin immunity seems to be mediated only by a two-component ABC transporter (*amIFE*), which is uncommon within the genus *Bacillus* (Figure 1.14) [100]. Using the platform antiSMASH 2.0 [78], the gene cluster of amylolysin was also identified in other *B. methylotrophicus* closely related strains: IT-45; LFB112; L-H15; L-S60 and NJN-6 [150]. Analysis of the flanking regions revealed the presence of a second *lanM* (KSO013300), located downstream of the *amIT* gene. The existence of possible ORF(s) between *amIT* and the second *lanM*, *amIM2*, was investigated and showed that three putative *lanA* genes (A2, A3 and A4) are present between these two genes, where the last two have 92% identity with each other and 40% similarity with LicA2 and HalA2. Thus, this second LanM enzyme can be responsible for modifying these additional structural genes, something that is characteristic of two-peptide lantibiotics [150]. Thus, in some strains of *B. methylotrophicus*, is more likely that amylolysin is the α -peptide of a two-peptide lantibiotic. More recently, the genome of the related strain *B. velezensis* CE2 was sequenced, revealing the presence of a two-peptide lantibiotic that was named velezensicidin. These peptide are 100% similar to amylolysin. Thus, we proposed that the name velezensicidin should not be used, since it is a synonymous of amylolysin.

A structure for amylolysin (M= 3318 Da) was proposed based on the molecular mass determination and homology with the α -peptides of lacticin 3147 and haloduracin [100]. Accordingly, amylolysin should contain three thioether bridges between amino acids 36-

34, 45-50 and 47-53, as well as two Dhb residues at position 55 and 57 (Figure 1.17A). As such, two free cysteines are present in its structure, a feature never reported before in lanthipeptides. Amylolysin is the first reported lantibiotic containing Cys residues that are not involved in the formation of Lan or MeLan rings. It is also possible that these two Cys react with the two Dhb residues to form MeLan bridges between amino acids 55-58 and 57-60, as shown in Figure 1.17B. Since both possibilities, among others, should be considered, further structural studies are required on amylolysin.

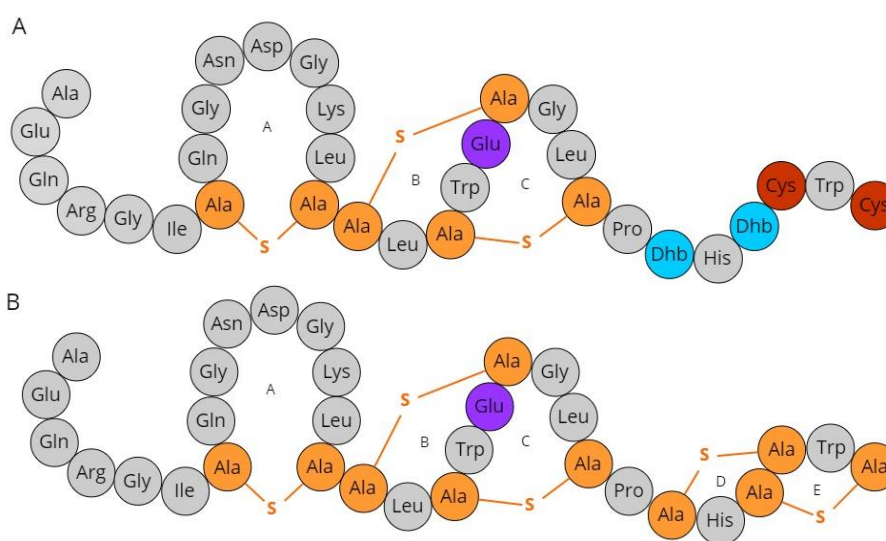


Figure 1.17. Structure of class II lantibiotic amylolysin produced by *B. methylotrophicus*. Amylolysin A and B correspond to the structure proposed by Arguelles Arias *et al* [100] and in this work, respectively. The Lan and MeLan rings are orange and dehydrated residues, blue. The conserved Glu motif is represented in

Amylolysin is heat and pH-stable, is bioactive against clinically relevant Gram-positive bacteria, such as MRSA (Table 1.2), and inhibits the proliferation of various *L. monocytogenes* isolated from poultry meat [100]. In contrast to nisin, amylolysin has a bacteriostatic effect against *L. monocytogenes* and is more resistant to the endogenous proteolytic enzymes of meat, which prevents its degradation in that environment. These properties illustrate the potential of this compound for use as a food preservative [100]. It was confirmed that amylolysin interacts directly with the peptidoglycan precursor lipid II, *in vitro*, thus inhibiting cell wall biosynthesis. Additionally, amylolysin may induce the formation of pores within the membrane, being the first class II lanthipeptide of *Bacillus* spp. with a dual mode of action [100]. Amylolysin was purified from cell-free supernatant of a large-scale (60 L) growth of *B. methylotrophicus* GA-1 in LB medium incubated for 10 h at 37°C [100].

1.8.3 Cerecidin.

Cerecidin was the first lantibiotic detected in the *B. cereus* group through genome mining of the extremophile *B. cereus* Q1 strain [77,165]. Thereafter, Wang *et al.* screened five different *B. cereus* strains for the presence of the *cerA* structural gene and also identified it in *B. cereus* As1.1846 strain [71]. Amplification and sequence determination of the complete *cer* gene cluster of As1.1846 revealed the presence of 17 ORF (Figure 1.14) [71]. The most distinctive feature is the seven nearly identical structural *cerA* genes (*cerA1* to *cerA7*; Figure 1.14). The leader peptides of CerA1, CerA2, CerA4 and CerA7 are identical and differ from the leader peptides of CerA3, CerA6 and CerA5 in the -25 residue (Figure 1.16). No variations in the core peptides of CerA1, A2, A3, A4, A5 and A6 were identified. However, there are two different amino acids between these and the CerA7 core peptide, the changes being: Leu12->Ile and Thr14->Ala (Figure 1.16 and Figure 1.18). Although 7 structural genes are present, the *cer* gene cluster encodes only one modification enzyme (CerM). Therefore, the biosynthesis of cerecidins should be different from that of two-peptide lantibiotics, which implies a LanM enzyme dedicated to each LanA. The *cer* cluster also contains the genes *cerR*, *cerT*, *cerP* and *cerFE* and the quorum sensing components *comQXPA* (Figure 1.14). It was demonstrated that CerT recognizes the characteristic class II GG/GA/GS-motif (in this case GA). The serine protease CerP is responsible for the removal of the hexapeptide SDVQPE that is conserved among all the CerA precursor peptides and is highly similar to the hexapeptide of lichenicidin LicA2 (Figure 1.16). The presence of *comQXPA* genes indicates that the production of cerecidin may be synchronized with other events of the late exponential/early stationary phases, namely competence development [71]. In addition, the orphan regulator gene *cerR* encodes an XRE-like protein with a HTH binding domain, suggesting that the regulation of cerecidin's biosynthesis should not be exclusively controlled by the *comQXPA* components. Finally, the self-protection of the cerecidin's producer should be mediated by an ABC transporter encoded by the genes *cerFE*. As with amycolysin, this transporter is composed of two proteins [71]. It was determined that all biosynthetic elements of the *cer* cluster, except *cerM*, are transcribed in *B. cereus* As1.1846 strain. As a result, this strain did not produce any of the peptides. To overcome this situation, the As1.1846 strain was complemented with a plasmid containing the *cerM* gene under the control of a constitutive promoter which resulted in the detection of mature CerA1 and/or CerA2 to CerA6 peptides (hereafter refereed A1; 1987.17 Da) by MS analysis [71]. However, the expected mass for the mature CerA7 (1958.03 Da) was not identified. This peptide was successfully produced using the co-expression of *cerA7* and

cerM genes in *E. coli*, followed by *in vitro* treatment with GluC and CerP proteases to remove the leader and the hexapeptide. The structure of cerecidin A1 and A7 was investigated by tandem MALDI-TOF MS analysis revealing that their structure resembles the β -peptides of two-peptide lantibiotics, such as Hal β and Bli β (Figure 1.18). Wang *et al.* [71] also performed SAR studies for cerecidin A7 core peptide using Ala scanning mutagenesis. The results showed that substitutions in thioether rings, and hydrophobic and positively charged residues resulted in impaired activity.

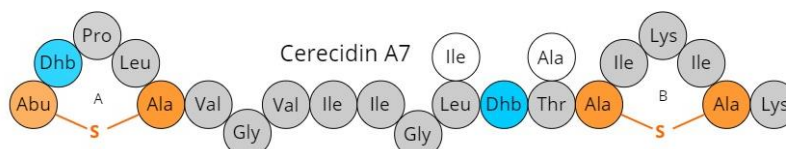


Figure 1.18. Structure of class II cerecidin A1 to A6 produced by *Bacillus cereus*. The residues that differ in cerecidin A7 are represented in white. The Lan and MeLan rings are orange, and dehydrated residues, blue.

When cerecidin A1 and A7 were administered independently the peptides inhibited the growth of *Micrococcus flavus* NCIB8166 strain and other Gram-positive bacteria [71]. In general, cerecidin A1 is less active than cerecidin A7 and shows no activity towards the MRSA 1-1 strain and the vancomycin-resistant *E. faecalis* V583 strain, which were inhibited by cerecidin A7. Additionally, cerecidin A7 is less potent than nisin against these two bacteria [71]. Excitingly, a variant of cerecidin A7 (Thr13Ala) resulted in a peptide with improved activity (2- to 4-fold) against *M. flavus* NCIB8166, *B. cereus* AS1.352 and the antibiotic resistant strains *S. aureus* 1-1 and *E. faecalis* V583. When applied at higher concentrations (20 μ g/mL), this variant of the A7 peptide was also able to affect the growth of two *Streptomyces* spp., rarely reported for other lantibiotics [71].

1.8.4 Pseudomycoicidin.

Pseudomycoicidin was discovered in *Bacillus pseudomycooides* DSM 12442 through a genome mining approach [72]. This lantibiotic has 26 residues (M = 2786 Da), four thioether and one disulphide bridge (Figure 1.19) and contains two C-terminus Ser-Trp-Ser-Cys motifs that had previously only been described in bacteriocins and not in lantibiotics. In addition to the modification enzyme PseM and the respective structural gene, *pseA*, the gene cluster also includes the ABC transporter containing the double-Gly peptidase domain (PseT), followed by three ORF (*pseFEG*) encoding the putative self-immunity enzymes, similar to what happens in the mersacidin gene cluster (Figure 1.14).

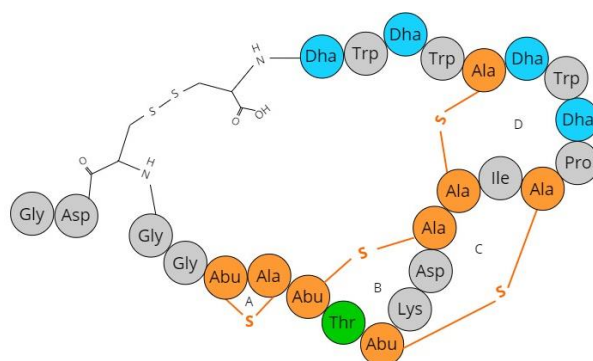


Figure 1.19. Proposed structure of class II pseudomycoicidin produced by *B. pseudomycooides*. The Lan and MeLan rings are orange, dehydrated and non-dehydrated Ser and Thr residues, in blue and green, respectively.

Its production was evaluated in different culture media, temperatures and incubation times. The best culture medium was TSB and the best growth condition was incubation at 30°C for 24h. The peptide is stable for 1h at pH 4 and pH 7, less than 1h at pH 10, and up to 4h at pH 2. Furthermore, its antimicrobial activity is stable between 20-100°C, at pH 2. Only isopropanol wash extracts from the cell pellet have activity against a large number of Gram-positive strains, but not against Gram-negative targets (Table 1.2). Pseudomycoicidin is thought to bind lipid II resulting in lysis of the target strains. Bioactive pseudomycoicidin was partially expressed *in vivo* in *E. coli*, through the co-expression of *pseA* and *pseM*, followed by *in vitro* cleavage of the leader sequence using Xa cleavage factor. To further clarify the ring topology of the peptide, the heterologous system was employed to produce six mutants where each cysteine was substituted by Ala. All the mutants were produced, although dehydration patterns were incomplete and they were inactive against *M. luteus*, unlike the native pseudomycoicidin. In addition, four of the mutants became sensitive to trypsin digestion, indicating that the rings were not correctly formed. The same study also reported that PseM is unable to modify a recombinant peptide in which PseA leader sequence is fused with the core peptide of another putative class II lantibiotic of *Caldicellulosiruptor bescii* DSM 6725 [72,166].

1.8.5 Ticins.

Whole genome sequencing of *B. thuringiensis* strain BMB3201 revealed the presence of two gene clusters encoding lanthipeptides [73]. The first cluster was expected to produce a class I lanthipeptide but no transcripts of the corresponding *lanB* gene could be detected. The second is a class II cluster that has four structural genes, *tinA1*, *tinA2*, *tinA3* and *tinA4*, sharing 88% similarity between them (Figure 1.16), followed by a single

modification enzyme, TinM. The designated *tin* gene cluster also encodes three genes corresponding to an ABC transporter, possibly involved with self-immunity (*tinEFG*), a transporter with a protease domain (*tinT1*), a secretion protein (*tinT2*) and a regulatory protein (*tinR*) (Figure 1.14). Only three structural genes were found to be expressed, originating the ticins A1 (M= 4062.9 Da), A3 (M= 4048.9 Da) and A4 (M= 4063.0 Da). All ticins possess 8 dehydrated Ser/Thr residues out of 12, indicating that the same modification enzyme is able to process these three peptides (Figure 1.20).

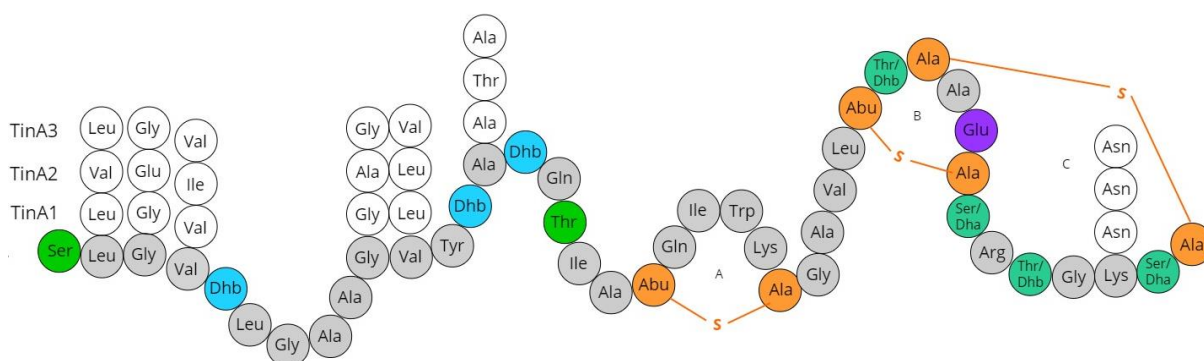


Figure 1.20. Structure of class II ticin produced by *B. thuringiensis*. The residues that differ in ticins are represented in white. The Lan and MeLan rings are orange, dehydrated Ser and Thr residues, in blue, and non-dehydrated, in green. Ser and Thr residues whose dehydration state could not be determined are represented in turquoise. The conserved Glu motif is represented in purple.

Xin and co-workers hypothesize that the differences in the expression of the four *tinA* genes are related to the transcription process, since each gene has its own promoter and terminator. The three peptides were purified and have similar characteristics regarding activity and stability. Ticin A4 is more stable than nisin in neutral - alkaline conditions, remaining 100% stable in pH 2 to 9, decreasing only at pH above 10. It also retains activity after 2 weeks at 28°C, 1h at 80-100°C and 30 min at 121°C. Ticin A4 is sensitive to the proteolytic enzymes, trypsin, chymotrypsin and papain and is resistant to α -amylase. Ticins have activity, sometimes higher than nisin, against various species of *Bacillus* and other Gram-positive bacteria (Table 1.2). Ticin A4 is bactericidal and this should also be the case of the other ticins [67].

1.8.6 Haloduracin.

Haloduracin was the first two-peptide lantibiotic (Figure 1.21) identified in the *Bacillus* spp. group. It was discovered by *in silico* bioinformatics analysis of *Bacillus halodurans* C-125 genome [34,167]. Its gene cluster comprises 11 open reading frames (Figure 1.14). It is a typical two-peptide lantibiotic cluster, encoding two precursor peptides (HalA1 and

HalA2) and two modification proteins (HalM1 and HalM2). *halT* encodes a LanT-like protein, responsible for the export and leader sequence cleavage of both antimicrobial peptides. Unlike other two-peptide lantibiotics already described, the gene cluster of haloduracin encodes two sets of immunity genes, *halF1G1E1* and *halF2G2E* [34,167].

Haloduracin is active against Gram-positive bacteria, including *Listeria*, *Streptococcus*, *Bacillus* and *Pediococcus* species and many lactococci [167]. In addition, this lantibiotic is able to inhibit *Bacillus anthracis* spore outgrowth with an efficacy similar to that of nisin [91]. As with other two-peptide lantibiotics, the two mature peptides Hal α (M= 3043,3 Da) and Hal β (M= 2330 Da) act synergistically to kill bacteria, and full activity of haloduracin is achieved at an optimal ratio of 1:1 of Hal α :Hal β [34,91]. When administered independently, Hal α and Hal β are still able to inhibit the growth of the highly susceptible *L. lactis* HP strain but a 50- to 100-fold decrease in activity was observed when compared to their combined use [91]. This independent bioactivity is explained by the mode of action of each peptide, where Hal α binds lipid II and prevents peptidoglycan biosynthesis, and Hal β binds to anionic lipids of the cell membrane and causes a transient formation of pore-like structures (Figure 1.5) [91]. It was also determined that Hal α binds to its substrate lipid II in a 2:1 stoichiometry, inhibiting the PBP1b (penicillin-binding protein) and this interaction is not inhibited by the presence of the leader peptide [95,161]. The proposed mechanism of action for haloduracin is similar to that of lacticin 3147 [92] which involves the formation of a complex by binding of Hal α to lipid II at the cell surface. Afterwards, Hal β recognizes and bind to the Hal α :lipid II complex causing membrane permeabilization along with depolarization and release of K⁺ ions [91,161]. It was suggested that this activity is achieved by a 1:2:2 lipid II:Hal α :Hal β stoichiometry [95,161]. Oman and co-workers [91] concluded that Hal α was highly stable, whereas Hal β showed moderate stability in a low buffer concentration at pH 7.5. It was found that the lower stability of Hal β may be due to oxidation, probably of the thioether bridges, since peptides with an increment of 16 Da were detected using MS analysis. The higher stability of Hal α can be due to fewer residues that can be oxidized in addition to its globular structure [91]. Even so, both peptides are more stable than nisin [91].

The biosynthesis of haloduracin was reconstituted *in vitro*, through the separate overexpression in *E. coli* of the structural genes and modification enzymes (*halA1*, *halA2*, *halM1* and *halM2*) followed by purification and incubation of the Hal α and Hal β with HalM1 and HalM2, respectively [34]. The system was used to investigate the substrate specificity of the HalM modification enzymes, to provide material to clarify the structure of the peptides, and to study the relationship between their structure and bioactivity [34,168].

The results showed that Hal α 's A-ring (Figure 1.21) is important but not essential for bioactivity. The Hal α is structurally similar to mersacidin and contains glutamic acid (Glu22) within its B-ring (Figure 1.21), which was also shown to be critical for bioactivity. Surprisingly, the formation of the MeLan residue that constitutes the B-ring of Hal α is not required for bioactivity. The A-ring of Hal β (Figure 1.21) is dispensable and the C- and D-rings are important but not essential for bioactivity. No conclusions could be drawn for the B-ring, since the loss of bioactivity observed could be due to the disruption of multiple rings caused by the substitution of Cys15 by Ala [56,168]. Cooper *et al.* [168] also investigated the proteolytic processing of the two peptides. The leader peptides of HalA1 and HalA2 are removed by the N-terminal protease domain of HalT at the GG/GA/GS-motif (Figure 1.16). As with mersacidin, the HalA2 also contains a hexapeptide that is removed to produce the mature Hal β peptide (Figure 1.16). It was determined that the bioactivity of haloduracin is unaffected if this hexapeptide is not cleaved. It is absent in the mature Hal β and it was concluded that it could be trimmed by a non-specific extracellular protease(s) secreted by the producing *B. halodurans* C-125 strain, since no ORF encoding a protease was found in the *hal* biosynthetic cluster [168].

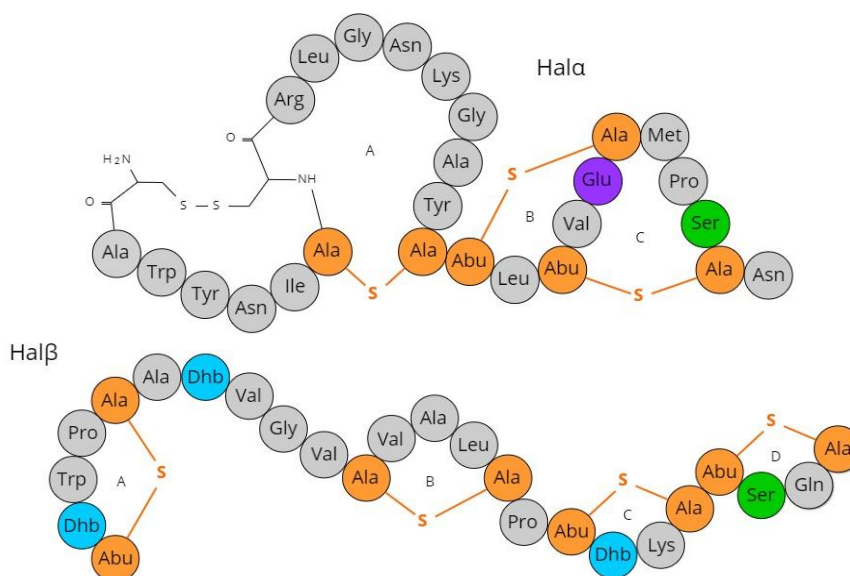


Figure 1.21. Structure of class II haloduracin, a two-peptide lantibiotic produced by *B. halodurans*. The Lan and MeLan rings are orange, dehydrated Ser and Thr residues, in blue, and non-dehydrated, in green. Ser and Thr residues whose dehydration state could not be determined are represented in turquoise. The conserved Glu motif is represented in purple.

Haloduracin production was observed in liquid or solid complex media. TY broth (pH 9.5) was used for liquid cultivation and haloduracin was purified from culture supernatants

after incubation for 20 h, at 37°C [167]. In solid medium, *B. halodurans* C-125 was grown for 96h at 30°C on Brain Heart Infusion agar plates [91]. More recently, a small scale fermentation using wheat bran as solid-state substrate was described. The authors investigated the effect of the addition of LB medium, MgSO₄, K₂HPO₄ and Na₂CO₃ on haloduracin yields [169]. It was found that the addition of LB decreased the production of haloduracin, whereas MgSO₄ or K₂HPO₄ addition resulted in three-fold increase in yield. Moreover, the optimal concentration of Na₂CO₃ was determined as 10% (w/w) for maximum haloduracin production. Once the conditions were optimized, the culture was allowed to grow for one week at 37°C and the highest activity was reached after 48 h of cultivation [169].

1.8.7 Thusin.

Thusin is a novel two-peptide lantibiotic of *B. thuringiensis* strain BGSC 4BT1, that was discovered by genome mining by Xin and co-workers (2016). It is composed of Ths α (M= 3928.9 Da) and Ths β (M= 2908.5 Da) or Ths β' (Ths β variant Ala14Gly) (M= 2922.5 Da) (Figure 1.22) [74]. Thusin gene cluster has approximately 10 kb consisting of eight genes organized in an unusual conformation (Figure 1.14): three structural genes, *thsA1*, *thsA2* and *thsA2'*, encoding each of the peptides; two modification enzymes, *thsM1* and *thsM2*; two putative immunity genes, *thsEF*; and an ABC transporter, *thsT*. ThsA1 has 43.8% similarity with LicA2 (from lichenicidin, section 1.9; Figure 1.16) whereas ThsA2 variants are similar to LtnA2 (from lacticin 3147). In Ths α , all the Ser and Thr residues are dehydrated and three thioether rings are proposed (Figure 1.22). As for Ths β peptides, these have eight out of ten dehydrations and also three rings (Figure 1.22). Thusin peptides are highly thermostable (100°C for 30 min), very stable to acidic conditions, but not to neutral or alkaline conditions. As with other two-peptide lantibiotics, such as lichenicidin and haloduracin, a 1:1 ratio of each of the peptides, is ideal for synergistic activity against Gram-positive bacteria, including *S. aureus* and *L. monocytogenes*. Although the peptides show bioactivity when applied separately, there is 4- to 16-fold increased efficacy when used at that molar ratio. A comparative study of thusin with thuricin 4A-4 and ticin A4 showed that thusin has greater activity against all strains tested. Thusin mechanism of action is similar to that described for haloduracin and lacticin 3147. The sequential interaction of the peptides has been confirmed: Ths β peptides can only exert their synergistic activity when added together or after addition of Ths α . Like haloduracin and nisin, thusin is capable of inhibiting the proliferation of *B. cereus* ATCC 14579 spores [74].

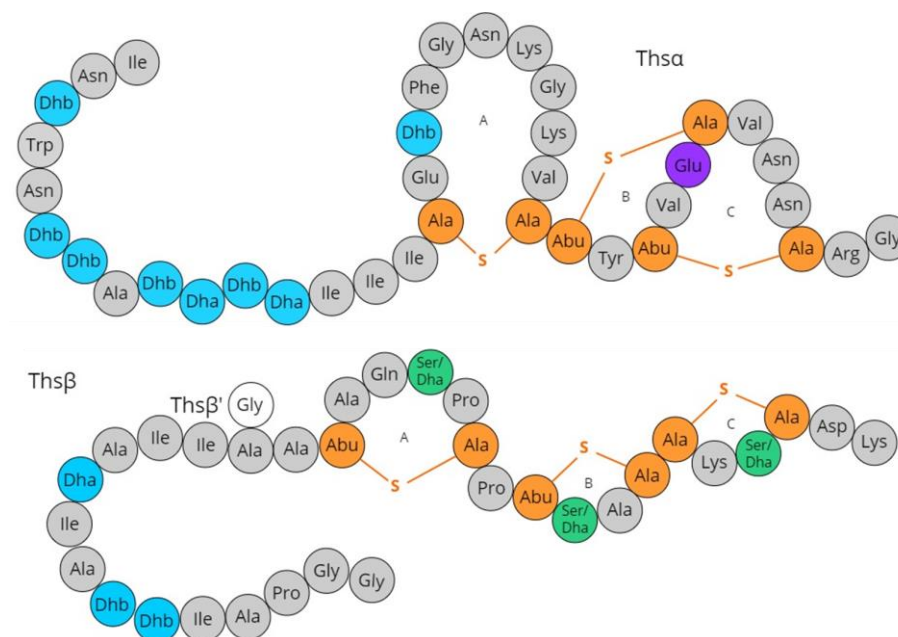


Figure 1.22. Structure of class II thusin, a two-peptide lantibiotic produced by *B. thurigiensis*. The residue that differ in thusin β' is represented in white. The Lan and MeLan rings are marked orange, dehydrated Ser and Thr residues, in blue, and non-dehydrated, in green. Ser and Thr residues whose dehydration state could not be determined are represented in turquoise. The conserved Glu motif is marked in purple.

1.8.8 Formicin.

Formicin is a two-peptide lantibiotic produced by *Bacillus paralicheniformis* APC 176 [75]. This bacterium was isolated from a mackerel gut. Formicin gene cluster was identified in the genome of this strain by Collins and co-workers (2016). The *frc* gene cluster is 17 kb long and the gene organization differs from that of other two-peptide lantibiotics. Genes are unidirectionally transcribed and each structural gene is followed by its modification enzyme LanM. (Figure 1.14). Thus, the structural genes, *frcA1* and *frcA2*, which give rise to Frc α (3254.3 Da) and Frc β (2472.1 Da) peptides, are interspersed with the respective modification enzymes, FrcM1 and FrcM2. Probably the transport and cleavage of both peptides is performed by FrcT, which has 52.5% homology with HalT of haloduracin. An additional protease, FrcP, is also encoded and has a similar proteolytic activity to that of LicP (lichenicidin), cleaving the N-terminal Frc β hexapeptide that still remains after cleavage by FrcT. Six other ORF encode two ABC transporters that are likely to contribute to the self-immunity of the producing strain. Between these, there is the

frcR gene that encodes the transcriptional regulator containing a helix-turn-helix XRE family domain. The gene cluster also includes a hypothetical protein with 28.4% homology with LicY of the lichenicidin biosynthetic gene cluster and whose function is still unknown, and a gene encoding a DNA damage-inducible protein (Figure 1.14) [75].

Formicin structures were predicted based on Hal α , Bli β and Lct β peptides. However, they have some structural differences that may indicate a different mechanism of action among the two-peptide lantibiotics. Frc α has a disulfide bond (Figure 1.23) that does not appear to be necessary for its activity but is thought to contribute to the stability of the peptide. However, the main differences are found on the nature of the residues within the peptide chain. Lan α peptides have an average of 9 hydrophobic amino acids, which are important for the interaction with the target membranes. The Frc α peptide has only five hydrophobic amino acids, which does not appear to compromise its bioactivity against some indicator strains, even in the absence of Frc β . Frc α has four positively charged and two negatively charged residues, something unusual among closely-related α peptides. This suggests that charged residues are responsible for interaction with the anionic bacterial membrane in the absence of hydrophobic interactions.

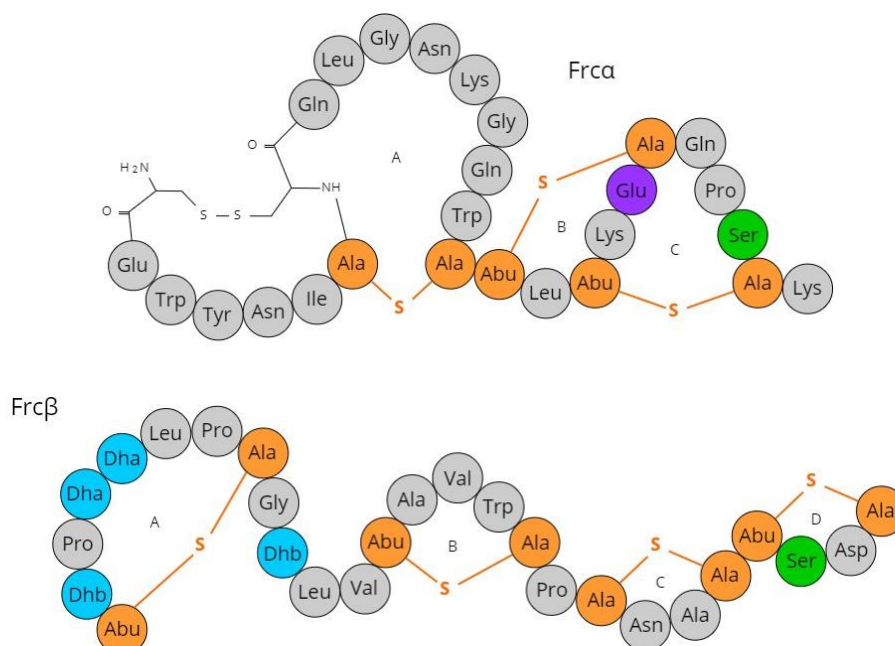


Figure 1.23. Structure of class II formicin, a two-peptide lantibiotic produced by *B. paralicheniformis*. The Lan and MeLan rings are marked orange, dehydrated Ser and Thr residues, in blue, and non-dehydrated, in green. The conserved Glu motif is marked in purple.

In general N-termini of β -peptides, contain hydrophobic amino acids, which are essential for their insertion through the bacterial membrane with consequent pore formation whereas the C-termini is positively charged and contain Lys and Arg residues.

Frc β , by contrast, has only one negatively charged residue at the C-terminal, an Asp (Figure 1.16). It is believed that β -peptide C-terminal interacts with the α -peptide [92]. It appears that since α -peptide is positively charged, the presence of the negatively charged residue at the C-terminal of Frc β may increase its affinity for Frc α , leading to the formation of a stronger complex when compared to other two-peptide lantibiotics. However, these changes render Frc β inactive when applied alone, in contrast to other β -peptides, such as Ltn β and Bli β [75].

Regarding its stability, formicin is more heat-sensitive than other lantibiotics, losing 28% of its activity after 30 minutes at 100°C and 100% after only 15 minutes at 121°C. It is sensitive to digestion by α -chymotrypsin and proteinase K. Formicin shows synergistic bioactivity against lactobacilli, enterococci and strains of medical relevance such as *S. aureus*, *S. mutans*, *L. monocytogenes*, *C. difficile* and *B. subtilis* [75].

1.8.9 Bicereucin, a special case.

Bicereucin was identified in *B. cereus* SJ1 genome. It was isolated after its partial *in vivo* biosynthesis in *E. coli* [76]. Bicereucin is an uncommon “lantibiotic”, because its activity results from the synergistic effect of a nonlantibiotic with a lantibiotic peptide. Since it does not fit completely into any of the classes described so far, it is considered a mixed two-peptide lantibiotic. Bicereucin gene cluster encodes three structural genes, *bsjA1*, *bsjA2* and *bsjA3*, which give rise to three peptides: Bsja1 and Bsja3, which have the same amino acid sequence and constitute Bsja α (3486 Da), and Bsja2, which originates Bsja β (4100 Da) (Figure 1.24). Bsja β has a single Cys residue at position 40 that forms a lanthionine ring with a dehydrated Ser at position 35, and which is essential for bicereucin activity. The two Bsja1 and Bsja3 peptides, lack Cys residues but are rich Ser/Thr residues that are dehydrated (Figure 1.16). All the three precursor peptides contain several D-amino acids, including D-Ala and D-Abu (Figure 1.24) [76]. This is not a common conformation, but D-amino acids seem to stabilize peptide structures by increasing protease resistance, which makes these peptides attractive for industrial applications [170].

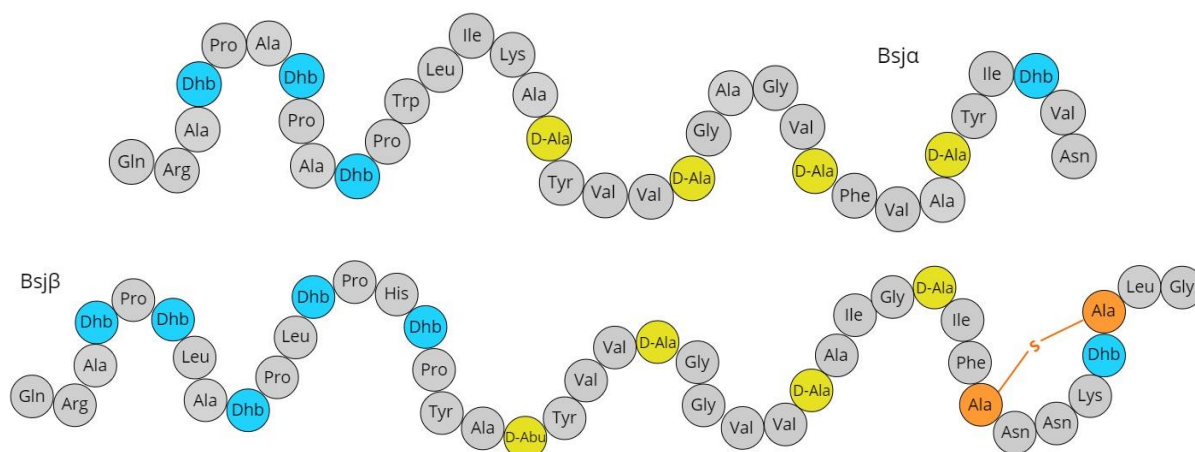


Figure 1.24. Structure of class II biceuricin, a two-peptide lantibiotic produced by *B. cereus*. The Lan ring is filled in orange and dehydrated Ser and Thr residues, in blue. Dehydrated residues in D-conformation are represented in yellow.

Thus, biceuricin gene cluster includes the class II lanthipeptide synthetase BsJM, which is responsible for the dehydration of the Ser and Thr residues and for the formation of the only lanthionine of this lantibiotic, and the dehydrogenase BsJB that reduces Dha and Dhb to D-Ala and D-Abu *in vivo* (Figure 1.14). Furthermore, the biosynthetic cluster contains a LanT-like peptidase-containing ABC transporter, BsJT, responsible for the transport and cleavage of the leader sequence at the conserved double Gly-like motif. A subtilisin-like protease (BsJP) is also encoded that, similarly to LicP, trims the oligopeptide constituted by three residues – Ala-Val-Glu – that remains attached to the core peptide after BsJT activity and which is common to all BsJA peptides. Interestingly, this Glu residue is conserved in other hexapeptides that undergo proteolysis by a dedicated protease after LanT processing, such as lichenicidin and the cercidins (Figure 1.16). Another peculiar characteristic of biceuricin is that the optimal molar ratio for its bioactivity is 2:1 Bsja:Bsjb. As Bsja is indeed the result of the expression of the two structural genes *bsjA1* and *bsjA3*, the optimal ratio is a direct reflection of the stoichiometry of the genes: 2:1 (*bsjA1* + *bsjA3*): *bsjA2*. At this ratio, the MIC of biceuricin against MRSA and VRE is 50 to 100-fold lower than the MIC of the independent peptides (Table 1.2). Interestingly, regarding the biceuricin mode of action, Huo and van der Donk determined that its effect is sequential, as for the majority of two-peptide lantibiotics: Bsja binds a target on the bacterial cell membrane and the resulting complex recruits Bsjb that is able to perform its synergistic effect [76].

1.9 THE CASE-STUDY OF LICHENICIDIN, A TWO-PEPTIDE LANTIBIOTIC PRODUCED BY *B. LICHENIFORMIS*

Lichenicidin was first described by two independent groups [77,171] using rational genome mining, wherein LanM proteins were used to screen the genomes of several microorganisms available in the databases. This lantibiotic was primarily found to be produced by *Bacillus licheniformis* ATCC 14580 [77] and DSM13 [171]. However, it is also produced by *B. licheniformis* VK21 [101] and *B. licheniformis* I89 [54,172], both isolated from hot springs. More recently, three marine isolates were also identified as lichenicidin-producers (*B. licheniformis* WIT 562, 564 and 566). These were the first known example of bacteria associated with algae to produce bacteriocins [36].

Lichenicidin produced by *B. licheniformis* I89 is a two-peptide lantibiotic constituted by Bli α (M= 3250 Da) and Bli β (M= 3020 Da) (Figure 1.25), which act synergistically to exert full antibacterial activity [101]. Its gene cluster includes the two structural genes, *licA1* and *licA2*, encoding the precursor peptides of Bli α and Bli β , respectively, and the modification enzymes, LicM1 and LicM2 (Figure 1.14). A gene encoding the transporter/protease protein LicT, typical of class II lanthipeptides is also present. Immediately downstream of *licT*, there is a gene that encodes an uncharacterized serine protease designated LicP. The LicA2 precursor peptide also contains the hexapeptide between the GG-motif and the first residue of the mature Bli β peptide (Figure 1.16), and LicP is the protease that catalyzes the removal of these 6 residues. Unlike Hal β , the Bli β peptide with the hexapeptide still attached to its N-terminal, the so-called Bli β ' peptide, does not have antibacterial activity [54]. A group of genes with homology with the immunity genes (*licFGEHI*) from other lantibiotics is also present in the *lic* cluster; these genes are putatively involved in self-protection of the producer strain [77,171]. In addition, a regulatory protein (LicR) is also encoded and it was previously demonstrated that the deletion of its gene (*licR*) resulted in the abolishment of Bli α production in *E. coli* [54]. However, further studies demonstrated that the lack of production was actually due to a mutation in *licM1* gene and not to the absence of *licR* [173]. Thus, the involvement of LicR in the regulatory processes of lichenicidin biosynthesis is still unknown. Since the absence of LicR does not affect lichenicidin production in *E. coli* (which is naturally resistant to this lantibiotic), this gene appears to be involved in regulating *lic* immunity genes, as previously reported for the two-peptide lantibiotic lactacin 3147 [174]. LtnR, like LicR, shares homology with transcriptional repressors of the XRE family-like proteins and negatively regulates the transcription of lactacin 3147 immunity genes but not the structural genes [174]. Furthermore, two other ORF are present in the gene cluster, *licX* and *licY*,

that do not have similarity with other proteins associated with the biosynthesis of lanthipeptides. Moreover, it is not possible to detect conserved domains in their encoded proteins making it difficult to predict their putative function [54].

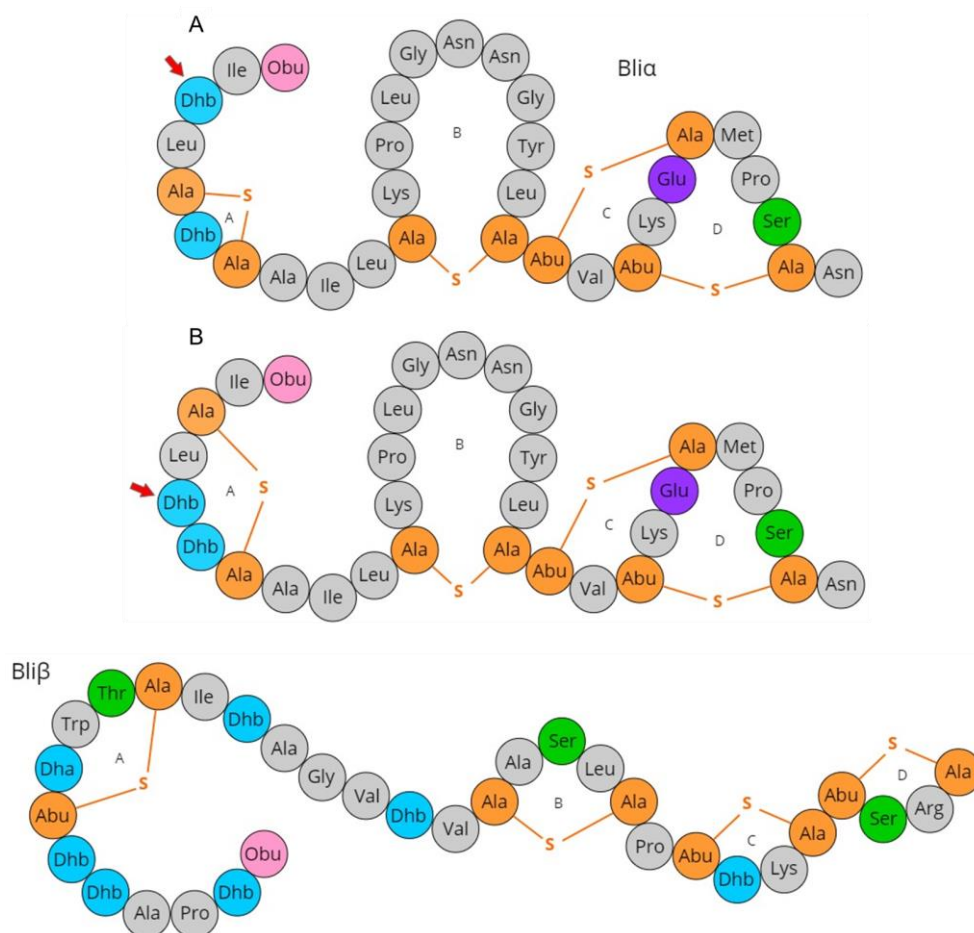


Figure 1.25. Structure of class II lichenicidin, a two-peptide lantibiotic produced by *B. licheniformis*. Two different structures are illustrated for Bli α peptide, according to Caetano *et al.*21 (A) and Shenkarev *et al.*103 (B). The Lan and MeLan rings are orange, dehydrated Ser and Thr residues, in blue and the non-dehydrated, in green. The Obu residue (pink) refers to 2-oxobutyl. The conserved Glu motif is represented in purple.

Lichenicidin I89 was the first lantibiotic to be produced totally *in vivo* in the Gram-negative host *E. coli* through the expression of the complete *lic* gene cluster in a fosmid under the regulation of *B. licheniformis* transcriptional elements [54]. This accomplishment facilitated the genetic manipulation of the gene cluster, since *B. licheniformis* is not naturally competent and is not amenable to transformation procedures. A *trans* complementation system was developed which enabled the expression of several LicA

mutant peptides generated by SDM [54]. Furthermore, optimized expression systems for the production of each peptide were developed, improving the production yields and enabling peptide purification [175]. The attainment of active lichenicidin in *E. coli* without the requirement of any further *in vitro* step prompted the development of an *in vivo* model for noncanonical amino acids (ncAAs) translation into lantibiotics [176]. In this latter study, Met and Pro residues of Bli α peptide were replaced by some of their ncAAs analogues [e.g., Hpg, Aha, Nle, Eth, (4*R*-OH)Pro, (4*R*-F)Pro, (4*S*-F)Pro, (S)Pro]. The antibacterial activity of Bli α (Met28Hpg) was slightly decreased when compared with the native peptide, was nearly equal for congeners containing Eth- or Nle-analogues and it was fully restored with the Aha-congener. Post-biosynthetic modifications using the click-reaction were successfully introduced by coupling 1-azido-1-deoxy- β -D-glucopyranoside and azido-fluorescein to the Bli α (Met28Hpg) congener peptide [176].

Kuthning and co-workers used an evolutionarily adapted *E. coli* strain to produce a Bli β variant containing the non-canonical Trp-analogue [3,2]Tpa (L- β -(thieno[3,2-*b*]pyrrolyl) alanine) [177,178]. After a long-term evolution experiment an *E. coli* able to incorporate [3,2] Tpa (instead of Trp) from TGG codons of the genome was isolated [179]. This strain was derived from the Trp-auxotrophic strain *E. coli* K12 W3110. Since Trp biosynthesis is abolished, Trp or its analogues have to be fed and enter the cell via transporter-mediated uptake [179]. Bli β was chosen for this study as it has a Trp residue in position 9 [177]. Bli β ([3,2]Tpa⁹) derivative was obtained and its synergistic activity with Bli α is comparable to the bioactivity of the native peptides. Thus, not only the *lic* machinery is able to cope with the insertion of this non-canonical amino acid, but also, this insertion does not impair bioactivity nor the interaction between both peptides [177]. This kind of studies is a step forward considering the development of biocontained cell factories for wide application in open systems [177]. As the strain is totally dependent on the feed of a particular amino acid or analog, its release and subsequent eradication in a certain environment can be controlled by feeding. Such adapted strains provide valuable tools for applications in open environments, including bioremediation approaches.

The *E. coli* host system was also employed to generate and analyze chimeric peptides, produced by in-frame fusion of lichenicidin leader peptides with haloduracin core peptides (which are the most closely related to lichenicidin) [180]. Results showed that the lichenicidin modification enzymes were not able to modify haloduracin peptides and vice-versa, contrarily to what was previously described for other lantibiotics [86]. The same study concluded that HalT, the transporter/protease protein of haloduracin, was able to remove the leader peptides and export Bli α and Bli β in the absence of LicT [180].

Two different methods were employed to characterize Bli α and Bli β structures: *i*) NMR spectroscopy combined with genomic data analysis [101], and *ii*) comparison of MS/MS fragmentation pattern of native and mutated peptides (where Ser, Thr and Cys residues were substituted by Ala) [54]. The results produced by both methodologies were consistent, except for the residues involved in the formation of the A-ring of Bli α as shown in Figure 1.25. Accordingly, Caetano and co-workers [54] hypothesized that the A-ring is formed between Ser5 and Cys7, while Shenkarev *et al.* [101] determined that this ring is formed with Thr3 and Cys7.

The outer membrane of Gram-negative bacteria creates a physical barrier to the entry of lichenicidin into the cell, which makes it ineffective against this group of bacteria [89]. In this study, an *E. coli* strain with a permeable outer membrane was employed (*E. coli* *lptD4213* – formerly *imp4213*) which proved to be slightly sensitive to lichenicidin when compared to the wild type *E. coli* MC4100 that was not affected by the lichenicidin peptides. Lichenicidin is active against several Gram-positive bacteria, including *B. subtilis*, *B. pumilus*, *Bacillus megaterium*, *S. aureus* (MSSA and MRSA) and *L. lactis* strains (Table 1.2) [101,171]. The MIC obtained when Bli α and Bli β produced by *B. licheniformis* VK21 were combined at a 1:1 molar ratio were much higher (nM range) than the sum of their individual MIC (μ M range), which highlighted their synergistic effect [101].

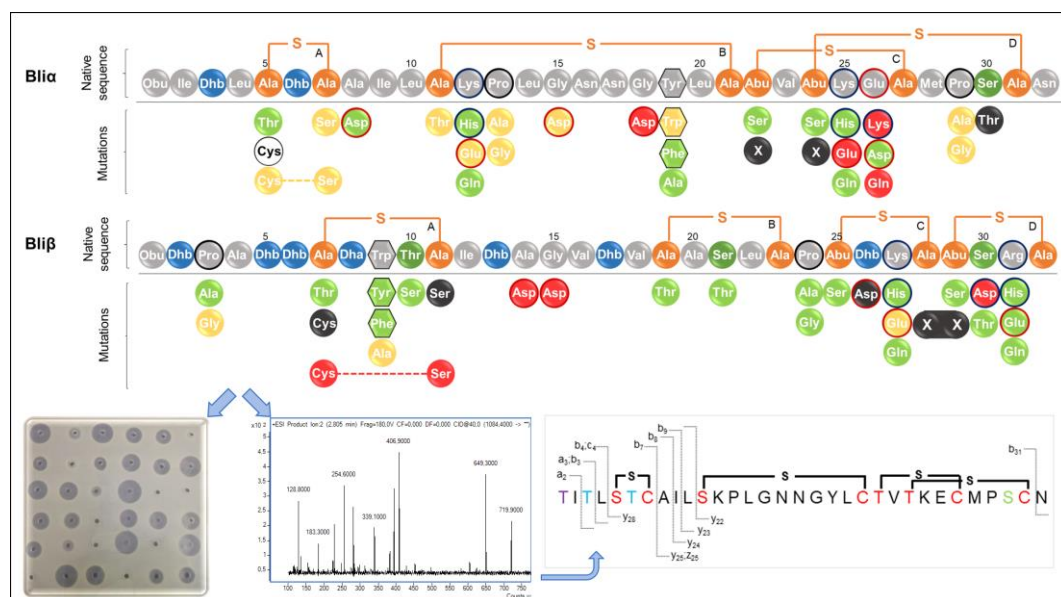
The production of lichenicidin by *B. licheniformis* strains was achieved in complex media such as medium M [172] and LB broth [77] as well as with C₂Mn medium (containing sucrose) [101], tryptone-yeast extract-glucose [77] medium and 2X BPM [171]. In 2X BPM medium lichenicidin was only detected in the cell-associated fraction and not in the culture supernatant. The incubation conditions described include the temperatures of 37°C [77,171], 45°C [101] and 50°C [172], with sampling times ranging from 6 h [101] to 48 h [171]. More recently, an extensive study to optimize the industrial production of several enzymes of *B. licheniformis* revealed that the transcription of the *lic* gene cluster starts in the early stages of the fermentation and tend to decline in the later stages [181]. The medium used in that particular case contained a complex nitrogen source and several salts with the pH stabilized at 7.9. Fermentation was performed at 39°C for 46 h in aerated 16 L fermenters containing 6 L of culture medium. When biphasic growth was observed, glucose was fed into the medium [182]. It was also found that the transcripts of lichenicidin structural genes *licA1* and *licA2* are highly abundant during the entire fermentation process [181]. The adaptation of bacteria to laboratory conditions (a process also termed domestication) is an important biological process that is often overlooked by

microbiologists. A recent study focused on the influence of this phenomenon in the production of lichenicidin [183]. It was found that the lichenicidin producer *B. licheniformis* I89 strain, originally characterized by colonies with a rough surface and strong adherence to the agar (R-type), acquired a distinct morphology during maintenance in laboratory. The new colonies were smooth and have poor adhesion to agar (S-type). Further experiments concluded that the S-type strain produced lower levels of the Bli β peptide than the original R-type strain resulting in a decrease in the synergistic activity of lichenicidin [183].

CHAPTER II

LICHENICIDIN RATIONAL SITE-DIRECTED MUTAGENESIS

LIBRARY



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2.1 INTRODUCTION

The dissemination of drug-resistant pathogenic bacteria led to an increased demand of new antibiotics, with specifically targeted activities. Lantibiotics – lanthipeptides with antibacterial activity – are natural antimicrobial peptides, which were firstly described in Gram-positive bacteria [42,51]. Typically, lantibiotics target other Gram-positive bacteria [23], but other targets have also been described, namely by the pinensins, that present antifungal bioactivity [17], and labyrinthopeptins, which exhibit anti-viral activity [18]. Most lantibiotics interact with lipid II, a bacterial cell wall synthesis precursor, while others are able to insert into the bacterial membrane, inducing pore formation [42]. Studies on lantibiotics have been intensified over the last few years, as these have proved to be promising candidates for the pharmaceutical industry due to some particular characteristics, namely, the activity against clinically important virulent strains, including methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant Enterococci (VRE) [23], sometimes with an even higher activity than conventional antibiotics like vancomycin [42,184]. Moreover, they have low toxicity towards mammals [23,37].

Lantibiotics are ribosomally synthesized peptides that undergo several post-translational modifications, until they reach the mature form, which include the formation of lanthionine (Lan) and methyllanthionine (MeLan) rings, characteristic of this type of compounds. Briefly, Ser and Thr residues that are present in the core peptide (the precursor of the mature peptide) are dehydrated to yield 2,3-didehydroalanine (Dha) and (Z)-2,3-didehydrobutyrine (Dhb), respectively. Then, an intramolecular Michael-type addition of Cys thiol side chains to Dha or Dhb residues with formation of Lan and MeLan rings, respectively [25]. For detailed characterization and biosynthetic pathways of lantibiotics, the readers are referred to [4,10,35]. Considering their synthesis, and also from a biotechnological perspective, lantibiotics are relatively easy to modify through mutagenesis approaches, as described by many authors [25,38,51,52,113,185–191]. This approach was employed to reveal information on structure-function relationship, to improve antimicrobial and pharmacological properties of lantibiotics and also for the rational design of these peptides. For example, site-directed mutagenesis (SDM) studies on nisin, revealed that the size of the hinge region between C- and D- rings is important for its bioactivity [192]. This was observed although amino acid exchanges in this region were well tolerated and, in some cases, even yielded higher bioactivities when compared to the native peptide [193]. The same was also observed for mutacin 1140, allowing to determine the ideal distance between A and B rings and also the size of the A ring [191].

With regard to lacticin 3147, along with the generation of improved variants [185,190], charged amino acids seem to play a significant role on bioactivity [185].

Lichenicidin is a two-peptide lantibiotic naturally produced by *Bacillus licheniformis* strains [36,54,77,101,171,172]. Bioactivity against target strains results from the synergistic activity of the two peptides, Bli α and Bli β [101]. Clinically relevant bacterial strains inhibited by lichenicidin include methicillin-resistant *Staphylococcus aureus* (MRSA), *Enterococcus faecium*, *Haemophilus influenza* and *Listeria monocytogenes* [194]. Like the other lantibiotics, all the genes required for lichenicidin synthesis are encoded in the *lic* gene cluster, which comprises the two structural genes, *licA1* and *licA2*, and the genes encoding the respective modification enzymes (LicM1 and LicM2) which give rise to both processed peptides, Bli α and Bli β . Additionally, the gene cluster includes genes for transport and proteolytic trimming (*licT*), Bli β processing (*licP*) and self-protection (*licFEGHI*) [54,77,171]. Lichenicidin was the first lantibiotic to be successfully produced totally *in vivo* in *Escherichia coli* [54]. The system developed allowed further manipulations of the biosynthetic machinery in the heterologous host, since these are particularly difficult to perform on the native producer, *Bacillus licheniformis* I89. In addition, a *trans* complementation system was developed which allowed the production of both peptides separately (either Bli α or Bli β) in the Gram-negative host [54]. The lichenicidin bioengineering systems were then employed to: *i*) determine the impact of Ser, Thr and Cys residues on the bioactivity of lichenicidin [54], *ii*) to insert non-canonical amino acid residues into the lichenicidin sequence and thus, expand its structural diversity [176] and *iii*) to elucidate the flexibility of the post-translational modification machinery, using chimeric genes lichenicidin-haloduracin [180]. More recently, an improved system for lichenicidin production in *E. coli* was developed to ease Bli α and Bli β purification [175]; the same vectors were employed for total *in vivo* insertion of toxic non-canonical amino acids using an evolutionarily adapted *E. coli* host strain [177].

The present study provides new insights into the role of specific amino acids in lichenicidin biosynthesis and bioactivity. It focuses on residues that were considered of interest and with possible impact on lichenicidin structure/activity, namely, amino acids containing aromatic side chains, charged residues, proline and ring-forming residues. Furthermore, lichenicidin was employed as a case study, to further broaden the understanding of structure-activity relationships (SAR) of lantibiotics in general. Additionally, the reliability of the full *in vivo* expression system to generate mutant libraries was assessed, employing different analytical methods, namely, HPLC-MS and MS/MS. At

the same time, we developed a quick and easy method to monitor antibacterial activity. The system was refined to screen mutant libraries of two-peptide lantibiotics.

2.2 RESULTS AND DISCUSSION

2.2.1 *Trans* complementation and bioactivity assay

A fosmid based *trans* complementation system was adapted for lichenicidin biosynthesis in *E. coli* [54]. It consists of a strain carrying the original fosmid lacking both lichenicidin structural genes (pLic5ΔA1ΔA2) that is complemented with a plasmid containing either *licA1* or *licA2* genes (Figure S2.1). This system greatly facilitates the production of lichenicidin mutants by simply using site-directed mutagenesis of each *licA* structural gene, as described in the experimental procedures. A total of 57 lichenicidin variants were generated: 29 for Bli α and 28 for Bli β (Figure 2.1) and the screening of their bioactivity was performed by antagonistic deferred bioassay against *Kocuria rhizophila* ATCC 9341 (formerly *M. luteus*). The bioactivity of lichenicidin results from the synergistic activity of both Bli α and Bli β peptides. But, in the *trans* complementation system used, individual colonies produce each peptide separately. Therefore, we employed a novel bioactivity test, tuned for a better applicability, which consists in supplying the complementary peptide in the agar medium. This is a very simple, inexpensive, user-friendly and timesaving method since it does not require the purification of the complementary peptides.

2.2.2 Substitution of aromatic amino acids

We tested the replacement of lichenicidin's aromatic amino acids by other aromatic residues and by Ala. The following mutants were generated: Bli α :Tyr19Trp, Bli α :Tyr19Phe, Bli α :Tyr19Ala, Bli β :Trp9Tyr, Bli β :Trp9Phe and Bli β :Trp9Ala (Figure 2.1). The supernatant extracts of *E. coli* expressing Bli α :Tyr19Ala retained its activity and also maintained its relative abundance, compared with the wildtype (Figure 2.2A). A decreased antagonistic effect was observed for Bli α :Tyr19Trp, Bli α :Tyr19Phe and Bli β :Trp9Ala extracts. The Bli β :Trp9Ala abundance accompanied this effect, since it decreased (Figure 2.2B). However, Bli α :Tyr19Trp and Bli α :Tyr19Phe peptides were more abundant than the wildtype, suggesting that they are probably less active (Figure 2.2A). The last two extracts (Bli β :Trp9Tyr and Bli β :Trp9Phe) presented a slightly increased bioactivity as well as a higher relative abundance (Figure 2.2B). Previous studies proposed that the activity of lantibiotics such as lacticin 3147 can be inversely related to the size of their aromatic

amino acids: Trp > Tyr > Phe [113,190]. Our results suggest that the substitution of Tyr or Trp by other aromatic amino acids can be advantageous in terms of peptide productivity in both Bli α and Bli β . However, for Bli β , this does not compromise the bioactivity as it does in Bli α (Figure 2.2).

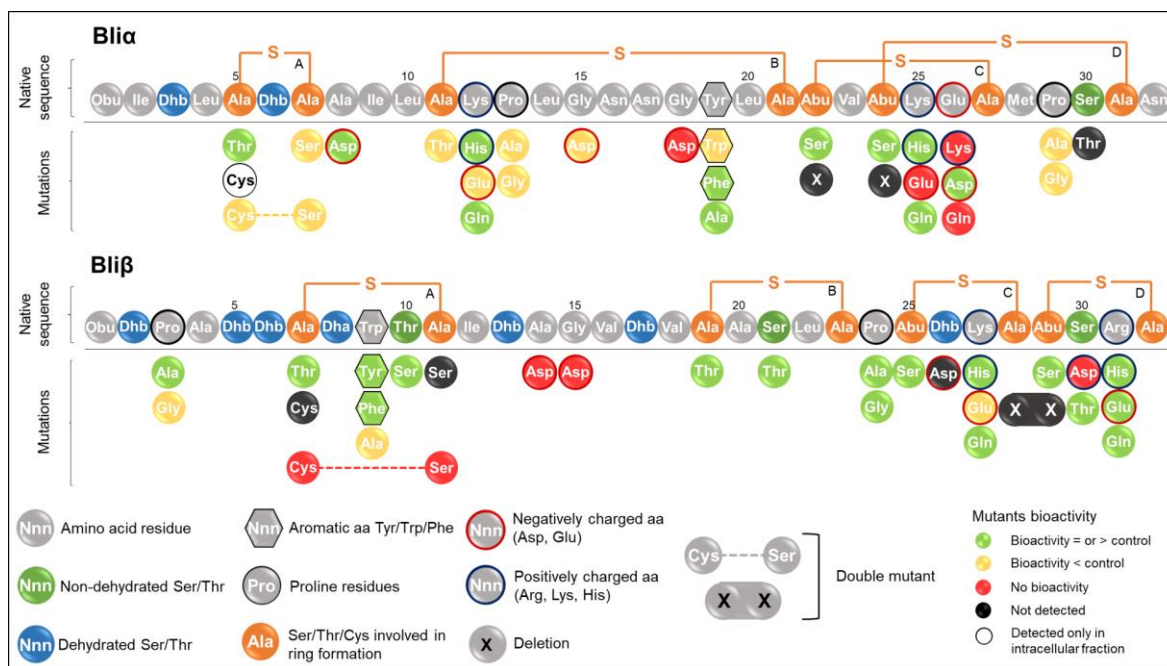


Figure 2.1. Bli α and Bli β amino acid sequences. Summary of the variants produced in this study and respective antagonist effect of crude extracts of the *Escherichia coli* mutant producers against *Kocuria rhizophila*.

Aromatic amino acids are important to promote hydrophobic interactions between antibacterial peptides and the cytoplasmic membrane at the lipid-water interface [195,196]. Previously, it was found that bioactivity of *Lactococcus lactis* expressing Ltn α :Phe6Ala, Ltn α :Trp12Ala and Ltn α :Trp18Ala was abolished and Ltn β :Tyr14Ala was decreased [113]. Additionally, the Ltn α mutant peptides were not detected, suggesting that *L. lactis* does not cope with the absence of these aromatic amino acids. Therefore, in lanthipeptides, aromatic amino acids are not only relevant for antibacterial activity, but also for biosynthesis. Interestingly, in our study, none of the expected deleterious effects of removing the aromatic amino acid was observed in Bli α :Tyr19Ala, once its bioactivity and production levels were both similar to the wild-type. In this case, the presence of an aromatic amino acid is not essential for Bli α 's production nor activity. The same is not true for Bli β :Trp9Ala, since a decreased abundance of this variant was observed. Nevertheless, lichenicidin biosynthetic machinery was able to cope with all the variants.

2.2.3 Substitution of positively charged amino acids

The overall charge of the mature lantibiotics characterized so far is usually neutral or positive, ranging from 0 in Ltn α to +7 in plantaricin W (Plw β). Mersacidin is an exception, since it has a negative net charge of -1 [185]. Both lichenicidin peptides have two positively charged amino acids (Bli α :Lys12, Bli α :Lys25, Bli β :Lys27 and Bli β :Arg31) and only Bli α has a negatively charged residue (Glu26) (Figure S2.2). Lichenicidin N-termini are chargeless due to the 2-oxobutyryl (Obu) modification, which results from spontaneous deamination of the otherwise unstable dehydrated Thr [197].

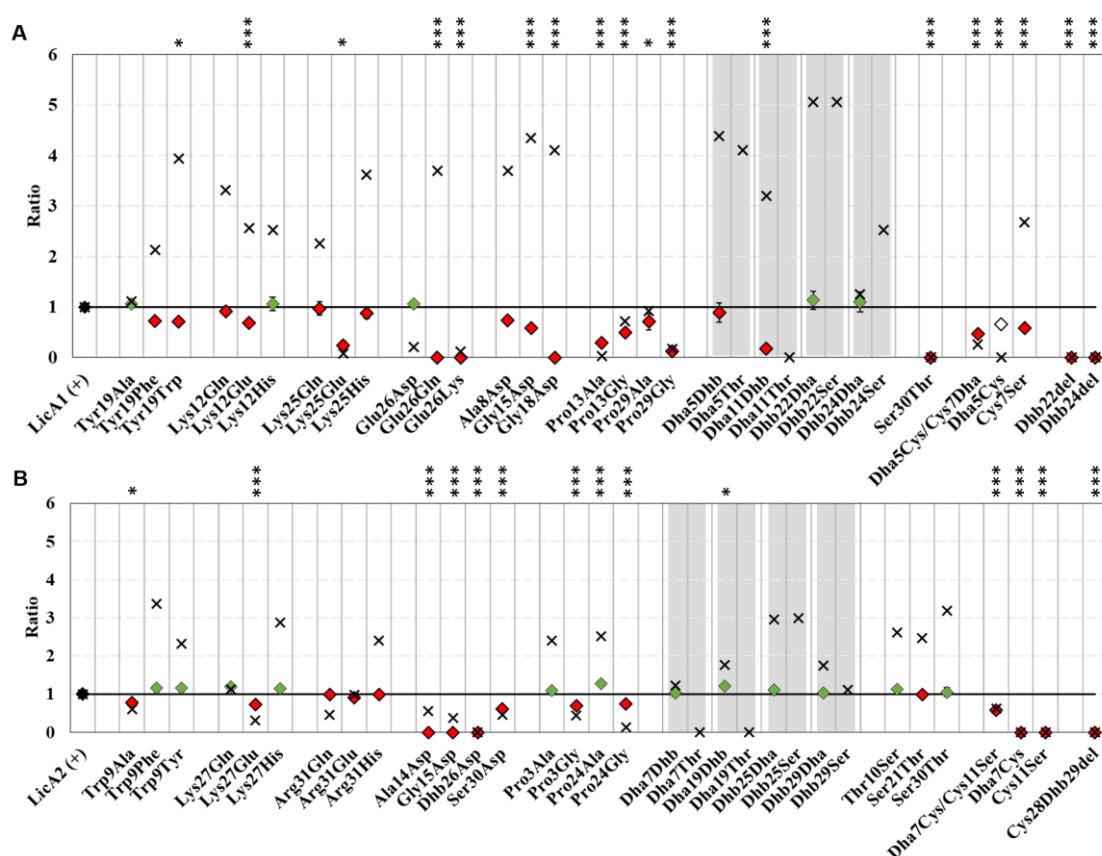


Figure 2.2. Analysis of the *Escherichia coli* extracts against *Kocuria rhizophila*, expressing (a) Bli α and (b) Bli β variants. The ratio between the bioactivity (♦) and relative quantification (x) of mutant and wildtype extracts is presented, considering the wildtype as 1. Mutants with increased bioactivity are represented in green and mutants with decreased bioactivity are represented in red. Grey areas indicate extracts in which the two variants were identified (bioactivity results from the mixture of both). Statistically significant differences of bioactivity ratio are also indicated: * = $p < 0.05$; ** = $p < 0.001$; *** = $p < 0.0001$. In bioactivity ratios, some error bars are not visible as standard deviations are too low to be visualized.

Herein, we replaced lichenicidin charged amino acids with residues of the same charge, with opposite charge or with neutral charge (Figure 2.1). Substitution of Lys by Glu in Bli α and Bli β resulted in a significant decrease in the bioactivity of *E. coli* extracts, especially for the strain expressing Bli α :Lys25Glu (Figure 2.2A). Bli α :Lys25Glu and Bli β :Lys27Glu were detected by MS, but in low abundances. Bli α :Lys12Glu was identified in a relative abundance superior to the wildtype, suggesting that the peptide produced can be less effective (Figure 2.2A).

The replacement of Lys by His (conservation of the positively charged state) or by a neutral residue (Lys to Gln) resulted in a slight decrease (Bli α :Lys12Gln, Bli α :Lys25Gln and Bli α :Lys25His), slight increase (Bli β :Lys27Gln and Bli β :Lys27His) or maintenance of the bioactivity phenotype (Bli α :Lys12His). In the supernatant extracts, all these variants but Bli β :Lys27Gln were identified in higher abundances than Bli β (Figure 2.2B). No major effects on bioactivity were observed for extracts from *E. coli* expressing Bli β peptides with Arg31 substituted by Glu, His or Gln (Figure 2.2B). Based on its relative abundance and bioactivity, it is highly probable that Arg31His mutation resulted in a less active peptide (Figure 2.2B). It is noteworthy that Bli β :Arg31Gln mutant resembles the haloduracin β C-terminus, which possesses a Gln in the exact same position [34].

In other studies, the effects caused by the substitution of positively charged residues in lantibiotics greatly diverged. For instance, in Ltn β , substitution of Arg27 by Asp led to complete abolishment of bioactivity [190]. On the contrary, for Ltn α and lactacin 481 (Lct), mutants of the same nature retained their bioactivity [198]. For mersacidin, negatively charged amino acids in Lys positions were not tolerated, while variants where Lys was replaced by His were produced in higher yields [52]. Finally, and contrary to the previous examples, Arg13Asp substitution in mutacin 1140, created a peptide with improved bioactivity against some target strains [191]. Overall, in our study, the reduction of the Bli α and Bli β charge to +1 or neutral, was not substantially beneficial to the peptide biosynthesis or bioactivity. However, for Bli β , the substitution of Lys27 by His or Gln (maintaining or reducing its charge to +1) appears to increase the abundance of peptide with no notable effect on antibacterial activity.

2.2.4 Substitution of negatively charged amino acids

In lichenicidin, only Bli α has a negatively charged amino acid (Glu26). In lichenicidin and in other lantibiotics (mersacidin, actagardine A, Hala α , Ltn α), this residue is essential for antibacterial activity [49,53,54,185,199]. However, its role in the binding to lipid II is still unclear [200]. In previous studies, Bli α :Glu26Ala variant was produced in higher

abundances by *E. coli*, but no bioactivity was observed [54]. Herein, the expression of another three Bli α :Glu26 variants was attempted (Figure 2.2A): Bli α :Glu26Asp, Bli α :Glu26Lys and Bli α :Glu26Gln. Similarly to Bli α :Glu26Ala, LC-ESI-MS analysis shows that Bli α :Glu26Gln was well produced, but its extract was not active (Figure 2.2). The relative abundances of Bli α :Glu26Lys and Bli α :Glu26Asp were low and similar. Yet, the bioactivity of Bli α :Glu26Asp extracts was similar to the wildtype, whereas Bli α :Glu26Lys was completely inactive (Figure 2.2A). This suggests that replacement of Glu26Asp can probably generate a more active peptide, albeit with decreased production. Some maintenance of bioactivity was also observed when Glu was replaced by Asp in Ltn α and actagardine A [185,189]. Similarly to what was observed in our study, a loss of mersacidin activity is detected when the Glu residue is replaced by a positively charged amino acid [53,54].

2.2.5 Substitution of neutral amino acids

Considering the cationic nature of lantibiotics and their interaction with bacterial cell membranes, the insertion of negatively charged amino acids into naturally neutral positions can strongly affect their bioactivity [38,189,190,201,202]. Herein, selected uncharged amino acids in Bli α and Bli β were replaced by negatively charged amino acids. Strains producing the following mutant peptides were generated: Bli α :Ala8Asp, Bli α :Gly15Asp, Bli α :Gly18Asp, Bli β :Ala14Asp, Bli β :Gly15Asp, Bli β :Thr26Asp and Bli β :Ser30Asp (Figure 2.1). The extracts of these strains were less active or completely inactive, when compared to the wildtype. All the Bli α and Bli β variants were detected, respectively, in higher or in lower abundances than the wildtype, except Bli β :Dhb26Asp. Despite produced in good amounts, Bli α :Gly18Asp is inactive, whereas Bli α :Gly15Asp retained some bioactivity. These substitutions have the same nature (Gly-to-Asp) and both residues are in close vicinity within Bli α 's B-ring. In Bli β extracts, the expected mass of Bli β :Dhb26Asp was not detected, suggesting that biosynthetic machinery was not able to cope with such alteration (Figure 2.2B). These results were similar to the ones obtained for the lantipeptide lactacin 3147, where most of the neutral to negatively charged substitutions resulted in peptides that could not be synthesised by its native produced *L. lactis* [190].

2.2.6 Substitution of proline residues

In the structure of each of the lichenicidin peptides there are two Pro residues. In Bli α , Pro13 and Pro29 are confined inside the B- and D-rings, respectively. Bli β has Pro3 and

Pro24, the last spatially separating B- and C-rings (Figure 2.1). In lantibiotics (specially β -peptides), Pro residues are frequently positioned at the N-termini and/or confined to the interior of a ring (usually A or B), except for Bli β and Ltn β . Pro is a unique amino acid that plays a specific role in the structure and function of peptides and proteins [203]. It restricts the peptide angles, imposing a characteristic secondary structure, such as turn structures, or acting as a disruptor of secondary structures [203,204]. It has been reported that Gly also influences the structural conformation of peptides [205]. Therefore, in lichenicidin, the Pro residues were substituted by Ala or by Gly (Figure 2.1). These substitutions did not have the same outcome in terms of the bioactivity of the producer's extracts. In general, the extracts of Gly substitutions were less active and the mutated peptides less abundantly identified than their Ala counterparts, exception made for Bli α :Pro13. This effect was especially evident for Bli β :Pro3. More specifically, all the extracts of Bli α variants and the extract of Bli β :Pro3Gly and Bli β :Pro24Gly presented a lower bioactivity and a lower peptide abundance in comparison with the wildtype (Figure 2.2A). The remaining Bli β Pro-to-Ala mutants (Bli β :Pro3Ala and Bli β :Pro24Ala) extracts slightly increased their antibacterial activity and the two peptides were also detected in higher abundances than Bli β (Figure 2.2B). In conclusion, the substitution of Pro in Bli α has a strong negative impact on the production that was not compensated by an increase in bioactivity since all the extracts were less active than the wildtype. On the other hand, in Bli β , replacement of single Pro by Ala (but not by Gly) can lead to an increase of its abundance in the extracts. For the α -like peptide mersacidin, it was also found, with respect to the production of peptides, that Pro is one of the least permissive amino acids to mutation [52]. In lacticin 3147, only the β -peptide has Pro residues (Pro3, Pro6 and Pro21). The alteration Pro to Ala of the two first prolines does not influence peptide's bioactivity. However, substitution of the third Pro by Ala resulted in β -peptide that produced a reduced synergy effect with the α -peptide [113].

2.2.7 Intersubstitution of threonines and serines involved in ring formation

In both peptides, all Ser and Thr residues involved in ring formation were replaced by either of their corresponding hydroxyl amino acids (Thr or Ser, respectively). Focusing on the dehydratase activity of LicM enzymes, it was found that the newly inserted residues were modified (Table S2.3). However, most of the extracts presented a mixture of dehydrated and non-dehydrated variants. This was found for all the extracts where the Thr involved in the formation of C- and D-rings of Bli α and Bli β were substituted by Ser and in the Bli α :Dha5Dhb/Dha5Thr extracts (Figure 2.2). Thus, these alterations induced an

incomplete dehydration reaction by the dedicated LicM enzymes. In the dehydrated variants, we investigated the formation of Lan and MeLan thioether rings by MS/MS analysis. The fragmentation pattern shows that the dehydrated Bli α :Dha5Dhb and Bli α :Dhb24Dha peptides were not cyclized (Table S2.3). Nevertheless, the bioactivity of their extracts and the abundance of the peptides was not significantly affected. So far, one cannot predict whether the cyclization was impaired due to the change in the amino acid sequence, or whether those variants underwent retro-Michael processing. This reversibility of ring formation is possible and was demonstrated for HalM2 enzyme in the biosynthesis of Hal β [206]. For Bli α :Dha5Dhb, the MS/MS fragmentation pattern point to this possibility (see peaks marked with “*” on Table S2.3). The extract with the most significantly decreased bioactivity was Bli α :Dha11Dhb, where the corresponding peptide was detected in higher abundance than the wildtype. On the other hand, dehydrated and non-dehydrated Bli α :Dhb22Ser and Bli β :Dhb5Ser were identified in relatively high abundances despite the bioactivity of their extracts being similar to the wildtype (Figure 2.2).

2.2.8 Intersubstitution of non-dehydrated serines and threonines

Bli α has 1 non-dehydrated Ser (Ser30; Figure 2.1) and Bli β has one non-dehydrated Thr (Thr10) and two Ser (Ser21 and Ser30; Figure 2.1). They were substituted by their corresponding hydroxyl amino acids. The Bli α :Ser30Thr extract was inactive and neither the expected peptide (where Thr30 is non-dehydrated) nor the possible dehydrated form were identified, indicating that lichenicidin biosynthetic enzymes are not able to cope with such alteration. The substitutions in Bli β (non-dehydrated Thr10, Ser21 and Ser30) resulted in the production of variants also with non-dehydrated residues (Table S2.3). Thus, LicM2 dehydratase activity was not strongly affected by the reciprocal substitution of Ser and Thr residues, meaning that its dehydration pattern is more likely a consequence of the amino acid position and/or its vicinity, rather than the residue itself. Rink and co-workers (2005) proposed that the presence of hydrophobic residues (particularly non-aromatic) around Ser or Thr should promote dehydration, whereas non dehydration will be favoured when these are surrounded by polar and/or negatively charged amino acids [207]. Our results demonstrate that this dehydration “code” is not universal: the vicinity of Bli β :Thr10 and Bli β :Ser26 comprises hydrophobic residues and even so they escape dehydration by LicM2 (including their intersubstitution variants). On the other hand, in Bli α , Ser30 is preceded by a hydrophobic amino acid and also escapes dehydration. The bioactivity of Bli β :Thr10Ser, Bli β :Ser21Thr and Bli β :Ser30Thr extracts

was equal or superior to the control and the abundances of the mutated peptides were always higher than the wildtype peptide (Figure 2.2).

2.2.9 Inversion and deletion of residues involved in ring formation

Lan and MeLan rings are usually formed between a Cys located at the C-terminus relative to a Dha or a Dhb. Only a few lanthipeptides enclose rings with an “inversed” orientation [207]. One example is Ltn α , whose A-ring is formed between Cys1 and Ser2 [208]. In this study, we generated a double mutant with the order of the ring-forming residues inverted for the A-ring of both lichenicidin peptides: Bli α :Dha5Cys/Cys7Ser and Bli β :Dha7Cys/Cys11Ser (Figure 2.1). Peptides corresponding to these variants were identified in the supernatant extracts of their producing strains, albeit in low abundances (Figure 2.2). As such, it was not surprising that the bioactivity of these extracts was impaired. More specifically, the strains expressing Bli α :Dha5Cys/Cys7Ser and Bli β :Dha7Cys/Cys11Ser mutations produced and exported to the supernatant peptides having a dehydrated Ser7 (Dha7) and with a non-dehydrated Ser11 (Ser11), respectively. Control variants with each of the single mutations (Bli α :Dha5Cys, Bli α :Cys7Ser, Bli β :Dha7Cys and Bli β :Cys11Ser) were also generated. The dehydrated variant of Bli β :Cys11Ser could not be detected in the supernatant extract of its producer (Figure 2.2B). Thus, the Bli α :Dha5Cys/Cys7Dha peptide had the requirements for the A-ring formation, whereas Bli β do not. The MS/MS fragmentation pattern of Bli α :Ser5Cys/Cys7Dha, indicates that the A-ring is correctly formed (Table S2.3). This result suggests that mechanistically, LicM1 enzyme is able to catalyse the formation of “inversed” thioether rings, as LtnM1. Interestingly, the predicted molecular mass of Bli α :Ser5Cys was detected in intracellular extracts and not in the supernatant extracts (as the double mutant or Bli α :Cys7Ser). Its MS/MS profile points out to the possible formation of a disulfide ring between the Cys5 and Cys7 residues, which perhaps can impair the export of the peptide (Table S2.3).

Deletion of some residues involved in the formation of the Bli α and Bli β C- and/or D-rings abolished the production of the expected peptides and their supernatant extracts were totally inactive (Figure 2.2 and Table S2.3). These results show that the post-translational machinery is unable to cope with severe structural changes involving rings closest to the peptides C-termini.

2.3 CONCLUSIONS

Our study shows, at the example of lichenicidin, that the generation of lanthipeptide mutants followed by their heterologous expression in *E. coli* is a fast and valuable tool for the screening and selection of biologically active variants. Herein, several mutants were generated and tested against a target bacterial strain using an economic, effective and user-friendly agar-based bioassay. The method was developed to be applied in the study of peptides that interact synergistically, such as two-peptide antibiotics. As proof of concept, the mutations tested were selected based on a comprehensive literature revision (mainly for class II antibiotics). Functional variants were obtained and the results are consistent with those already reported for other antibiotics, suggesting that the host used does not influence the results obtained. Due to its simplicity, the bioassay can be scaled up and applied in the primary screening of large mutant libraries of two-peptide antibiotics. However, factors such as differences in the solubility of the peptides (that may directly influence their diffusion) or the peptide's production levels can affect the bioactivity observed. Thus, it is important that this bioassay is complemented with MS analysis.

The work performed contributes to a better understanding of the flexibility of antibiotics biosynthetic enzymes, opening new perspectives for future application on peptide's production and improvement. Furthermore, it gives new guidelines for the rational design of this important class of bioactive compounds, with the aim of developing more effective weapons to combat bacterial resistance to antibiotics worldwide.

2.4 MATERIAL AND METHODS

2.4.1 Construction of a *trans* complementation system

To compare the production and bioactivity of each mutant variant, a *trans* complementation system was constructed. The system was based on the fosmid pLic5ΔA1 [54], which has all the genes necessary for lichenicidin biosynthesis, except *licA1* structural gene. *licA2* gene was substituted by an apramycin resistance cassette giving rise to a new fosmid pLic5ΔA1ΔA2:apra. The deletion procedure was performed as described by Gust *et al.* [209] and Caetano *et al.* [54]. The following primers were used for the

gene	deletion:	lanA2_fw:	5'-
TGCAAGGATGGATTCTTTTGAATTTTTTATGATTCCCTAGCTAGCATTCCGGGGATCC			
GTGCACC-3'	and	lanA2_rv:	5'-
ATTCGATAGTTTGCCCGTTCTAGGAGGTGAGAATCATGGCTAGCTGTAGGCTGGAG			

CTGCTTC-3'. The pLic5ΔA1ΔA2:apra fosmid was transformed into *E. coli* BL21 Gold chemically competent cells, originating the BLic5ΔA1ΔA2 strain. To allow the expression of the native peptides, the system was then complemented with pLicA1 or pLicA2 plasmids. Briefly, *licA1* and *licA2* structural genes were cloned independently into pET24a+ vector, as described by Caetano and co-workers [54] and used to transform BLic5ΔA1ΔA2 chemically competent cells. The resulting strains are BLic5ΔA1ΔA2_pLicA1 and BLic5ΔA1ΔA2_pLicA2 that produce only Bli α or Bli β , respectively.

2.4.2 Production of mutants by site directed mutagenesis

Mutations were performed by SDM on both lichenicidin structural genes, using pLicA1 and pLicA2 plasmids as templates. A two-step PCR reaction was used as described in Caetano *et al.* [54]. First, two different reactions were prepared to allow independent amplification from each primer (forward and reverse). Each reaction was performed in a total volume of 25 μ L, containing 1X KAPA HiFi Fidelity Buffer, 1.5 μ L of 10 mM KAPA dNTP Mix (final concentration 0.3mM each), 0.3 μ M of primer, 1.25 μ L DMSO, 1U of KAPA HiFi HotStart DNA polymerase and 50-100 ng of template DNA (pLicA1 and pLicA2 for Bli α and Bli β variants, respectively). The amplification parameters were as follows: one step of denaturation at 95°C for 2 min followed by 5 cycles of denaturation at 98°C for 20 s, primer annealing at the required temperature for 15 s and extension at 72°C for 2 min and an ultimate extension step at 72°C for 5 min. In the second step, forward and reverse reactions for each mutation were mixed and the amplification proceeded as described for 20 additional cycles. Specific primers for each mutation were designed using the web-based PrimerX tool and are listed in Table S2.1. After amplification, the parental methylated pLicA1 or pLicA2 DNA was digested with 10 U of DpnI (Fermentas), for 1h at 37°C. Then, 5 μ L of this product were used to transform 50 μ L of chemically competent DH5 α *E. coli* cells by heat shock. Cells were spread onto LB agar containing kanamycin (50 μ g/mL) as selective marker. After overnight incubation at 37°C, three different colonies were randomly selected, the plasmids were extracted using GeneJet Plasmid extraction kit (Thermo Scientific), according to the manufacturer's instructions, and the presence of the desired mutation was confirmed by sequencing the structural gene using T7term primer. After confirmation of the correct mutation, 2 μ L of the plasmid DNA were used to transform 50 μ L of BLic5ΔA1ΔA2 chemically competent cells that were then spread onto LB agar containing kanamycin (50 μ g/mL) and chloramphenicol (12.5 μ g/mL). The mutated structural genes were sequenced again, to confirm the presence of the desired mutation.

2.4.3 Peptide detection and analytics

For peptide detection, 40 mL of M medium (1% of tryptone, 1% of NaCl, 1% KH_2PO_4 , 0.5% of yeast extract, pH 6.5 [172]) were inoculated with 400 μL of an overnight culture of the desired strain. 1 mL of bacterial culture was centrifuged at 10 000 g for 5 min. The cell-free supernatant was pre-purified by addition of an equal volume of ethyl acetate followed by shaking for 10 min at room temperature and centrifugation for phase separation and removal of undesired compounds. The organic layer was discarded and an equal amount of *n*-butanol was added to the aqueous phase for peptide extraction. After shaking for 10 min at room temperature, phases were separated by centrifugation and the organic layer was dried in vacuum. When necessary, intracellular extracts were prepared using the pellet resulting from the centrifugation of the bacterial culture. Briefly, each pellet was suspended in methanol, sonicated for 10 min, followed by centrifugation to remove the cell debris. Methanol phases were collected and dried in vacuum. All the dried extracts were then suspended in 200 μL 70% ACN and 10 μL of each sample were analysed by ESI-Triple-Quadrupole-MS, 6460 Series, (Agilent Technologies, Germany) coupled to an Agilent 1290 Infinity HPLC system (Agilent Technologies). Separation was performed with a Poroshell 120 EC-C8 (2.1 x 50 mm, 2.1 μm) column with a precolumn Poroshell 120 EC-C8 (2.1 x 5 mm, 2.1 μm), using the following gradient: from 5 % to 20 % of solvent B over 0.5 min, increased to 70 % of B at 4.5 min, followed by 100% B at 6 min with a flow rate: 0.5 mL/min. The solvents used were: H_2O with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B). For peptide detection, a MS2scan of each sample was performed and the predicted masses were identified, mainly $[\text{M}+3\text{H}]^{3+}$ and $[\text{M}+3\text{Na}]^{3+}$ adducts (Table S2.2). The predicted masses for each variant were detected (Table S2.2), unless otherwise stated. For relative quantification of the peptides it was assumed that the behaviour of the mutated and native peptides is similar in MS, as only one amino acid (or, in a few cases, two) was changed. The MS2scan peaks corresponding to the detected masses were integrated and considered proportional to the relative amount of peptide in the extract. Comparisons were only performed with control peptides from the same run and expressed as a ratio (mutant peptide peak area)/(native peptide peak area). Intracellular and supernatant extracts were not compared to each other, as regards the amount, since the extraction procedures are different. For the MS/MS experiments, the identified mass was selected from the previous MS2scan and spectra were obtained using four different collision energies: 20, 30, 40 and 45 eV. Thus, four different spectra were obtained for each peptide containing fragments with different

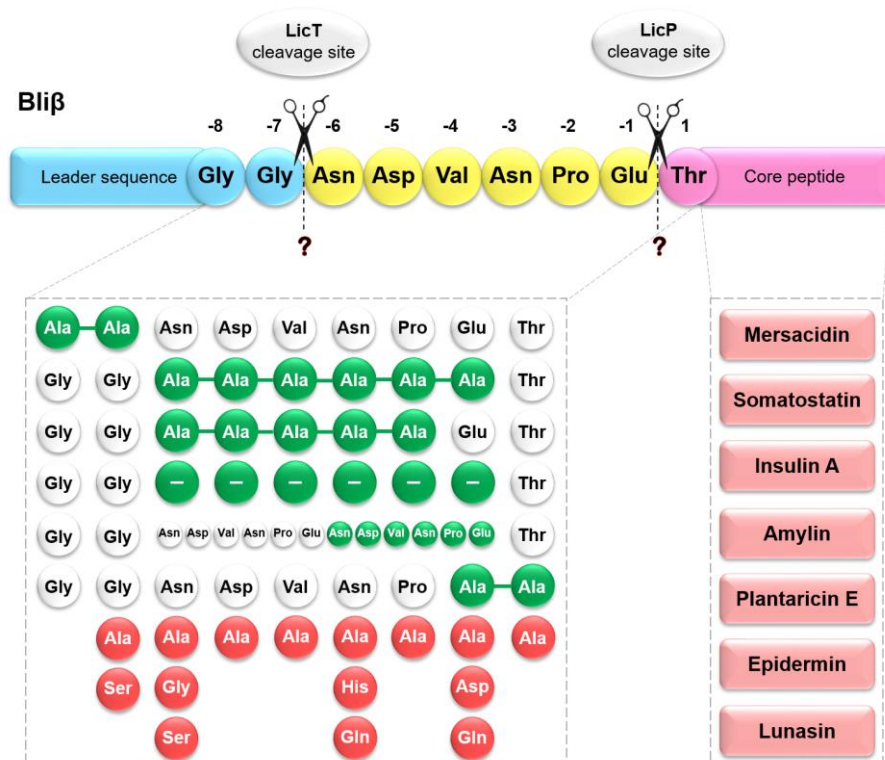
sizes that, when combined, provide detailed information on the fragmentation pattern of each part of the peptide.

2.4.4 Antagonistic deferred bioassay

Bioactivity of the extracts was tested using an antagonistic deferred bioassay against the indicator strain *Kocuria rhizophila* ATCC 9341. Bli α and Bli β have to act synergistically for full lichenicidin bioactivity. Mutant colonies produce only the peptide variant and not its complementary native peptide. Therefore, the latter needs to be incorporated into the agar medium assay plates (Figure S2.3). This procedure requires cell free supernatants of Blic5 Δ A2 (producing only Bli α) or BLic5 Δ A1 (producing only Bli β) strains that were obtained as follows: 200 mL of medium M were inoculated with an overnight culture of the desired strain and allowed to grow for 24h at 37°C, 180 rpm. After centrifugation for 5 min at 10000 \times g, the supernatants were collected and filter sterilized with a 0.2 μ m polyethersulfone membrane. *Kocuria rhizophila* ATCC 9341 (formerly *M. luteus*) was grown in TSB (Merck, Germany) at 37 °C for 24h with aeration (180 rpm). Bioassay plates were prepared by mixing the Blic5 Δ A2 or BLic5 Δ A1 cell free supernatant (depending on the peptide mutants to test) with TSA (Merck, Germany) in a proportion of 1:8 (vol/vol) and the indicator strain to a final OD_{600nm} 0.02. To test the butanol extracts, wells with 0.5 cm of diameter were made on the plates and filled up with 50 μ L of the extract (Figure S2.3). The bioassay plates were grown for 16h at 37°C and the resulting inhibition radii were measured (Figure S2.3 and Figure S2.4). Three biological replicates of each extract were tested in different batches. Since the concentration of peptides in the supernatants can vary slightly between experiments, the same complementary supernatant was used to test each batch of extracts. Data was analysed with GraphPad Prism 5. Statistical analyses were performed with one-way ANOVAs. To avoid correct the values and increase the accuracy of the analysis, Bonferroni's Multiple Comparison post-test was applied. The level of significance defined for all the analyses was $p < 0.05$.

CHAPTER III

UNDERSTANDING AND EXPLORING THE ROLE OF LICHENICIDIN BLI β LEADER PEPTIDE.



3.1 INTRODUCTION

Lantibiotics are lanthipeptides have been the subject of intense research due to their recognized antimicrobial activity against clinically relevant strains and their low toxicity towards mammals [4,23,42,210,211]. During their processing, the installation of the characteristic lanthionine (Lan) and methyllanthionine (MeLan) rings is catalysed by dedicated modifying enzymes, which have different mechanisms for installing these modifications depending on the class of lanthipeptide. In class I and II, after Lan/MeLan have been installed by LanBC or LanM enzymes, respectively, the N-terminal region (the so-called, leader peptide) is removed and the modified and core peptide is transported to the extracellular environment [4,10,35]. In class II lantibiotics, LanTs are the bifunctional enzymes that cleave the leader peptide immediately downstream of the GlyGly motif (GlyGly/GlyAla/GlySer) and promote the transport of the active lantibiotic [4,212]. Class II lantibiotics include the two-peptide lantibiotics, in which two different peptides, LanA1 and LanA2, are produced and modified by two dedicated LanM. The same LanT removes the leader peptides and secretes them, giving rise to the active α - and β -peptides. However, in the β -peptide a short oligopeptide tag remains attached to its N-terminus which is subsequently trimmed by a subtilisin-like extracellular serine protease (LanP) [54,71]. Two-peptide lantibiotics act synergistically to inhibit the growth of several bacteria and include, for example, lichenicidin (Bli α and Bli β) and haloduracin (Hal α and Hal β) [91,101,167]. Generally, the oligopeptide tag of β -peptides is composed of 6 amino acids and is, therefore, referred to as hexapeptide. The LanP enzymes can be encoded in the lantibiotic biosynthetic cluster, as it is the case of lichenicidin, or may be of unknown origin, as with haloduracin [101,168,171]. The *in vitro* activity of the lichenicidin LanP (LicP) was investigated and it was found that it does not require prior cleavage by LicT or the installation of Lan/MeLan modifications to exert its proteolytic activity. In addition, the Glu-1 residue of the lichenicidin hexapeptide (Figure 3.1) was found to be critical for LicP processing [212]. Apart from being recognized by LanP, the exact role of the hexapeptide remains unclear. It has been suggested that it can have a role in the recognition of the post-translational processing machinery, since it is part of the leader peptide. In addition, it may contribute to the immunity of the producing strain, since its presence keeps the β -peptide inactive until it reaches the extracellular environment, where it can interact with the α -peptide. Thus, lantibiotic producers are also natural sources of new proteases with novel specificities and, possibly, several stability-related features [212]. In addition, they are sources of different secretion systems directed by specific leader peptides. As such,

these systems can be exploited to develop novel “*in vivo* biocatalytic factories” entailing microorganisms capable of recombinant DNA technology, such as *Escherichia coli*.

The present work contributes to the knowledge on LanP and LanT activities involved in the biosynthesis of two-peptide lantibiotics, applying a totally *in vivo* approach. First, we aimed to understand the role of the hexapeptide and each of its residues in the effectiveness of proteolysis. We then investigated the ability of LanT and LanP to act as exporters and proteases of other peptides, namely other lantibiotics and peptides with medical application such as insulin A, amylin and lunasin. The study was conducted with the enzymes (LicT and LicP) and Bliβ peptide of lichenicidin (Figure 3.1) using *E. coli* as the heterologous expression host. This was possible because lichenicidin was the first lantibiotic to be produced fully *in vivo* in *E. coli* [54], and we had the system optimized to carry out the various process steps.

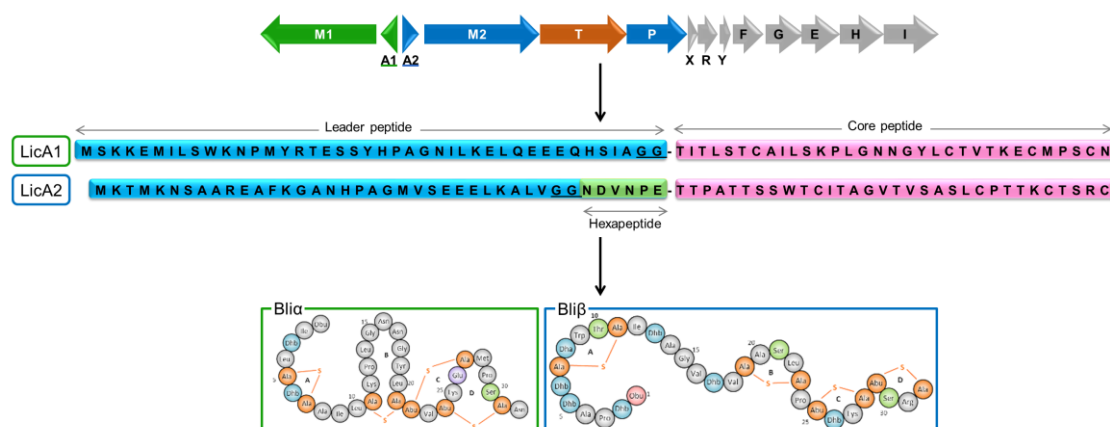


Figure 3.1. Lichenicidin gene cluster containing the genes essential for the production of Bliα (green) and for the production of Bliβ (blue); LicT is responsible for cleaving the precursor peptides C-terminally of the double-Gly motif (underlined) and for the transport of both peptides out of the cell. LicP cleaves the remaining hexapeptide attached to Bliβ in the extracellular space; in grey, are other genes encoding putative regulatory enzymes and self-immunity genes, not essential for lichenicidin production in *E. coli*. Adapted from [54].

3.2 RESULTS AND DISCUSSION

3.2.1 Is the hexapeptide required for Bliβ maturation *in vivo*?

One of the first objectives was to better understand whether the hexapeptide is required for the production of Bliβ. To clarify this, two mutants were constructed: one without the hexapeptide and another with the hexapeptide residues replaced by Ala (NoHexa and HexaAla mutants, respectively; Figure 3.2).

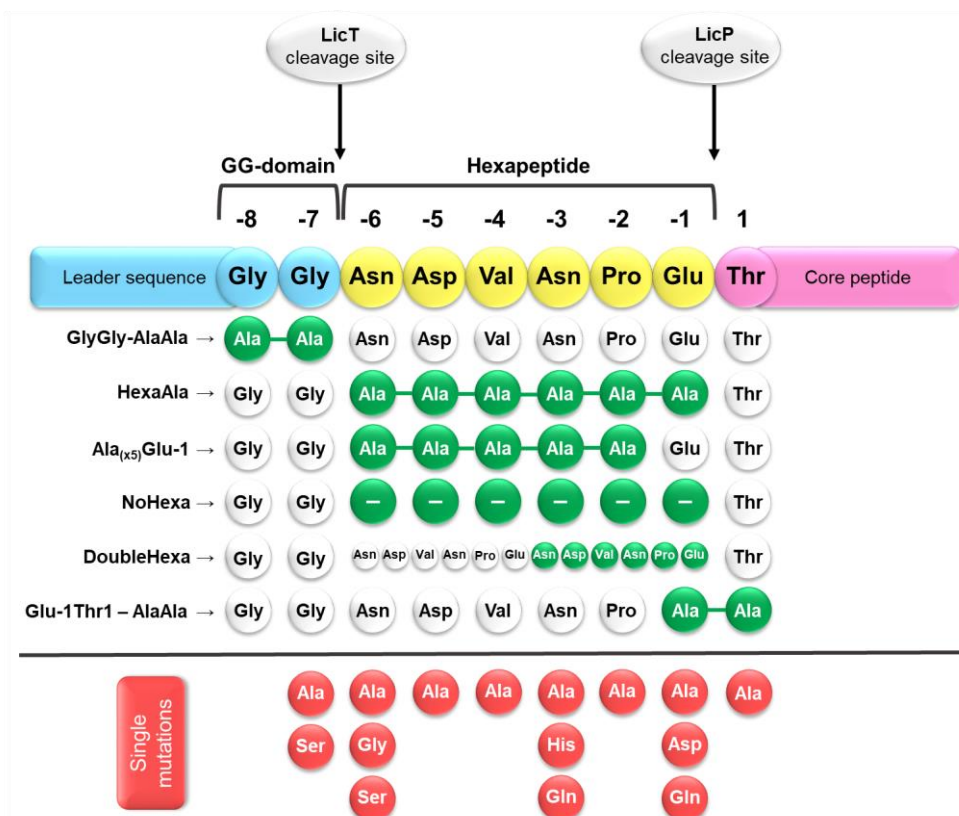


Figure 3.2. Schematic representation of the mutations inserted into Bliβ's hexapeptide. The native sequence is shown on top: hexapeptide in yellow, core peptide in pink and the remaining leader sequence, in blue. The mutations inserted are represented in green when multiple amino acids were replaced or in red for single amino acid mutations. Deletion of an amino acid is represented by a circle containing a “–”. The cleavage sites of LicT and LicP enzymes are also represented.

In the extracts of *E. coli* producing the NoHexa mutant no Bliβ peptide was detected by HPLC-ESI-MS, even when the significantly more sensitive MRM mode was employed. This result suggests that the absence of the hexapeptide impairs the removal of the leader peptide by LicP and/or the dehydration of Ser/Thr residues by LicM2. Surprisingly, the *E. coli* extracts retained 50% of their bioactivity (Figure 3.3), implying that a Bliβ variant should still be produced. However, no lichenicidin-like peptide with reasonable molecular masses were detected by HPLC-ESI-MS. In the extracts of *E. coli* producing the HexaAla variant, only a small amount of Bliβ was detected. However, the bioactivity of these extracts was similar to that of the control. Thus, as for the NoHexa mutant, a not fully processed LicA2 peptide may be produced that still retains bioactivity, although it couldn't be detected.

The impact of hexapeptide size was also evaluated by duplicating its sequence (DoubleHexa mutant; Figure 3.2). This alteration caused a significant decrease in Bliβ production and, consequently, on the bioactivity of the *E. coli* extracts (Figure 3.3).

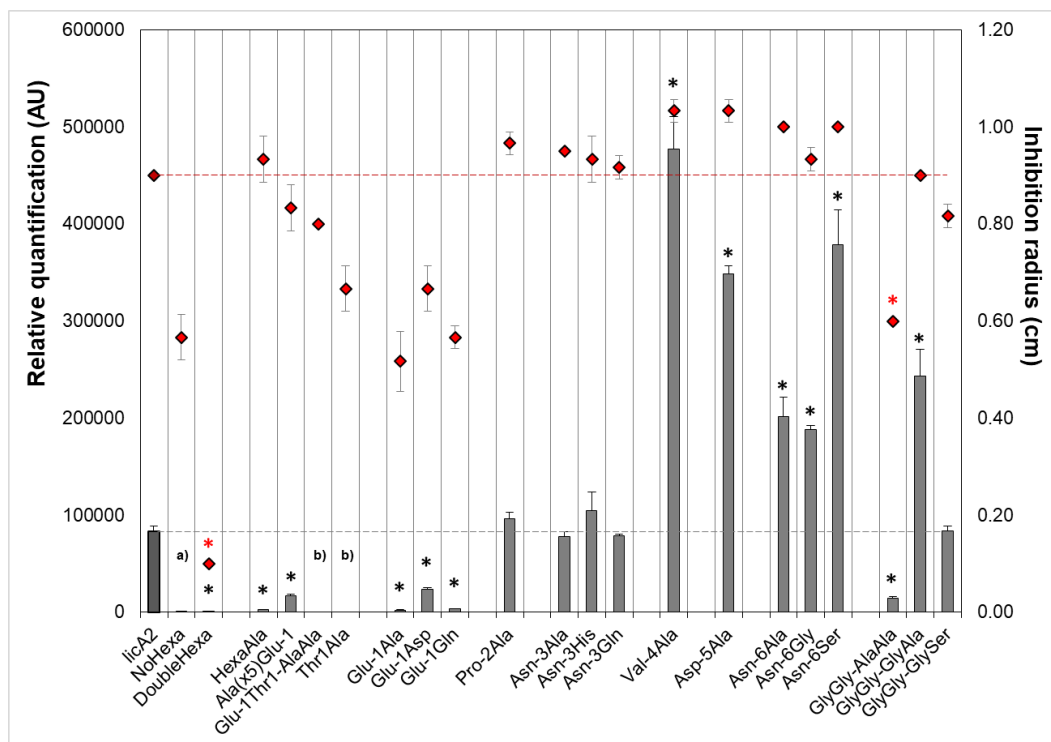


Figure 3.3. Quantification of peptide (grey bars) and bioactivity against *Kocuria rhizophila* (red diamonds) of Bliβ hexapeptide mutants. Native Bliβ activity (LicA2) is represented in dark grey/dark red; * indicates statistically significant differences in bioactivity (red) and quantification (black) compared to the control ($p < 0.05$). a) not detected; b) quantification not performed by the MRM method, since the first amino acid is different.

Although NoHexa and HexaAla extracts retained bioactivity, the mass spectrometric analytics indicated that the posttranslational modifications did not occur as expected, particularly as regards the dehydration patterns. In fact, variants with masses corresponding to incomplete dehydration of the peptides – only 6 or 10 dehydrations out of 11 in the native peptide, within NoHexa and HexaAla extracts respectively – could be identified, that were not present in the control extracts (data not shown). However, the exact nature of such alterations could not be assessed so far, as the variants were still not recovered in the amounts required to perform further studies. Therefore, our results raise questions whether the hexapeptide function goes beyond a recognition site for LicP. This sequence may be determinant for LicM2 post-translational modification of LicA2 core peptide. It may be a tag that helps distinguish LicA1 and LicA2 by their dedicated LicM enzymes, contributing to peptide specificity. However, this hypothesis is still under investigation.

3.2.2 Impact of hexapeptide's amino acid sequence on Bli β production

Other objective was to understand which amino acids in the hexapeptide sequence are essential for the production of Bli β . For this, an Ala-scan was performed (Figure 3.2).

Glu-1 is particularly important because it is the position where LicP protease cleaves the hexapeptide (Figure 3.1). *E. coli* expressing Glu-1Ala was the only mutant in which Bli β production decreased significantly, accompanied by a drop in bioactivity (Figure 3.3). Similar results were obtained when the Glu-1 residue was replaced by the isosteric uncharged Gln, or by Asp, which maintains the negatively charge nature of this position. It should be noted that Gln is found at position -1 of the hexapeptides of various other lantibiotics, such as haloduracin A2 (HalA2; Figure 3.4). The substitution of Glu by Asp was better tolerated than Glu-1Ala or Glu-1Asn mutations. This suggests that the introduction of the negative charge appears to be important for LicP proteolysis (Figure 3.3). Interestingly, Tang and co-workers (2015) also observed that LicP did not tolerate Glu-1 substitutions in an *in vitro* reaction. Furthermore, they were able to establish some interactions between Glu-1 and specific residues from the catalytic site of LicP [212].

The Glu-1Ala substitution raised some doubts about the results obtained for the HexaAla mutant: was the change observed in processing due to the replacement of Glu-1 by Ala, or was it due to the substitution of the entire hexapeptide by Ala? To answer this question, a new mutant was generated where all the residues of the hexapeptide, except Glu-1 were replaced by Ala (Ala_(x5)Glu-1). By comparing the extracts obtained with the expression of HexaAla and Ala_(x5)Glu-1 mutants, it was found that the latter had higher amounts of Bli β , although a slight, but not significant decrease in bioactivity was observed. Thus, the presence of a Glu residue at position -1 ensures a higher production of the mature Bli β [212].

For the remaining individual residue substitutions, it was observed that the replacement of residues in positions -4, -5 and -6 by Ala led to a significant increase in Bli β yields. (Figure 3.3). This result was unexpected for Val-4 and Asp-5, because *in vitro* assays showed that these residues are important for the recognition and interaction of LicP with LicA2. [212]. Tang *et al.* suggest that the residue at position -4 appears to interact with a pocket within LicP structure that is suitable only for small hydrophobic amino acid, as it is the case of both Val and Ala [212]. Therefore, our results suggest that, *in vivo*, LicP tolerates better the changes introduced in proximity of its active site, corresponding to the replacement of a Val-4 by an Ala residue. In addition, the Val-4Ala mutation makes the hexapeptide more similar to the LicP linker sequence (Asn-Thr-**Ala**-Val-Asn-Glu), a

sequence found between the pro- and the catalytic domains of LicP. This linker is cleaved in an autocatalytic process to yield the active LicP catalytic domain [212].

Substitutions at intermediate positions, -2 and -3, had no impact on the Bli β production or bioactivity (Figure 3.3). Particularly the Pro-2Ala substitution did not affect Bli β production. This result is consistent with the report by Tang and co-workers, who have identified this residue as not essential for LicP recognition of the LicA2 hexapeptide [212]. In fact, other lantibiotics have either Pro or Ala at this position (Figure 3.4).

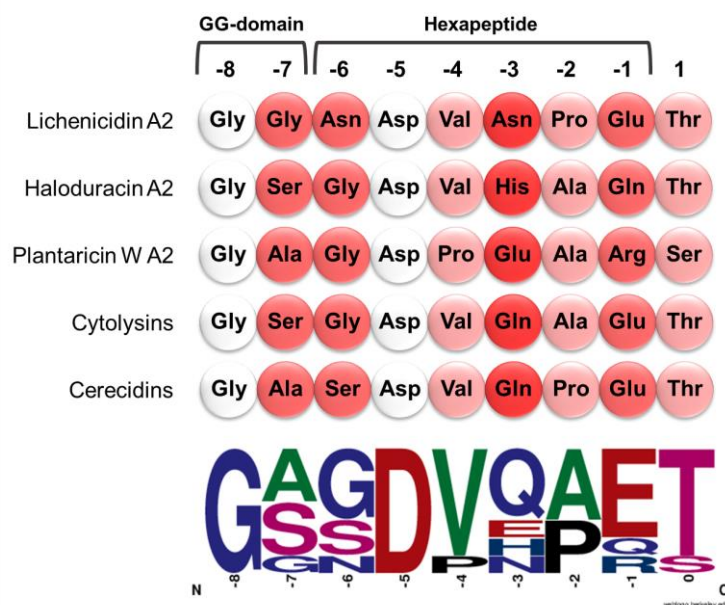


Figure 3.4. Alignment of LicA2 with hexapeptides from other closely related lantibiotics: haloduracin A2, plantaricin W A2, cytolysins and cecidins. White circles represent amino acids conserved among all the sequences, while the decrease in the conservation level is represented by red circles: light red for higher conservation and dark red for lower. At the bottom, representation of the relative frequency of hexapeptide amino acids (generated using WebLogo [276]).

3.2.3 Can LicP recognize hexapeptides other similar lantibiotics?

Residues -3 and -6 are the least conserved amino acids in the hexapeptides of lantibiotics most closely related to lichenicidin (Figure 3.4). For this reason, we replaced Asn-3 by His and Gln, and Asn-6 by Gly and Ser. No significant differences in bioactivity or Bli β abundance were observed for Asn-3His and Asn-3Gln mutants (Figure 3.3). As for the extracts of Asn-6 derivatives, they showed significantly higher production yields of Bli β , compared to wildtype extracts. The Asn-6Ser substitution was particularly beneficial as the abundance of Bli β was approximately 3 times higher than that of the wildtype

(Figure 3.3). The results on other variations of residues -2 and -1 were already discussed in the previous section.

3.2.4 What is the impact of proteolysis sites for LicP and LicT trimming?

The maturation of the precursor peptide LicA2 implies two proteolysis reactions: one at the C-terminal of the leader sequence performed by LicT, after the GlyGly-motif, and the other between the Glu-1 and Thr1 residues, performed by LicP. Thus, we tested and compared the outcome of the following substitutions: Glu-1Thr1→AlaAla, Glu-1Ala and Thr1Ala. The bioactivity of the extracts of Glu-1Thr1→AlaAla mutant decreased slightly and that of the other two mutants also decreased compared to the wildtype (Figure 3.3). Glu-1Thr1→AlaAla and Thr1Ala were not quantified by ESI-MS (MRM mode) as this method is optimized for the native LicA2 sequence and both variants have an alanine in position 1 instead of the native modified threonine (Figure 3.2). However, the molecular masses corresponding to the variant resulting from these two mutants were identified by ESI-MS and their quantification in the extracts is expected to be lower than that of the native peptide producer. Changing the exact LicP cleavage site, seems to be detrimental to Bliβ maturation, although it is not completely impaired.

The GlyGly-motif of LicA2 was mutated to AlaAla, which disrupts the recognition motif of LicT, and to GlyAla or GlySer, sequence motifs found in other class II lanthipeptides (Figure 3.4). Disruption of the GlyGly-motif caused a significant decrease in the abundance of Bliβ, accompanied by a significant decrease in bioactivity (Figure 3.3). Thus, albeit in very low amounts, Bliβ was identified by HPLC-MS (Figure 3.3). This suggests that LicP is able to interact and process the hexapeptide if it is still attached to the leader peptide, as previously demonstrated *in vitro* by Tang and co-workers [212]. Nonetheless, this reaction is favoured if LicT is able to perform the proteolysis reaction. Disruption of lactacin 481 GlyAla-motif (to AlaAla) severely affected the production of the wild-type peptide, that was detected in residual amounts. However that manipulation originated other peptide that is a truncated version of the native one, lacking the five N-terminal residues [213]. The production of Bliβ significantly increased when the GlyGly was changed to GlyAla, and remained the same when changed to GlySer (Figure 3.3). Thus, the proteolysis domain of LicT recognizes all the three GlyGly-motifs described so far for class II lanthipeptides (GlyGly, GlySer and GlyAla). Surprisingly, it seems to have a preference for GlyAla, at least when the biosynthesis occurs in *E. coli*. A GlyGly→GlyAla substitution was previously tested with mutacin II (also a class II lantibiotic), but in this case, the production of the mature peptide was abolished because the dehydrated

premutacin accumulated in the cell membrane [214]. Other lantibiotics, such as plantaricin W A2, cerecidins (Figure 3.4) and lacticin 481, possess GlyAla as recognition motif for LanT cleavage [215]. In particular, lacticin 481 GlyAla was intensively mutated and the only mutation tolerated was GlyAla→GlyGly, thus, resembling the straight double Gly-motif [215].

3.2.5 Can LicT and LicP process other core peptides?

Other aim of the present study was to understand whether LicP and LicT are able to remove the LicA2 leader peptide fused to other core peptides using a fully *in vivo* approach. As a proof of concept, seven peptides were selected in view of their potential medical or industrial application. All of them except mersacidin were previously reported to be expressed in *E. coli*. (Table 3.1 and Figure 3.5).

Table 3.1. List of genes used in this study and their respective masses.

Peptide	Origin	Amino Acid Sequence	Peptide mass (Da)	Applications	Reference
Insulin A	Human	GIVEQCCTSICSLYQ LENYCN	2485	Diabetes therapeutics	[216–218]
Epidermin	<i>Staphylococcus epidermidis</i>	IASKFICTPGCAKTG SFNSYCC	2164	Antimicrobial agent (lantibiotic)	[219,220]
Amylin	Human	KCNTATCATQRLAN FLVHSSNNFGAILSS TNVGSNTY	4005	Inhibits secretion of growth hormone	[221–223]
Plantaricin E	<i>Lactobacillus plantarum</i>	FNRGGYNFGKSVR HVVDAIGSVAGIRGI LKSIR	3644	Antimicrobial agent (bacteriocin, not lantibiotic)	[224–226]
Lunasin	Soy, barley, wheat	SKWQHQQDSCRKQ LQGVNLTPEKHIM QKIQRGDDDDDDDD DD	5125	Chemopreventive peptide	[227]
Somatostatin-14	Human	AGCKNFFWKFTTSC	1639	Prevention of aging-associated diseases, including Alzheimer's disease and type II diabetes	[228,229]
Mersacidin	<i>Bacillus</i> spp.	CTFTLPGGGGVCTL TSECIC	1825	Antimicrobial agent (lantibiotic)	[164,230]

Of these seven peptides, epidermin and mersacidin are class I and class II lanthipeptides, respectively. Herein, these two peptides were expected to be linear and non-dehydrated, because their dedicated LanMs were not included in the expression system. The application of these enzymes constitutes a valuable tool, not only to expand the number of available proteases with known recognition sites, but also to ease the

purification of products of interest, namely by promoting their excretion to the extracellular environment. Moreover, the addition of a tag – in this case, the leader sequence – might promote the solubilisation of otherwise insoluble peptides whose purification would have to be achieved through laborious procedures involving their extraction from inclusion bodies.

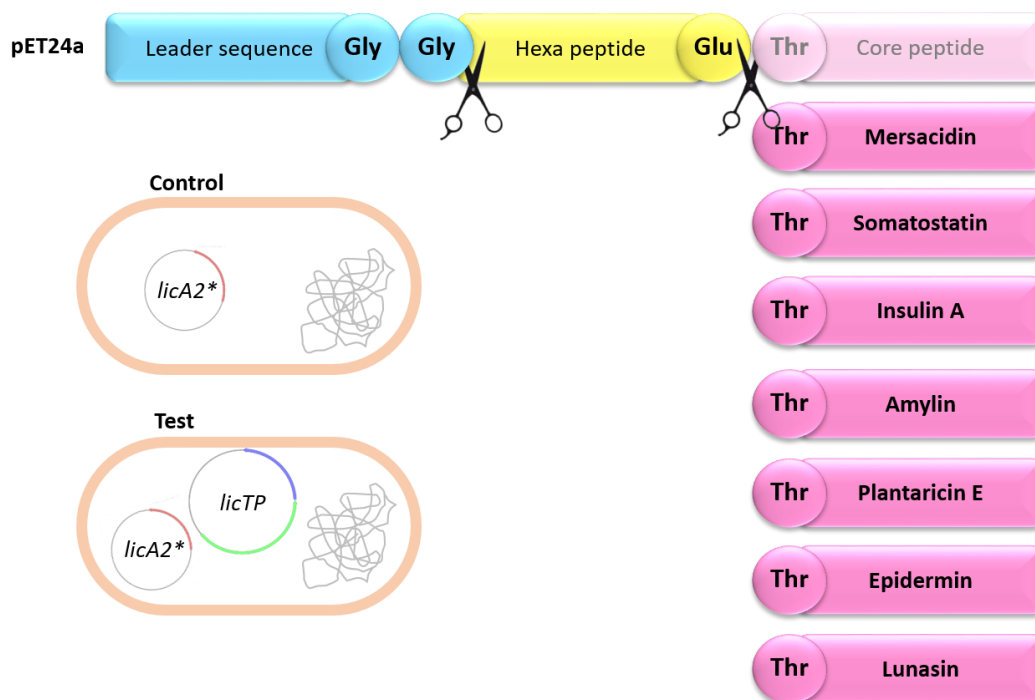


Figure 3.5. Representation of constructions made with chimeric genes. In blue, LicA2 leader sequence with double Gly motif highlighted; in yellow the hexapeptide with a Glu residue in position -1; in pink the core peptide is indicated, which is replaced by various core sequences; position 1 was mutated to Thr if required, to maintain the cleavage site. Conditions tested: control (Ctl), LicT and LicP combined activity (licT.P).

Insulin A, amylin and epidermin were identified by HPLC-ESI-MS in the supernatant, without LicA2 leader peptide attached. These three peptides were not found in the supernatant of the control (expression without LicT and LicP; Table 3.2, Table S3.3 and Table S3.4).

Therefore, LicP and LicT were able to cleave and secrete insulin A, amylin and epidermin. The three peptides, as well as their fused versions (leader peptide and/or hexapeptide), were also identified in the soluble cellular fraction (Table 3.2). This was surprising for amylin, which is a naturally aggregation-prone peptide that is generally recovered from inclusion bodies when expressed in *E. coli* [221–223,231,232]. The fusion of plantaricin E with LicA2 leader peptide was not sufficient to induce peptide secretion, as

it was detected only in soluble cellular fractions (Table 3.2, Table S3.3 and Table S3.4). However, proteolysis by LicP and LicT did take place because plantaricin without leader sequence and plantaricin with the hexapeptide attached were produced (Table 3.2).

Table 3.2. Peptides and their derivatives identified in the soluble cellular fractions and in the supernatant of *E. coli*, in the presence (+LicTP) or absence of LicTP (-LicTP). ND indicates that none of the peptides was detected.

Peptide	+ LicTP		- LicTP (Control)	
	Soluble cellular fraction	Supernatant	Soluble cellular fraction	Supernatant
Insulin A	Leader+Hexa+InsA Hexa+InsA InsA	Leader+Hexa+InsA Hexa+InsA InsA	Leader+Hexa+InsA	Leader+Hexa+InsA
Epidermin	Leader+Hexa+Epi	Leader+Hexa+Epi Hexa+Epi Epi	Leader+Hexa+Epi	Leader+Hexa+Epi
Amylin	Leader+Hexa+Amy Amy	Leader+Hexa+Amy Amy	ND	ND
Plantaricin E	Leader+Hexa+PlnE Hexa+PlnE PlnE	ND	Leader+Hexa+PlnE	ND
Lunasin	Leader+Hexa+Lun	Leader+Hexa+Lun	Leader+Hexa+Lun	Leader+Hexa+Lun
Somatostatin-14	ND	ND	ND	ND
Mersacidin	ND	ND	ND	ND

The fusion of lunasin with LicA2 leader peptide promoted its solubility, but prevented the action of the enzymes LicP and LicT, because in all fractions only lunasin fused to the leader peptide was detected (Table 3.2 and Table S3.3). It should be noted that fused, and thus unprocessed peptides, were detected in the supernatant of the control expression system, where LicTP are missing. This is most probably due to leakage throughout the outer membrane, a phenomenon observed in the optimization of the *E. coli* fermentation conditions [233]. This process is still unclear but it is believed to occur at a basal level and depends largely on culture conditions, including cell growth and stress factors [234,235]. Finally, somatostatin-14 and mersacidin could not be detected (Table 3.2). Taken together, although some optimization is still required, the results presented show that the addition of the LicA2 leader sequence to other peptides may direct the proteolytic activity of LicP and LicT, as well as secretion of peptides into the extracellular environment. However, the success of these reactions depends on the nature of the peptide. *In vitro*, LicP also showed some flexibility with respect to the C-terminal sequences attached to LicA2 recognition sequence [212].

3.3 CONCLUSIONS

LicT and LicP proteases are trimming enzymes involved in lichenicidin biosynthesis. The LicA2 leader peptide provides a recognition and anchoring motif for both enzymes. Particularly, *in vitro*, LicA2 hexapeptide is important for LicP recognition and activity, and in the present study similar results were obtained *in vivo*. The length of the hexapeptide and the presence of a negatively charged residue at position -1 (preferably Glu) are determinant for Bli β maturation. Our results raise the important question of whether the hexapeptide is an additional recognition tag for LicM2, and has direct influence on the dehydration or the cyclization pattern, and this aspect deserves further investigation. Furthermore, we also demonstrated that LicT and LicP processing activity can be improved, *in vivo*, by replacing the residues Val-4 and Asp-5 with Ala or by altering the GlyGly-motif to GlyAla, respectively. The application of LicT and LicP to produce and secrete other peptides in *E. coli* was also investigated. It has been shown that the LicA2 leader peptide can direct the cleavage of industrially relevant peptides such as insulin A, amylin and epidermin. The LicA2 leader peptide can also function as a solubilisation tag, especially for amylin and lunasin. Although the expression systems need further optimization, the results show that it is possible to expand the use of lanthipeptide biosynthetic enzymes to other biotechnological applications, contributing to the increased application of microorganisms as “*in vivo* microbial cell factories”.

3.4 MATERIAL AND METHODS

3.4.1 Construction and analysis of the hexapeptide mutants

Hexapeptide and GlyGly-motif mutations were performed by site directed mutagenesis (SDM) of the *licA2* gene cloned into the pET24a+ vector (Figure 3.2) as previously described by Barbosa *et al.* [236]. A total of 18 hexapeptide and 3 double Gly-motif mutants were obtained. Specific primers for each mutation were designed using the web-based PrimerX tool and are listed in Table S3.1. *E. coli* BL21(DE3)Gold containing a fosmid encoding the entire *lic* gene cluster, except the structural genes, was transformed with the plasmid containing each of the desired mutations. The host was allowed to grow and the supernatant was extracted with 1-butanol, as previously described by Barbosa *et al.* [236]. Extracts were then suspended in 100 μ L 70% ACN. 15 μ L were applied for HPLC-MS analysis, using multiple reaction monitoring (MRM) mode on an ESI-

Triple-Quadrupole-MS 6460 series (Agilent Technologies, Germany) coupled to an Agilent 1290 Infinity HPLC system (Agilent Technologies, Germany). Separation was performed with a Poroshell 120 EC-C8 (2.1 x 50 mm, 2.1 μ m) column with a precolumn Poroshell 120 EC-C8 (2.1 x 5 mm, 2.1 μ m) and with the following gradient: from 5% to 20% of solvent B over 0.5 min, increased to 70% of B over 4.5 min, followed by 100% B over 6 min with a flowrate: 0.5 mL/min. The solvent A was H₂O with 0.1% formic acid and solvent B was acetonitrile with 0.1% formic acid. For quantification of lichenicidin peptides, [M+3H]³⁺ adducts were used as precursor ions. For MRM, mass transitions for Bli β m/z 1007.8 \rightarrow 1302.0 and m/z 1007.8 \rightarrow 264.9 were used and compared with a standard calibration curve obtained with pure Bli α and Bli β (Figure S3.1). When required, a MS2scan of the sample was also performed for identification of masses, other than the native peptides. The bioactivity of all the extracts was assessed by deferred antagonistic bioassay against the Gram-positive indicator strain *Kocuria rhizophila* ATCC 9341, where the complementary peptide required for synergistic bioactivity was incorporated in the bioassay plate as described by Barbosa *et al.* [236]. All the experiments were performed in triplicate and the statistical analyses of bioactivity and quantification were performed with one-way ANOVAs. Whenever the data failed to meet the normality, one-way ANOVAs on Ranks were used. The resulting data was analysed with SigmaPlot 11.0 and the significance defined was p<0.05, for all the analyses.

3.4.2 Construction of the recombinant genes

The recombinant genes containing the leader sequence of the *licA2* gene fused upstream of the peptide of interest were synthesized by General Biosystems (USA). Each recombinant gene was amplified, digested with *NdeI* and *XhoI*, and cloned into pET-24a vector (containing a kanamycin resistance cassette). *licT* and *licP* individual genes were amplified from *B. licheniformis* I89 genomic DNA and *licT.P* amplicon was amplified from pHP β vector, developed by Kuthning *et al.* [175] which contains an optimized promotor and an ATG starter codon in the *licP* gene. The amplicons were digested with *SaI* and *NcoI* and cloned into pCDFDuet-1 vector (streptomycin resistance cassette). All primers used in this study are listed in Table S3.2. *E. coli* BL21(DE3)Gold chemically competent cells were transformed with two plasmids: one containing the recombinant gene and the other encoding the enzymes LicT and LicP. *E. coli* expressing only the recombinant gene was used as control for each condition. The expected peptides and respective masses are listed in Table 3.1. All the recombinant genes were cloned in frame with a C-terminal His₆-

Tag, to facilitate purification, regardless of the proteolytic cleavage the peptide may have undergone.

3.4.3 Recombinant peptide extraction using Ni-NTA affinity beads

Recombinant peptides were extracted with Ni-NTA beads (Qiagen, Germany), due to their high affinity to the C-terminal His₆-Tag. *E. coli* cultures expressing the recombinant genes grew overnight, at 37°C and 180 rpm, in a final volume of 3 mL Luria-Bertani broth (LB) containing the appropriate selective markers. 1 mL of this overnight culture was used to inoculate 100 mL of LB and the cells were allowed to grow for 24h under the same conditions. Cells were harvested by centrifugation at 4°C, 3220 × g for 20 min. The supernatants were kept on ice until extraction with the Ni-beads. The cellular pellets were weighted and dissolved in 10 mL of binding buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, adjusted to pH 8.0 with NaOH solution) containing DNase I (5 µg/mL). The cells were lysed with 10 mg/mL of lysozyme and sonicated for 15 min on ice. Then, lysates were incubated on ice for 15 min and centrifuged at 4°C, 10 000 × g for 1h. The intracellular, soluble fraction was collected and placed on ice. The adequate amount of Ni-NTA resin was washed and equilibrated with the binding buffer, according to the manufacturer's instructions, and added to the supernatants and to the intracellular soluble fractions. The mixture was incubated at 4°C for 4h with gentle shaking, and then centrifuged at 3220 × g at 4°C for 1 min, to collect the Ni-beads. The beads were washed twice with 10 mL of binding buffer, centrifuged as abovementioned and the supernatant was discarded. After washing, the beads were transferred to a new centrifuge tube, using 1 mL of binding buffer. After centrifugation for 1 min at 4°C, 6000 × g, the supernatant was discarded and the peptides were eluted with elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, adjusted to pH 8.0 with NaOH solution). The elution step was repeated 5 times, collecting all the supernatant for further analysis.

3.4.4 Analytics of recombinant peptides

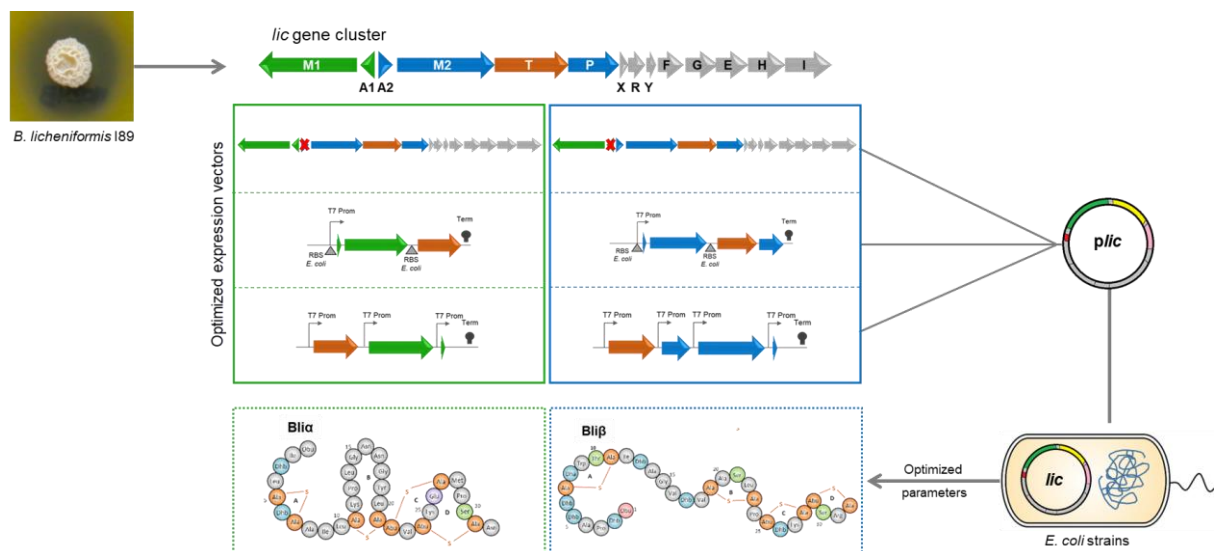
Before analysis, samples were desalted to remove excess of salts using the drop dialysis technique, with cellulose-ester membrane discs (Millipore, Germany). A 10 µL aliquot of the desalted samples was analyzed in a 6530 Accurate Mass Q-ToF (Agilent Technologies, Germany) coupled to an Agilent 1260Infinity HPLC system (Agilent Technologies). Separation was performed with a C5 Supelco Bio Wide Pore column (100 × 2.1 mm, 5 µm) using the following gradient: from 5% to 100% of solvent B over 10 min, followed by 1 min at 100% of B and decreasing again to 5%, with a flow rate of 0.5

mL/min. The following solvents were used: H₂O with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B). The Q-ToF spectrometer is equipped with a Dual AJS ESI Source and was set to the following parameters: gas temperature 300°C, drying gas 8 L/min, sheat gas 350°C, capillary voltage 3500 V, nozzle voltage 0 V. The samples were run in scan mode and the predicted molecular masses were identified (Table S3.3).

CHAPTER IV

IMPROVEMENT OF LICHENICIDIN PRODUCTION IN

ESCHERICHIA COLI



Part of this Chapter's results are also included in Chapter V, that is submitted for publication.

4.1 INTRODUCTION

Many producers of natural products of microbial origin are not amenable to genetic manipulation in the laboratory. In these cases heterologous expression in other reliable hosts is an effective solution. Whenever possible, *E. coli* is the organism of choice due to its easy and low-cost handling, as well as the rapid growth rate and, most importantly, flexibility of genetic manipulation. A vast number of genetic tools and expression methodologies have been developed over the years, aiming to simplify the use of *E. coli* as host [237]. Regarding lanthipeptides, a number of peptides were partially produced in *E. coli*, including prochlorosins, haloduracin, nisin [238], actagardine variants [239] and nukacin ISK-1 [240]. In these cases, the structural genes were co-expressed with the respective modification enzymes, yielding fully dehydrated and cyclized peptides. After purification, the leader sequences of these peptides were proteolytically removed in an *in vitro* reaction. To the best of our knowledge, lichenicidin was the first, and so far the only, lantibiotic to be heterologously produced, totally *in vivo*, in the Gram-negative host *E. coli* [54]. In this case, the entire lichenicidin gene cluster was expressed by *E. coli*, thus directing the production and secretion of the fully matured peptides. The system developed, enabled the manipulation of the *lic* cluster and allowed to investigate the involvement of each of the *lic* genes in the biosynthetic pathway of this lantibiotic (Figure 4.1; [54,89]) and enabled the establishment of a *trans* complementation system to generate lichenicidin variants [54,176,183]. More specifically, manipulation of *lic* gene cluster allowed the production of each lichenicidin peptide separately, which facilitates downstream processing of lichenicidin (such as the purification of each peptide). Herein, several attempts have been made to improve the yields of lichenicidin production in this host, either by co-expressing both peptides or producing each peptide separately in a differently optimized host. Lichenicidin production levels and bioactivity were compared between the original producer, *B. licheniformis* I89, and the different heterologous systems for production of both peptides. Two different strains of *E. coli* were used in combination with different expression vectors and the result was compared by antagonist deferred bioassay and MS analysis.

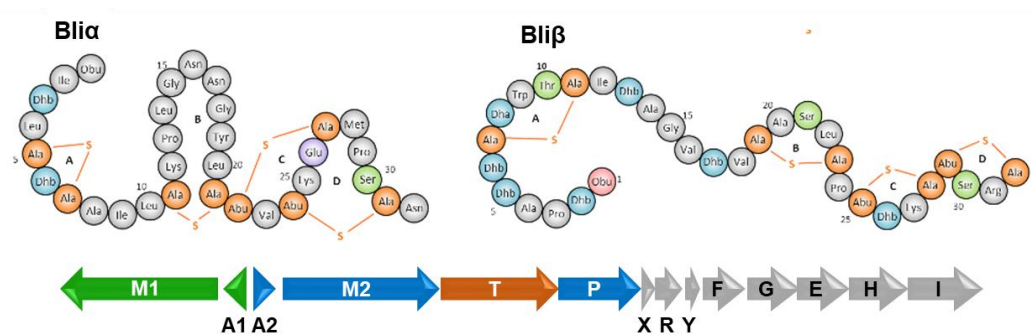


Figure 4.1. Schematic representation of lichenicidin peptides and biosynthetic gene cluster. The essential genes for the biosynthesis of each peptide in *E. coli* are presented: green arrows indicate genes required for Bli α production, blue arrows, for Bli β and the orange arrow for both. Grey arrows represent non-essential genes for lichenicidin production in *E. coli*. Adapted from [54].

4.2 RESULTS AND DISCUSSION

4.2.1 Lichenicidin production yields in *B. licheniformis* I89 and *E. coli*

The heterologous expression presents a viable alternative for the production of peptides with high yields, for genetic manipulations and for potential industrial applications, when the original producer strain lacks this ability. *E. coli* is the most widely used host for the heterologous expression of proteins and other products, given its ease of manipulation and well established purification protocols [238,241]. The fosmid pLic5 includes the entire *lic* gene cluster, where the transcription of all genes is directed by the original *B. licheniformis* I89 promoters (Figure S4.1; [54]). When this vector was used for the first time, the yields of lichenicidin in *E. coli* were not compared with those of the native producer. Additionally, only *E. coli* BL21(DE3)Gold was tested as a host. Herein, an additional strain was tested, *E. coli* BL21(DE3)Star, that has improved stability of the mRNA transcripts [242]. The bioactivity and lichenicidin production levels of *E. coli* and *B. licheniformis* I89 were compared. The antagonistic activity assay revealed that the extracts of *B. licheniformis* I89, the original producer, present significantly higher bioactivity ($p < 0.05$) than those obtained with the two *E. coli* strains (Figure 4.2). However, so far, it is not known if lichenicidin is the only antimicrobial produced by *B. licheniformis*. Anyhow, the analysis of the same extracts by MS showed that it produces significantly higher amounts of Bli α and Bli β than the two *E. coli* strains (Figure 4.2). On the other hand, no significant differences were observed in the relative abundances of Bli α and Bli β between *E. coli* extracts (Figure 4.2).

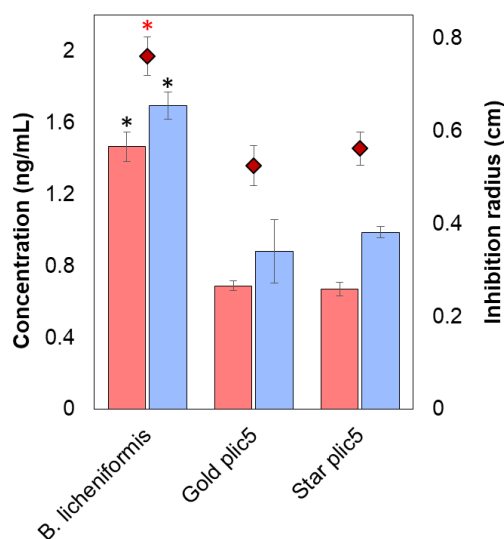


Figure 4.2. Bioactivity (red diamonds) and quantification (bars) of lichenicidin extracts from *B. licheniformis* and *E. coli* producing strains. Bli α quantification is shown in pink and Bli β , in blue. * indicates statistical significance ($p < 0.05$).

4.2.2 Separate production of lichenicidin peptides

The production of Bli α and Bli β peptides separately is useful since it will simplify downstream processing procedures. It can also be advantageous to the host, since the strain can dedicate its metabolism to the production of only one peptide. Thus, we tested different systems to compare the yields of Bli α and Bli β , when produced separately in *E. coli*. The first couple of vectors uses pLic5-based fosmids without *licA1* (pLic5 Δ A1) or *licA2* (pLic5 Δ A2), directing the expression of Bli β and Bli α , respectively [54]. It should be highlighted that, in these fosmids, transcription is directed by *B. licheniformis* promoters. We constructed two plasmids containing only the genes required for the biosynthesis of each peptide under the control of *E. coli* promoters and RBS (Figure S4.1 and Figure S4.2): pA1M1T for Bli α and pA2M2TP for Bli β . At the same time, a similar system was developed by Kuthning and co-workers ([175]; Figure 4.1 and Figure S4.2). This system is composed by plasmids pHP α and pHP β , responsible for the production of Bli α and Bli β , respectively. In the two plasmids, each one of the genes required for the biosynthesis of lichenicidin peptides were cloned under the regulation of a T7 promoter in the pET-24a(+) vector. Using the system of Kuthning and co-workers, no significant differences on bioactivity and MS measurements were observed between the two *E. coli* strains tested (Figure 4.3). Bioactivity was slightly increased for pA1M1T- and pHP β -expressing strains

(Figure 4.3). Peptide quantification corroborated these results since significantly higher amounts of Bli α and Bli β peptides were produced by *E. coli* Gold expressing pA1M1T (~16 ng/mL) and pHP β (~14 ng/mL), respectively (Figure 4.4).

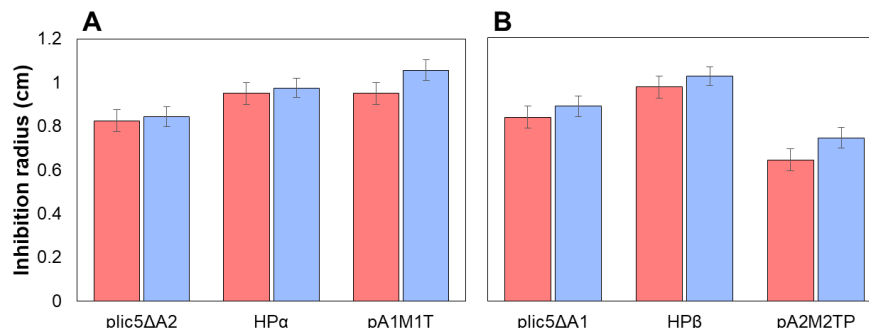


Figure 4.3. Bioactivity of Bli α (A) and Bli β (B) producers extracts against *K. rhizophila*. In pink, *E. coli* BL21Gold strain and in blue, *E. coli* BL21Star.

For comparison purposes, the peptide yields obtained with the expression systems previously applied in different lichenicidin studies were also investigated (pLic Δ A1 Δ A2+plicA1 and pLic Δ A1 Δ A2+plicA2). The production of peptides was lower than that of strains expressing pA1M1T or pHP β . However, it was slightly higher (~6 ng/mL) or equal (~2 ng/mL) to the first expression system used for the production of Bli α and Bli β separately (i.e. pLic5 Δ A2 (~1 ng/mL) and pLic5 Δ A1 (~2 ng/mL), respectively; Figure 4.4). Despite the lower yields, pLic Δ A1 Δ A2 with plicA1 or plicA2 is still a very useful expression system because it allows for the easier manipulation of the structural genes. Each of the genes is encoded by an independent plasmid, which facilitates procedures such as SDM.

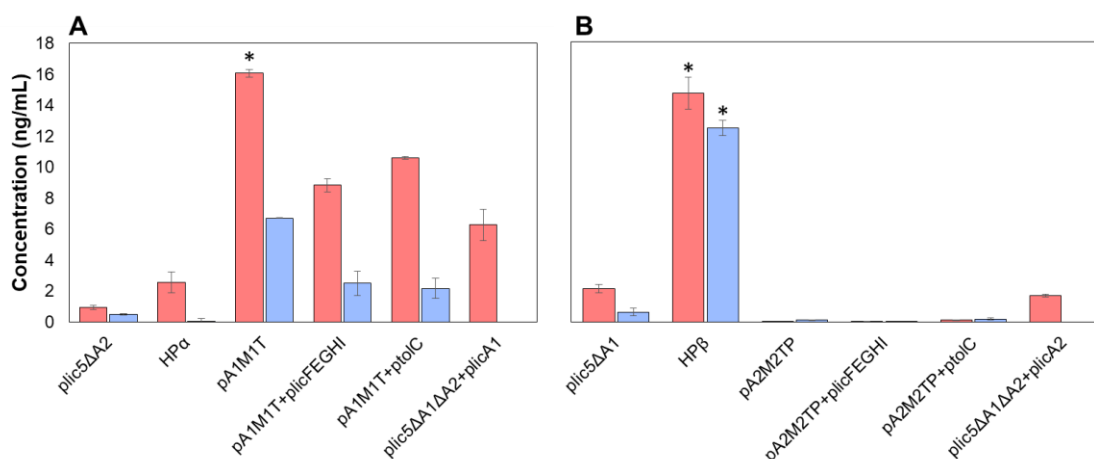


Figure 4.4. Quantification of Bli α (A) and Bli β (B) peptide in extracts of all the expression systems compared during the present work, using MS. *E. coli* BL21Gold extracts quantification is presented in pink and BL21Star's, in blue. *indicates statistical significance ($p < 0.05$).

We selected the constructs with best production levels to purify Bli α (*E. coli* Gold with pA1M1T) and Bli β (*E. coli* Gold with pHP β) peptides needed for Chapter V and Chapter VI.

4.2.3 Contribution of the immunity and *tolC* genes for lichenicidin production

The peptide yield may be influenced by various factors, such as toxicity to the producer organism and transport to the extracellular environment. The immunity genes *licFGEHI* are not required for lichenicidin production in *E. coli* [89]. However, Caetano and co-workers suggested that the absence of these genes could affect negatively the host fitness and, consequently, lichenicidin production levels. We investigated if these genes could benefit lichenicidin production by expressing *licFGEHI* genes in strains with pA1M1T and pA2M2TP vectors (Figure S4.1). The results showed that the presence of the immunity genes does not increase the production yields (Figure 4.4). Also, the bioactivity of the extracts was not affected, as shown for pA2M2TP+*plicFGEHI* (Figure 4.5). *E. coli* is naturally resistant to lichenicidin due to the presence of the outer membrane that constitutes a natural barrier for its entry into the cell, preventing the interaction with the lipid II and the cytoplasmic membrane. In Gram-positive bacteria, the introduction of additional copies of the immunity genes resulted in increased lantibiotic production, as demonstrated for subtilin [133]. However, in that study, the native producer (like other closely-related bacteria) presented some natural sensitivity to subtilin. Therefore, additional copies of the immunity genes contribute to increased resistance of the producing organism to higher amounts of the lantibiotic, thus allowing increased production levels.

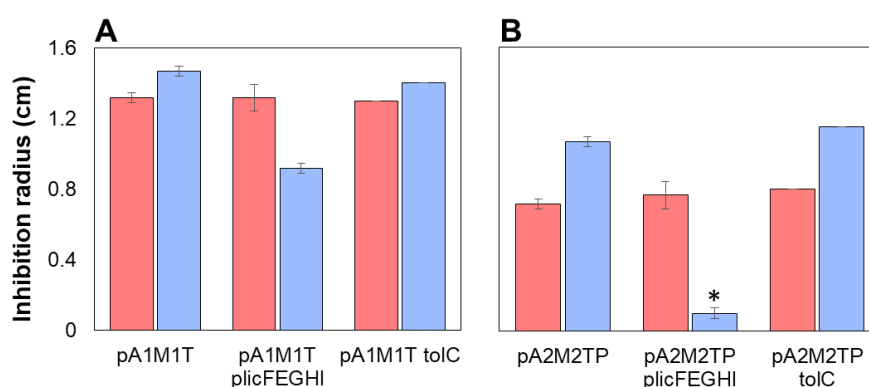


Figure 4.5. Bioactivity of Bli α (A) and Bli β (B) producers extracts containing additional genes, against *K. rhizophila*. In pink, *E. coli* BL21Gold strain and in blue, *E. coli* BL21Star. *indicates statistical significance ($p < 0.05$).

Another strategy for increasing lichenicidin production may be to improve peptides excretion. Our group demonstrated that, in *E. coli*, lichenicidin excretion across the outer membrane is by active transport mediated by TolC [54]. This protein is also essential for microcin J25 peptide excretion in *E. coli* MC4100 [243]. Here, we introduced additional copies of the *tolC* gene into the strains already containing the pA1M1T and pA2M2TP vectors. This approach did not result in increased yields and bioactivity (Figure 4.4 and Figure 4.5), suggesting that the export through the outer membrane is not the limiting step in the amount of peptides present in the extracellular environment. Indeed, TolC-related proteins are ubiquitous in Gram-negative bacteria, and specifically in *E. coli*, and their efflux substrates comprise a wide range of molecules, including enzymes, detergents, toxins and antibacterial drugs [244]. In this study, it was shown that the *E. coli* intrinsic amounts of TolC (encoded on the chromosome) are sufficient to guarantee excretion of lichenicidin, since the addition of extra copies of *tolC* did not result in higher production yields (Figure 4.5).

4.2.4 Induction tests of selected strains

Since the vectors used are inducible, the influence of IPTG concentration on lichenicidin yields was also evaluated in the selected best expression system for each peptide. Surprisingly, the highest concentrations of peptides were detected without IPTG and were even more significant for Bli α , at all the timepoints tested (Figure 4.6). Kuthning and colleagues obtained twice as much Bli α when their system was induced with IPTG as compared to the use of an autoinduction medium. For Bli β , similar amounts were obtained in both cases [175]. However, this study did not analysed the peptide yields in the absence of induction (either IPTG or autoinduction medium). So, at this regard, no comparisons with our study can be performed.

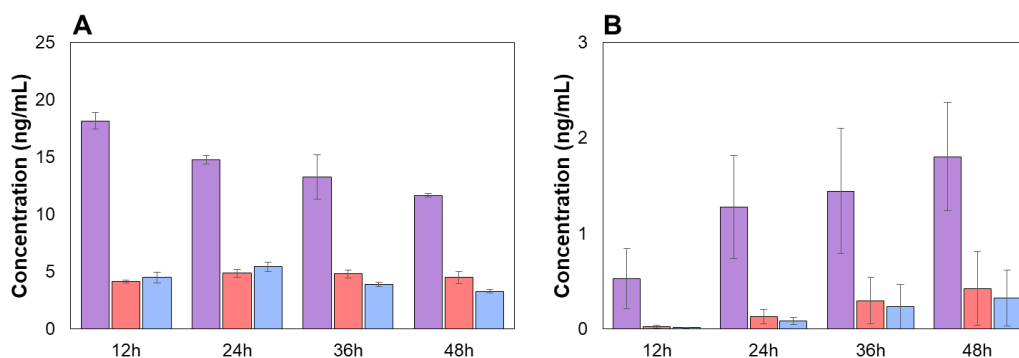


Figure 4.6. Quantification of Bli α (A) and Bli β (B) produced by Gold pA1M1T and HP β , respectively without IPTG induction (purple) and with 0.1mM (pink) or 1 mM (blue) IPTG added to the growing media.

Additionally, and for purification purposes, it appears that 24h is the best time for supernatant collection and extraction of both peptides: for Bli α the amount of peptide appears to decrease slightly over the course of fermentation, probably due to degradation and/or aggregation; for Bli β , its production does not increase significantly after this growth period. Thus, this seems to be the appropriate time for a balanced yield of both peptides and time/energy consumption during the fermentation process. These results are in agreement with those reported by Kuthning and co-workers [175], since the highest yields of both peptides were obtained between 16 h and 24 h, which correspond to the end of the exponential growth phase of the host. Longer expression times may result in nutrient and oxygen depletion in the culture medium, leading to cell death and thus, inhibition of expression. Kuthning and co-authors also suggested a decrease on the peptide amount due to possible degradation [175].

4.2.5 Effect of LicP proteolysis in Bli β production

During this study, it was observed that Bli β amounts are generally more variable between replicates than those of Bli α . In addition, as regards absolute quantification, in some cases Bli β yields are also lower than those of Bli α . Production of mature Bli β requires an additional proteolytic trimming step of the remaining hexapeptide by LicP, after LicT transport and proteolysis [54]. This additional step might represent a higher metabolic effort for the producing host and is time-consuming for the cell, compared to that of the hosts producing Bli α . In addition, the efficiency of LicP proteolysis or even its availability in the extracellular environment can be different in the heterologous host in comparison with the original producer. To understand if LicP trimming constitutes a limiting step on Bli β production, the presence of the Bli β ' was assessed and a relative quantification was performed over time for the immature peptide. As expected, it was observed that the presence of Bli β ' in the extracts decreases over time, whereas the presence of mature Bli β increases (Figure 4.7) and this should be the reason why Bli β concentration increases over time, unlike Bli α (Figure 4.6). The insertion of an additional copy of *licP*, using the same approach described for the immunity genes, did not increase the amounts of processed Bli β (data not shown). Another approach was attempted, in which a defined amount of purified Bli β ' peptide was added to an *E. coli* strain expressing only LicP. Since LicP is an extracellular protease, its presence in the extracellular medium was enough to process Bli β ', producing fully mature Bli β after 24h of incubation (Figure S4.3). This preliminary test still requires optimization as the reaction rate has yet to be clarified.

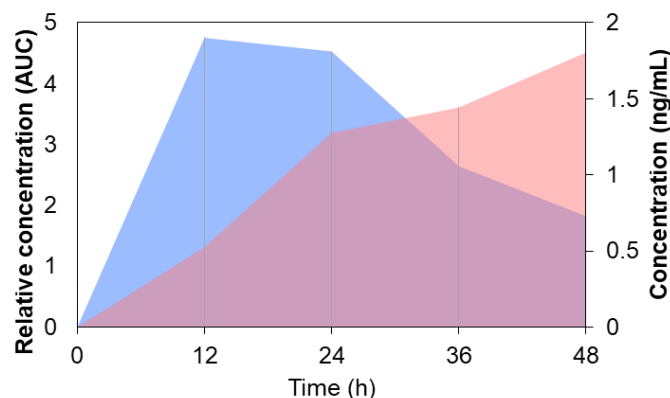


Figure 4.7. Relative concentration of LicA2 with the hexapeptide attached (blue) comparison with concentration of Bliβ (pink), produced by Gold HPβ, over time.

4.3 CONCLUSIONS

When working with antibacterials, bioactivity determination against indicator strains is a rapid method for screening and evaluating production levels prior to MS analysis. For two-peptide lantibiotics, the use of this method can be challenging because the inhibition phenotype requires the interaction of two compounds. Herein, a good correlation was generally obtained between the bioactivity of extracts and the relative quantification of the peptides by MS. Thus, the bioassay method described here is a valuable tool for making decisions about additional MS analysis, especially when studies involve a large number of samples. Furthermore, our results suggest that in *E. coli* hosts, the production of the two-peptide lantibiotic lichenicidin totally *in vivo* is favoured by the inclusion of optimized *E. coli* transcriptional regulator elements, as previously reported. The best systems to produce Bliα and Bliβ are Gold pA1M1T and Gold HPβ, respectively. Separate production of each peptide is advantageous for peptide purification. However, it is worth mentioning, that other optimization strategies can be used, contributing to achieve higher levels of lichenicidin production using *E. coli* as a heterologous host.

4.4 MATERIAL AND METHODS

4.4.1 Culture media, strains and vectors

The media Luria Bertani Agar (LA), tryptic soy agar (TSA) and Luria Bertani (LB), for strains maintenance and growth were purchased from Liofilchem and prepared according to the manufacturer's instructions. For lichenicidin production, M medium was prepared as

described by Mendo *et al.* [172]. 1-Butanol was purchased from VWR Chemicals and acetonitrile from Romil Pure Chemistry. The *E. coli* strains used for lichenicidin expression were BL21(DE3)Gold and BL21(DE3)Star; *E. coli* DH5 α was employed in the intermediate cloning steps when necessary. A list of vectors and respective selective markers can be found in Table S4.1. *Kocuria rhizophila* ATCC 9341 was used as indicator strain in the bioactivity tests.

4.4.2 Construction of plasmids

The construction of pET-based systems involved a two-step cloning of PCR products (Figure S4.2). First, *licA1M1* and *licA2M2* genes were amplified and cloned into pET-24a+ to give the plasmids plicA1M1 and plicA2M2, respectively. Then, the *licT* gene and the *licTP* genes were amplified and inserted in the previously digested plicA1M1 and plicA2M2, to produce the plasmids pA1M1T and pA2M2TP, respectively. All the amplifications were performed in a 50 μ L reaction, containing 25 mM dNTPs, 5 X Herculanase II Buffer, 1 μ L of DMSO, 10 pmol/ μ L each primer, 100-400 ng of *B. licheniformis* I89 total DNA and 5 U of Herculanase II DNA polymerase. The primers, annealing temperature and extension time for each set of genes are listed in Table S4.2. All the digestion reactions were carried out in a final volume of 40 μ L containing 1000 ng of insert or 700 ng of plasmid DNA and the appropriate enzymes and reaction buffer (Thermo Scientific; Table S4.2). Digestions were performed at 37°C for 1h and purified with NZYGelpure kit (NZYtech), according to the manufacturer's instructions. Ligation reactions were performed in a total volume of 20 μ L, containing 50 ng of plasmid DNA, 150 ng of DNA insert, 1 X T4 DNA ligase buffer and 5 U of T4 DNA ligase (Thermo Scientific). The reactions were incubated at 22°C for 1 h and immediately used to transform chemically competent *E. coli* DH5 α cells. Once obtained, the final plasmids pA1M1T and pA2M2TP were purified and used to transform chemically competent *E. coli* BL21(DE3)Gold and BL21(DE3)Star cells. The transformants were selected on LA plates with 50 μ g/mL of kanamycin (Kan). Positive clones were selected by colony-bioassay (see the following section), followed by amplification and sequencing of the inserts using the T7 promoter and T7 terminator universal primers. For additional analysis, the four strains (Gold and Star/pA1M1T and Gold and Star/pA2M2TP) were transformed by heat-shock with the pFGEHI or ptoIC (Table S4.1) plasmids. pFGEHI was constructed as above using the conditions provided in Table S4.2. The *licFGEHI* genes were cloned into pUC19a, a vector derived from pUC19 (Thermo Scientific), which has the *Nco*I and *Nhe*I restriction sites next to the ATG codon of its *lacZ* gene. Strains containing pET-based plasmid and

pFGEHI or ptoIC were selected in LA plates with 50 µg/mL of Kan and 100 µg/mL of ampicillin (Amp).

4.4.3 Colony deferred antagonistic bioassay

For the colony deferred antagonistic bioassay, four different colonies of each system tested were grown overnight in 5 mL of LB medium supplemented with the appropriate antibiotic(s) for 16h at 37°C and 180 rpm. 50 µL of these pre-cultures were used to inoculate 5 mL of medium M (without selective marker) and grown for 24h under the same conditions. 3 µL of each of these cultures were used to inoculate medium M with 1.75% agar plates. After incubation overnight at 37°C, the plates were UV-irradiated for 15 min and overlaid with TSA medium containing the indicator strain *K. rhizophila* (at a final OD_{600nm} of 0.02) and, when required, the supernatant of the strain expressing the complementary peptide, prepared as described in Appendix 2, Figure S2.3A. The cell-free supernatant obtained was mixed with 30 mL of TSA (in a proportion 1:8) containing the indicator strain. This complemented medium was then poured on top of the previous layer, which contains the irradiated producing-colonies. Since the concentration of peptides in the supernatants can vary slightly between experiments, the same culture batch was used for each set of tests, to avoid variations. The inhibition zones were evaluated after overnight incubation at 37°C.

4.4.4 Preparation of peptide extracts

For the preparation of 1-butanol extracts, pre-cultures from three different colonies were prepared for each of the systems. For this, each colony was grown in 3 mL of LB medium containing the appropriate selective marker, for 16h at 37°C and 180 rpm. 500 µL of this culture were used to inoculate 30 mL of medium M in 100 mL erlenmeyers. The cultures were allowed to grow for 24h, at 37°C and 180 rpm. Then, the cultures were centrifuged for 5 min at 10 000 × *g*. 20 mL of each supernatant were mixed with 3 mL of 1-butanol and shaken for 1h at 180 rpm, centrifuged for 5 min at 10 000 × *g* and 1 mL of the upper 1-butanol phase (containing the peptide(s)) was collected. This phase was subdivided in two aliquots (one for bioassay and the other for MS analysis) that were evaporated in the SpeedVac system (UNIVAPO 100 H, UniEquip, Germany) and the pellets were kept at -80°C until further analysis.

4.4.5 IPTG induction

The selected strains were grown, in triplicates, in 3 mL of LB medium containing the appropriate selective marker, for 16h at 37°C, 180 rpm. 1.5 mL of this culture were used to inoculate 150 mL of medium M and allowed to grow for approximately 3-4h, until it reached an OD_{600nm} of 0.5-0.6. Then, 45 ml of the culture were transferred to three smaller erlenmeyer flasks, induced with 0 mM, 0.1 mM and 1 mM of IPTG and incubated under the same conditions. Samples of 1 mL were taken immediately after induction and after 12, 24, 36 and 48h of cultivation. Samples were centrifuged at 10 000 × *g* for 5 min. The cell-free supernatant was pre-purified by addition of 1 mL ethyl acetate followed by shaking for 10 min at room temperature and centrifuged for phase separation and removal of undesired compounds. The organic layer was discarded and an equal amount of 1-butanol was added to the aqueous phase for peptide extraction. After shaking for 10 min at room temperature, phases were separated by centrifugation and the organic layer was vacuum dried. The dried samples were kept at -20°C until further analysis.

4.4.6 Antagonistic deferred bioassay

For bioactivity testing of 1-butanol extracts, TSA plates (0.75% agar) previously inoculated with the indicator strain, *K. rhizophila*, were prepared. 0.5 cm diameter wells were made in the agar and filled with 50 µL of a solution that resulted from the suspension of the 1-butanol extracts (after evaporation) in 200 µL of 70% acetonitrile:water. The supernatant of the strain producing the complementary peptide was incorporated into the agar, in a proportion of 1:8 (vol/vol) for a final volume of 50 mL TSA. The plates were incubated overnight at 37°C and the inhibition zones were measured. This procedure is described in detail in Appendix 2, Figure S2.3.

4.4.7 Mass spectrometry analysis

For MS analysis, the same samples that were previously suspended in 70% ACN were used. 3 µL were injected and analysed by HPLC-MS, using multiple reaction monitoring (MRM) mode on an ESI-Triple-Quadrupole-MS 6460 series (Agilent Technologies, Germany) coupled to an Agilent 1290 Infinity HPLC system (Agilent Technologies, Germany). Separation was performed with a Poroshell 120 EC-C8 (2.1 x 50 mm, 2.1 µm) column with a precolumn Poroshell 120 EC-C8 (2.1 x 5 mm, 2.1 µm), using the following gradient: from 5 % to 20 % of solvent B over 0.5 min, increased to 70 % of B over 4.5 min, followed by 100 % B over 6 min with a flowrate: 0.5 mL/min. The

solvent A was H₂O with 0.1% formic acid (H_{Fo}) and solvent B was acetonitrile with 0.1 % H_{Fo}. For relative quantification of lichenicidin peptides, [M+3H]³⁺ adducts were used as precursor ions. Mass transitions for Bli α m/z 1084.6 \rightarrow 1302.2 and m/z 1084.6 \rightarrow 1153.5, for Bli β m/z 1007.8 \rightarrow 1302.0 and m/z 1007.8 \rightarrow 264.9 and for hexa-LicA2 m/z 1230 \rightarrow 1427.6 and m/z 1230 \rightarrow 835.1 were used. For quantification, a standard calibration curve was obtained by running each peptide at different concentrations (Figure S4.4).

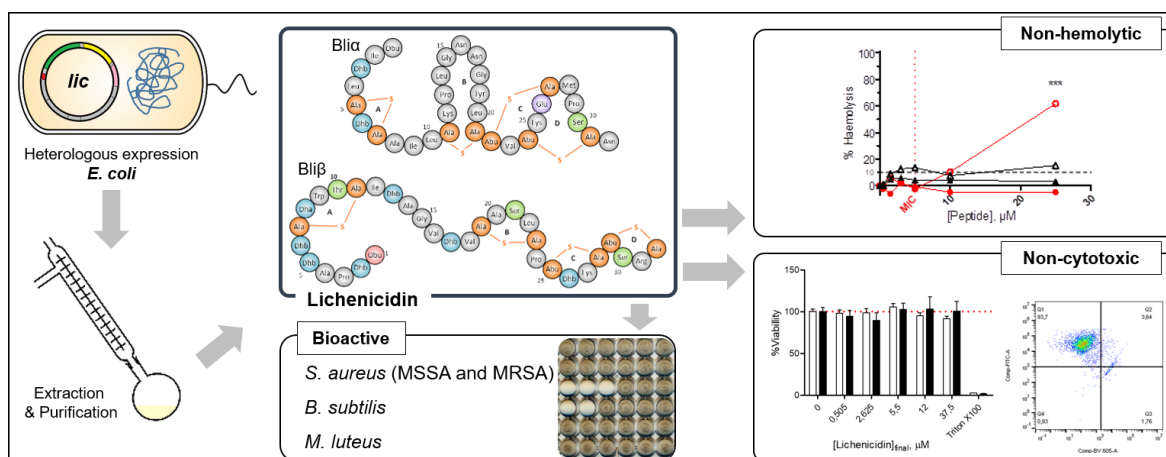
4.4.8 Statistical analysis

The statistical analyses were performed using one-way ANOVAs and one-way ANOVAs on Ranks, whenever the data failed to meet the normality. The data obtained was analysed with SigmaPlot 11.0 and the level of significance defined for all the analyses was $p < 0.05$.

CHAPTER V

LICHENICIDIN, A PROMISING THERAPEUTIC AGENT:

PRODUCTION, BIOACTIVITY AND TOXICITY



This chapter has been submitted to the **Applied Microbiology and Biotechnology Journal** as:

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Lichenicidin, a promising therapeutic agent: production, bioactivity and toxicity

January 2020

5.1 INTRODUCTION

The demand for new antimicrobials is a priority of the academic and medical communities, considering the growing number of multi-drug resistant microorganisms [245]. Lantibiotics – lanthionine-containing antibiotics – appear as a promising therapeutic alternative to the currently used antibiotics, given their diverse applications in human and veterinary medicine and in the food industry. Lantibiotics were first isolated from Gram-positive bacteria [42], they typically target other Gram-positive bacteria [23] and have been reported to be active against clinically relevant strains, namely, methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant Enterococci (VRE) [23]. In addition to these, other bioactivities have also been described as the antifungal and antiviral exhibited by pinensins [17] and labyrinthopeptins [18], respectively. Lantibiotics are ribosomally synthesized and undergo several posttranslational modifications until they become fully bioactive. Generally, the enzymatic machinery required for the biosynthesis of a lantibiotic is encoded in a single gene cluster. Therefore, from a biotechnological perspective, lantibiotics are easier to handle and more suitable for the application of directed evolution techniques, as demonstrated by the studies reporting their bioengineering [25,38,113]. For further information regarding lantibiotic biosynthesis and the installation of PTM, readers are referred to the following reviews: [4,10,35].

Lichenicidin is a lantibiotic naturally produced by various *Bacillus licheniformis* strains, although structure of the peptides may vary depending on the producer concerned [36,54,77,101,171,172]. Lichenicidin inhibits the growth of clinically relevant strains, including methicillin-resistant *Staphylococcus aureus* (MRSA), *Enterococcus faecium*, *Haemophilus influenza* and *Listeria monocytogenes* [194]. Shenkarev and co-workers determined that lichenicidin activity is enhanced at a ratio of 1:1 Bli α :Bli β [101]. Lichenicidin is a class II lanthipeptide [35,88]. More specifically, it is a two-peptide lantibiotic, where two mature peptides, Bli α and Bli β , interact to produce full bioactivity against the target strains [54]. A dual mechanism of action was proposed for the two-peptide lantibiotics, based on studies involving lacticin 3147 and haloduracin: in a first step, the α -peptide binds to the peptidoglycan precursor lipid II, inhibiting the cell wall synthesis; then, the complex Lan α :lipid II recruits the β -peptide that inserts into the membrane, leading to pore formation and leakage of the intracellular content [91,92,94,95]. Lichenicidin was the first lantibiotic to be heterologously produced, totally *in vivo*, in the Gram-negative host *Escherichia coli* [54]. This host, which is more amenable to manipulations than the original producer (*Bacillus licheniformis*), together with the system developed, enabled the investigation of the role of each *lic* gene in the

biosynthetic pathway of lichenicidin [54,89] and allowed the establishment of a *trans* complementation system to generate lichenicidin variants [54,176,180,236]. More recently, Kuthning and co-workers employed a plasmid-based system to produce each lichenicidin peptides separately, in *E. coli* [175]. This strategy facilitates the extraction and purification processes required for large-scale production of lichenicidin.

Lantibiotics have been claimed to have low toxicity to mammals due to their mode of action. However, and so far, this effect was only demonstrated for a limited number of peptides [246–249]. With respect to the two peptide lantibiotics, cytolysin was the only lantibiotic characterized in terms of its toxicity. It was hemolytic and cytotoxic against human cell lines, while exhibiting its antimicrobial activity and inducing virulence in Enterococci strains. To date and to the best of our knowledge, there are no reports of non-cytotoxic two peptide lantibiotics. In the present study, we present the results of a study that characterized the antibacterial activity and cytotoxicity of lichenicidin I89, in order to explore its possible application in human health. For this, we used a system optimized for the *in vivo* production of lichenicidin peptides. The peptides were separately expressed and, after purification, their bioactivity was tested against different bacteria (including methicillin-sensitive and methicillin-resistant *S. aureus*) and their effect on human cells (erythrocytes and fibroblasts) was evaluated. The results showed that lichenicidin I89 has bactericidal activity and that it is a membrane lysing agent. In addition, we showed that lichenicidin is neither hemolytic nor cytotoxic to human fibroblasts. Given these results, lichenicidin is a strong candidate for future clinical applications.

5.2 RESULTS AND DISCUSSION

5.2.1 Best system for lichenicidin expression and purification

In general, chemical synthesis of lantibiotics is difficult because the *in vitro* installation of the characteristic posttranslational modifications, such as (methyl)lanthionine bridges are difficult to achieve. In addition, the use of toxic compounds during the process is harmful to the environment. Semi synthetic or biological synthesis are currently the best alternatives [250]. Another disadvantage is that the both peptides have almost similar retention times and both are required for full bioactivity [101]. Lichenicidin was the first lantibiotic to be produced fully *in vivo* in the heterologous host *E. coli* (Figure 4.1) [54]. Taking advantage of the heterologous expression system developed, the genetic machinery was adapted to produce each peptide separately, facilitating the purification procedure [54]. Herein, we developed and tested three different expression systems

(Figure S4.2) to further increase the yield of lichenicidin peptides [175]. The production was optimized in *E. coli* BL21(DE3)Gold and BL21(DE3)Star strains (see SI P1 and P2). The production was optimized in *E. coli* BL21(DE3)Gold and BL21(DE3)Star strains. The former had already been tested in previous studies and the latter has improved stability of the mRNA transcripts. The concentration of Bli α and Bli β was determined by MS using standard curves with defined concentrations (Figure S4.4). The results showed that higher yields of Bli α and Bli β were obtained with plasmids pA1M1T (this study) and pHP β (Kuthning et al., 2015) [175], respectively, when expressed in *E. coli* BL21(DE3)Gold (Figure 5.1). The maximum titres on the raw extracts were approximately 4 mg for Bli α and 3 mg for Bli β , per litre of bacterial culture. After purification, approximately 1 mg/L of Bli α and 0.4 mg/L of Bli β were obtained, which were then employed on further tests regarding lichenicidin bioactivity.

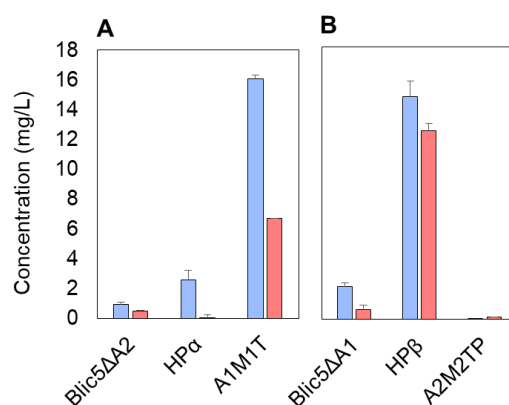


Figure 5.1. Quantification of Bli α (A) and Bli β (B) present in extracts using the respective *E. coli* expression systems constructed. Blue represents the expression in *E. coli* Gold and red the expression in *E. coli* Star. For standard calibration curves, see Figure S4.4. Error bars represent the standard deviation of three independent replicates; when these are not visible, standard deviation is too low to be noticed.

5.2.2 Antibacterial activity of lichenicidin

Lichenicidin bioactivity against several target strains was previously assessed by Shenkarev and co-workers. These authors showed that lichenicidin has activity against all the tested Gram-positive bacteria and that maximum activity is achieved by the synergistic activity of both peptides, with an optimal ratio of 1:1 Bli α :Bli β [101]. Yet, the structure of Bli α produced by *B. licheniformis* VK21 is slightly different from that of the peptide produced by *B. licheniformis* I89 which was used in the present study: In VK21 strain, Bli α A-ring is formed between Cys7 and Dhb3, while in I89 variant, it is between Cys7 and Dha7 [54,101]. Thus, the MIC of lichenicidin produced by *B. licheniformis* I89 (hereafter called lichenicidin I89) was assessed in the present study.

The MIC of lichenicidin I89 was determined for Gram-positive (including MSSA and MRSA) and Gram-negative strains. As reported for the majority of lantibiotics, lichenicidin I89 does not inhibit any of the Gram-negative strains tested [23]. Considering the Gram positive strains, when applied separately, the two peptides were active against *M. luteus* (Table 5.1) and Bli β had a MIC of 32 mg/L against *B. subtilis* (Table 5.1). For these two bacteria, Bli β was found to be more active than Bli α . In addition, the synergy of both peptides was clear: the MIC of combined Bli α and Bli β was lower than the MIC of Bli α and Bli β applied separately. This synergistic effect was observed for *M. luteus*, *B. subtilis* and *S. aureus* (MRSA and MSSA). It was determined that higher amounts of lichenicidin I89 are required to inhibit the MRSA strain, for which the MIC was between 64 and 128 mg/L, whereas for *S. aureus* ATCC 29213 the MIC was between 16 and 32 mg/L. As far as lantibiotics are concerned and with the exception of amycolysin [100] and gallidermin [247], previous studies showed that, in general, similar or higher amounts of the peptides are required to inhibit MRSA strains [4]. When compared to other class II lantibiotics, such as mersacidin or the two-peptide haloduracin, the values for similar strains are within the same range [4]. However, the MIC of lichenicidin I89 was found to be higher than MIC of lichenicidin VK21. Interestingly, the MIC of the lichenicidin I89 peptides tested separately against *B. subtilis* and *M. luteus* is lower than the MIC of lichenicidin VK21 peptides (Table 5.1) [101].

Table 5.1. MIC of lichenicidin, Bli α and Bli β against selected Gram-positive target strains.

	<i>M. luteus</i> DSM1790	<i>B. subtilis</i> DSM10	<i>S. aureus</i> ATCC 29213	<i>S. aureus</i> MRSA
Bliα	8 μ g/mL 1.25 μ M	> 256 μ g/mL > 40 μ M	> 256 μ g/mL > 40 μ M	> 256 μ g/mL > 40 μ M
Bliβ	4 μ g/mL 0.625 μ M	32 μ g/mL 5 μ M	> 256 μ g/mL > 40 μ M	> 256 μ g/mL > 40 μ M
Lichenicidin	2 μ g/mL 0.313 μ M	8 μ g/mL 1.25 μ M	16-32 μ g/mL 2.5-5 μ M	64-128 μ g/mL 10-20 μ M

A time-kill assay was performed with *S. aureus* ATCC 29213 using two concentrations of lichenicidin I89: 32 mg/L (MIC) and 64 mg/L (2x MIC) and 160 mg/L (5x MIC). At MIC, the *S. aureus* strain was completely killed in less than 3 h (Figure 5.2). At 2x MIC, the kill-time decreased to less than 45 min, and at a 5x MIC complete death occurred in less than 5 min (Figure 5.2). Thus, independently of the concentration, lichenicidin I89 induces more than a 3 log₁₀ reduction of bacterial cell counts, which is indicative of a bactericidal activity [251]. Moreover, it causes a rapid cell death (within 30 min), which, according to Bakhtiari

et al., is typical of membrane lysing agents [94]. The same effect in a comparable time was obtained with lacticin 3147, a two-peptide lantibiotic, against *Lactococcus lactis* subsp. *cremoris* HP, however, concentrations much higher than the MIC were tested [92,94]. The effect of lichenicidin VK21 on *S. aureus* growth was evaluated at different concentrations. However, it is not possible to compare it with lichenicidin I89 since no cell counts are available for lichenicidin VK21. Concluding, the mechanism of action of lichenicidin I89 is associated with cell lysis, which is in accordance with the synergistic mode of action proposed for two-peptide lantibiotics [91,92,200]. When nisin, the best studied lantibiotic, is used at a concentration 10 times higher than the MIC, a significant reduction on the viability of an exponentially growing culture of *S. aureus* AH2547 is observed after 5 min of incubation [252]. A comparative study, using nisin A and salivaricin B, also showed a rapid killing curve when other target strains (*Streptococcus pyogenes* ATCC 1234 and *M. luteus* ATCC 10240) were incubated with 10-fold MIC, and 90% cell death was achieved after less than 3 h of incubation [253]. A recent report on the mode of action of the two-peptide lantibiotic lacticin 3147 against *Lactococcus lactis* subsp. *cremoris* HP, indicates that lacticin A1 (LtnA1) has a bacteriostatic effect at concentrations much higher than the concentration at which 50% growth inhibition can be observed [94,254]. However, lacticin A2 (LtnA2) alone is bactericidal. Thus, we suggest that the same may be true for the activity, of each separate lichenicidin peptides, observed against the most sensitive strains *B. subtilis* DSM10 and *M. luteus* DSM1790.

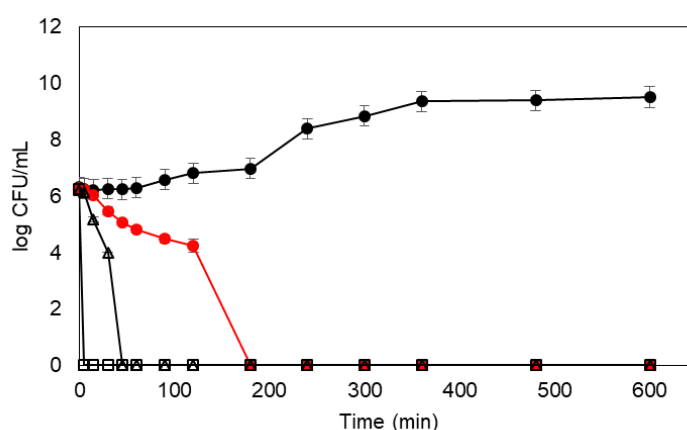


Figure 5.2. Lichenicidin killing time curve. *S. aureus* ATCC 29213 growth curve is shown in black full circles. Three lichenicidin concentrations were tested, corresponding to the MIC value (5 μ M; red full circles), 2-fold MIC (10 μ M; empty triangles) and 5-fold MIC (25 μ M; empty squares). All the conditions were followed for 24 h, although only the first 10h are shown. Error bars represent standard deviation of three independent replicates.

5.2.3 Hemolytic activity of lichenicidin

Investigation of the cytotoxicity of novel bioactive compounds is critical before they could be considered for pharmacological applications. The *in vitro* toxicity of lichenicidin was tested in human cells by evaluating its hemolytic activity and by cell viability assays. For the hemolytic assay, ampicillin was used as a non-hemolytic control, and, as expected, it did not induce significant hemolysis (Figure S5.1). The same was observed for the solvent in which lichenicidin was dissolved (methanol; Figure S5.1). After 1 h of incubation, no hemolysis was observed on lichenicidin-treated cells at any of the concentrations tested (Figure 5.3).

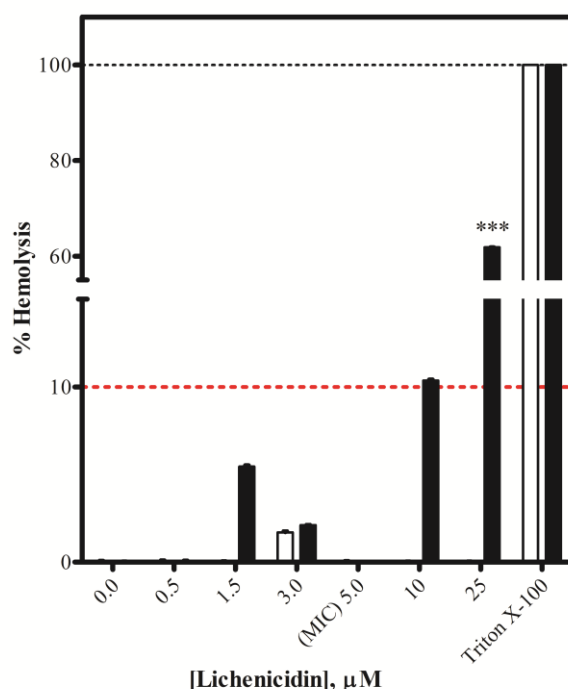


Figure 5.3. Percentage of hemolysis caused by different lichenicidin concentrations after 1 h (white) and 24 h (black) of incubation. Error bars represent standard deviation of three independent replicates; when these are not visible, standard deviation is too low to be noticed. Triton X-100 was used as positive hemolytic control. The MIC value is indicated in the xx axis. The 10% hemolysis threshold, above which a compound is considered hemolytic, is depicted as a red dashed line. *** shows a statistically significant ($p < 0.001$) increase in hemolysis for that specific sample in comparison to all the other conditions tested. Controls with ampicillin, which was used as non-hemolytic agent, and methanol are shown in Figure S5.1.

After 24 h, hemolysis was significantly higher only at 25 μM ($p < 0.001$). This concentration is 20x and 5x higher than the MIC of lichenicidin determined for *B. subtilis* and *S. aureus* ATCC 29213, respectively. Overall, except for 25 μM concentration, the

percentage of hemolysis observed was about or below 10%. According to Pagano and Faggio, if the tested compound induces a percentage of hemolysis up to 9%, it is considered non-toxic [255]. Accordingly, lichenicidin can be considered non-hemolytic at the clinically relevant MIC concentrations for *B. subtilis* and *S. aureus*. Nisin, the first lantibiotic described, does not cause hemolysis of human erythrocytes [256,257]. Likewise, the lantibiotic gallidermin is non-hemolytic at a wide range of concentrations, after 1 h incubation [247]. To date, other lantibiotics have been tested, such as penisin and paenibacillin (against rabbit blood samples) [249,258], thusin [74] and ticins [73] and none of them is hemolytic. Cytolysin constitutes as exception, as this particular two-peptide lantibiotic was shown to be hemolytic against human, horse, cow and rabbit erythrocytes but not sheep and goat [259]. To the best of our knowledge, this is the first report demonstrating the non-hemolytic effect of a two-peptide lantibiotic.

5.2.4 Viability of human fibroblasts treated with lichenicidin

The results of the exposure of human fibroblasts (HFF-1 ATCC SCRC-1041) to different concentrations of lichenicidin for 2 and 24 h, assessed by the XTT assay and by flow cytometry analysis are shown in Figure 5.4. It can be seen that lichenicidin does not affect the metabolic activity of human fibroblasts at any of the concentrations tested (Figure 5.4). Similarly, the exposure time had no influence on cell's viability (Figure 5.4).

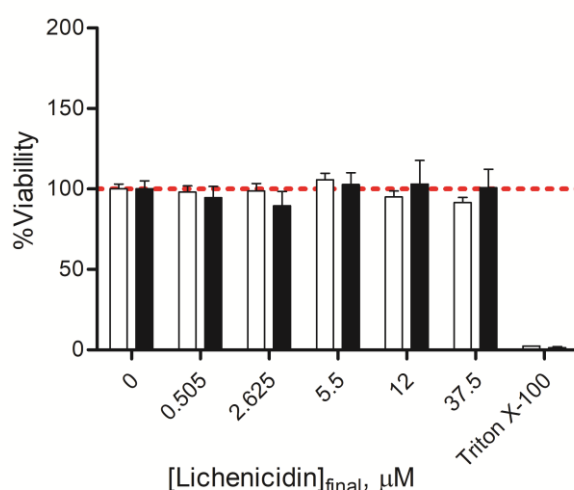


Figure 5.4. Percentage of viability of HFF-1 human fibroblasts after 2 h (white bars) and 24 h (black bars) of incubation with different lichenicidin concentrations, using XTT cell proliferation assay. Control samples containing the same percentage of methanol that was used to dissolve lichenicidin can be found in Figure S5.2. Error bars represent standard deviation of three independent replicates; when these are not visible, standard deviation is too low to be observed.

Surprisingly, fibroblasts treated with methanol, the solvent of lichenicidin, showed higher viability than the untreated control condition and the corresponding lichenicidin exposed samples (Figure S5.2). This effect was more evident after 2 h of incubation (Figure S5.2). Thus, a protective effect driven by the methanol present in the samples should be considered. Recently, Ren *et al.* reported that methanol helps in the adhesion of mouse embryonic fibroblasts that are used as the feeder layer to other cells that require, for example, additional growth factors or receptors [260]. This layer of feeder cells consists of cells that are adhered to the plaque and which produce active substances that support the growth of the remaining cells. The same author also described that treatment with methanol or solutions containing methanol prevents the proliferation of these feeder cells, without affecting their viability and activity [260]. Therefore, although high concentrations of methanol were used to fix mouse fibroblasts, we cannot exclude the possibility that, in the shorter incubation time, the presence of small amounts of methanol may improve the adhesion of HFF-1 cells, without affecting their metabolism.

Viability of cells was also evaluated by flow cytometry, using the live/dead viability assay kit. Calcein AM and ethidium homodimer-1 were used as probes to distinguish between live and dead cells, which cluster in distinct populations when plotted by the emitted fluorescence. HFF-1 fibroblasts were incubated with the previously tested concentrations of lichenicidin and the respective methanol controls and then incubated with the live/dead kit reagents. The percentages of cells in each group are shown in Figure 5.5 and Figure S5.3. The percentage of live cells was significantly higher than the dead cells in all tested conditions. Also, this percentage remained constant regardless of the lichenicidin concentrations used, as well as in the methanol controls (Figure S5.4). In this assay, calcein is metabolized and retained only by the living cells, whereas EthD-1 binds to the nucleic acids of cells whose membranes have been damaged or compromised in some way. Although unlikely, our results suggest the existence of a considerable, but not significant, group of cells that are double-stained. One possible explanation for that may be that some fibroblasts, although viable and active, may have their membranes compromised in such a way that EthD-1 can enter and bind to the nucleic acids. This suggests that these cells may be entering apoptosis. Overall, flow cytometry results are in agreement with those obtained in the XTT assay, showing that lichenicidin is not cytotoxic for HFF-1 human fibroblasts. Since the 1980's, lantibiotics have been considered non-toxic to mammal cells, based on the effects observed for a single peptide (nisin) and before its approval for human consumption as a food preservative.[246]

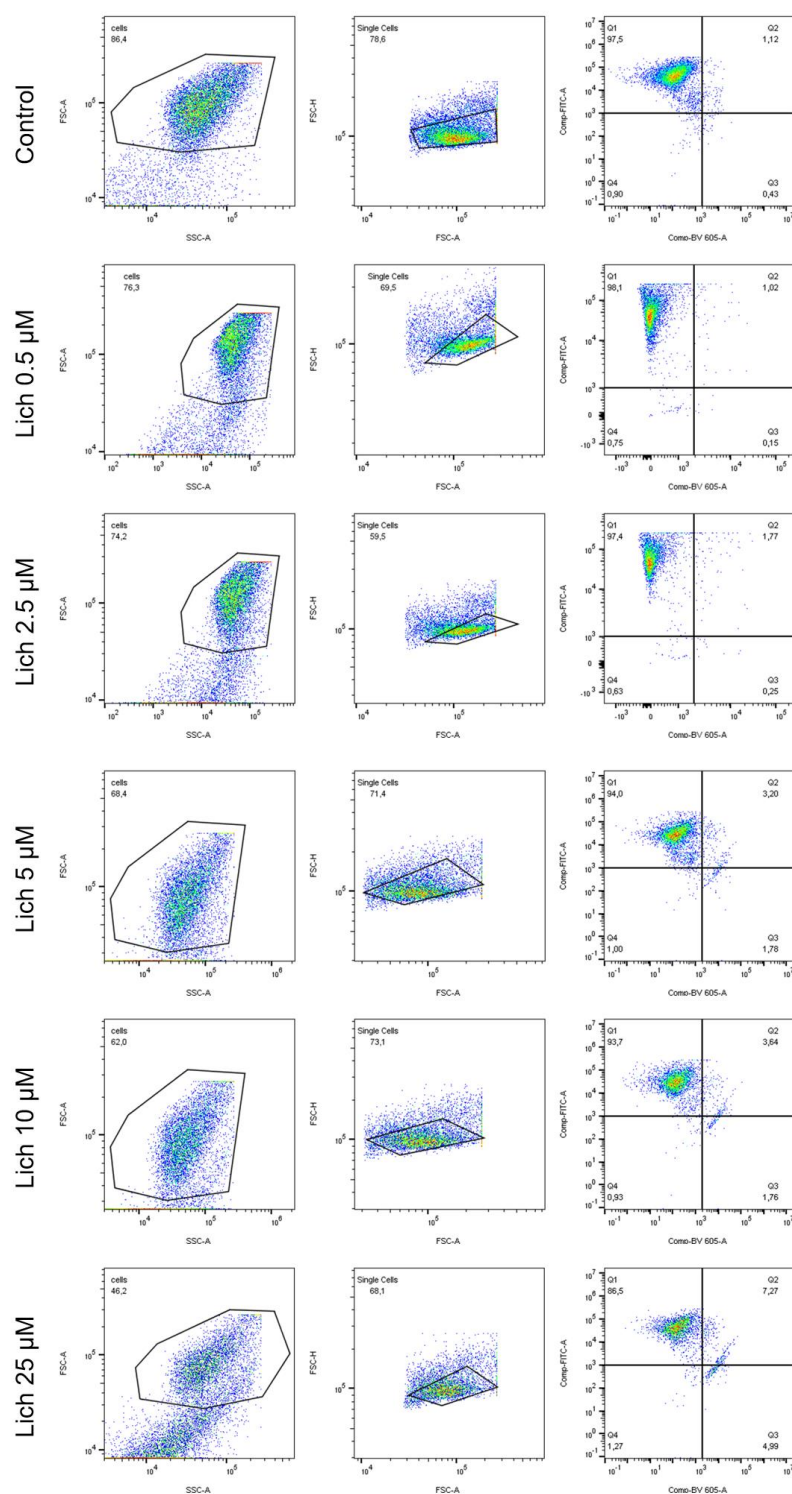


Figure 5.5. Flow cytometry analysis of HFF-1 fibroblasts treated with increasing lichenicidin concentrations and respective gating strategy. Controls, including those with methanol, are shown in Figure S5.3.

Only very recently other lantibiotics were examined at this respect: *i)* paenibacillin, which was tested for a wide range of concentrations in human embryonic kidney and mouse macrophage cell lines [249]; *ii)* gallidermin, which does not affect primary human dermal fibroblasts, except at high concentrations, after 24 h of incubation [247]; and, *iii)* mutacin 1140, which showed low levels of toxicity towards different cell lines [248]. None of these peptides belong to the two-peptide group of lantipeptides, such as lichenicidin. The two-peptide cytolsin was shown to be toxic against several eukaryotic cells, including the human intestinal epithelial cell line HT29 [259]. The results of the present study show that lichenicidin is non-toxic to mammalian cells, constituting the first report of a non-toxic two-peptide lantibiotic, opening new perspectives for its use for therapeutic purposes.

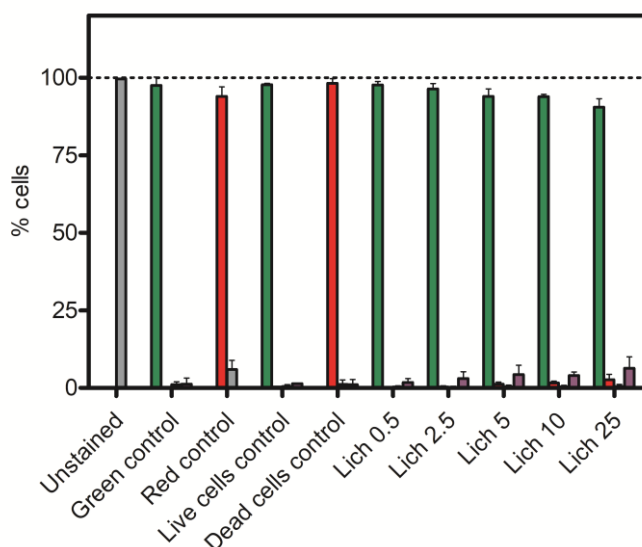


Figure 5.6. Viability assay by flow cytometry of HFF-1 human fibroblasts after 2h of incubation with different lichenicidin concentrations. Unstained cells are represented in grey; live cells, marked with calcein, are shown in green; dead cells, marked with ethidium homodimer-1, are shown in red; double stained cells are represented in purple. Controls with the same percentages of methanol are shown in Figure S5.4. Error bars represent standard deviation of three independent replicates; when these are not visible, standard deviation is too low to be noticed.

5.3 CONCLUSIONS

Herein, we have characterized, for the first time, the antibacterial activity and cytotoxicity of the two-peptide lantibiotic lichenicidin to further investigate its possible future therapeutic applications. We selected the best expression systems to produce lichenicidin. Pure peptides were then employed in the following steps of the work. We

have found that lichenicidin has a strong bactericidal effect against *S. aureus* (MSSA). It induces cell lysis at the highest concentration, in less than 30 min. We also suggest that this lytic effect is enhanced by Bli β , which is congruent with the pore formation activity that has been proposed and described for some β -peptides of two-peptide lantibiotics. Moreover, lichenicidin is not toxic to human erythrocytes and fibroblasts and is, therefore, a promising therapeutic agent to be applied in human medicine.

5.4 MATERIAL AND METHODS

5.4.1 Lichenicidin expression systems

Lichenicidin was the first lantibiotic to be fully expressed *in vivo* in *E. coli*, by our group. A fosmid was constructed containing the whole gene cluster required for lichenicidin synthesis [54] (Figure 4.1). To obtain pure lichenicidin, and to ease the purification process, this system was optimized in order to express each peptide separately. Briefly, we generated two fosmids without each of the *licA* genes (pLic5 Δ A1 and pLic5 Δ A2 to produce only Bli β or Bli α , respectively) and two plasmids expressing the *licA1M1T* (pA1M1T) or *licA2M2TP* (pA2M2TP) genes (Supporting Information, Figure S4.1 and Supplementary Procedures). Additionally, we also evaluated the performance of two other plasmids previously constructed by Kuthning *et al.* [175]: pHP α and pHP β .

Each fosmid/plasmid was transformed into chemically competent *E. coli* BL21(DE3)Gold and BL21(DE3)Star cells. For the analysis of the expression systems, pre-cultures from three different colonies were prepared for each system. Each colony was grown in 3 mL of LB medium (Liofilchem, Italy) containing the appropriate selective marker, for 16h at 37°C and 180 rpm. 300 μ L of this culture were used to inoculate 30 mL of medium M using 100 mL Erlenmeyers. Cultures were allowed to grow for 24h, at 37°C and 180 rpm and then centrifuged for 5 min at 10 000 $\times g$. 20 mL of each supernatant were mixed with 3 mL of 1-butanol and shaken for 1h at 180 rpm. After centrifugation for 5 min at 10 000 $\times g$, 1 mL of the upper 1-butanol phase (containing the peptide) was collected. This phase was divided in two aliquots that were evaporated in a SpeedVac system (UNIVAPO 100 H, UniEquip, Germany). After drying, the extracts were suspended in 100 μ L of 70% acetonitrile (ACN) and analysed by mass spectrometry (MS). For this, 3 μ L were injected in an Agilent 1290 Infinity HPLC system (Agilent Technologies, Germany) coupled to an ESI-Triple-Quadrupole-MS 6460 series (Agilent Technologies, Germany) and analysed using the multiple reaction monitoring (MRM) mode. Separation was performed with a Poroshell 120 EC-C8 (2.1 \times 50 mm, 2.1 μ m) column with a

precolumn Poroshell 120 EC-C8 (2.1 × 5 mm, 2.1 µm), using the following gradient: from 5% to 20% of solvent B over 0.5 min, increased to 70% of B over 4.5 min, followed by 100% B over 6 min, with a flowrate: 0.5 mL/min. Solvent A was H₂O with 0.1% formic acid (HFO) and solvent B was acetonitrile with 0.1% HFO. For the relative quantification of lichenicidin peptides, [M+3H]³⁺ adducts were used as precursor ions. Mass transitions for Bliα m/z 1084.6 → 1302.2 and m/z 1084.6 → 1153.5 and for Bliβ m/z 1007.8 → 1302.0 and m/z 1007.8 → 264.9 were used as qualifier and quantifier in the MRM method.

5.4.2 Purification of lichenicidin peptides

After selection of the best expression system for each peptide, batches of 6 L of *E. coli* culture supernatant were pre-purified via extraction with 1/4 volume of ethyl acetate to remove byproducts and finally extracted with 1/5 volumes of 1-butanol. The organic phase was vacuum dried. Purification of the peptides was performed via preparative HPLC: the dried extracts were dissolved in 70% ACN and loaded onto a RepoSil XR120 C8 (150 × 25 mm, 10 µm) column from Dr. Maisch (Ammerbuch-Entringen, Germany). The sample was eluted with the following gradient: 5-25% ACN in 1 min, increasing to 42% ACN in 13 min, and finally increasing to 100% ACN in 2 min, with a flowrate of 50 mL/min. Peptides were eluted between 32 and 42% ACN. The respective fractions were collected and vacuum dried. As final purification step, samples were diluted in as less as possible 70% ACN and precipitated in ice-cold acetone. The precipitate was spinned down by centrifugation, acetone was removed and the sample was dried by lyophilisation. The purity of the samples was confirmed by ESI-Orbitrap-MS Exactive, 1200 Series HPLC (Thermo Scientific) coupled with an analytical HPLC and Proton NMR (Bruker Avance III 700 MHz) (Figure S5.5).

5.4.3 Determination of Minimal Inhibitory Concentration (MIC)

MIC of lichenicidin was determined for *E. coli* BW25113, *E. coli* DSM1116, *Salmonella typhimurium* TA100 (Gram-negative strains), *Bacillus subtilis* DSM10, *Micrococcus luteus* DSM1790, *Staphylococcus aureus* ATCC 29213 (MSSA) and a MRSA clinical isolate (Gram-positive strains). MIC was determined by the broth microdilution method in triplicate for each strain and according to International Standard ISO 20776-1:2006(E), as recommended by EUCAST (ISO, 2006). Lichenicidin stock solution was prepared in 50% ACN or 30% methanol (only for *S. aureus* strains) and diluted in Mueller-Hinton broth (MHB, Oxoid, UK). Bacterial suspensions were prepared as follows: strains were grown at 37°C in tryptic soy broth (TSB, Oxoid, UK) at 180 rpm until OD_{625nm} > 0.13. The OD_{625nm} of

the cultures was adjusted to approximately 0.1 with TSB (equivalent to the 0.5 McFarland standard). To obtain an inoculum with a final concentration equal to 1×10^6 colony-forming units per millilitre (CFU/mL), the cultures were diluted 1:100 in TSB. Following the recommendations of ISO, this dilution was spread onto agar plates to confirm that the bacterial culture was at a concentration of 5×10^5 CFU/mL. 50 μ L of the culture were added to 50 μ L of each lichenicidin dilution to test. Bacterial growth was visually checked after 24h of incubation at 37°C for all strains. Ciprofloxacin and erythromycin were used as quality controls (QC) and the assay was validated based on QC breakpoint tables established by EUCAST (version 6.0) and CLSI (CLSI, 2013). Solvent controls were also tested.

5.4.4 Time-kill assay

Isolated colonies of *S. aureus* ATCC 29213 were used to inoculate TSB and the culture was allowed to grow overnight at 37°C. 100 μ L of this culture were used to inoculate 5 mL of TSB and the new suspension was incubated at 37°C, 180 rpm, for approximately 1.5h, until it reached a concentration of 3×10^8 CFU/mL. Time-kill assays were performed in triplicate to determine the time of action of lichenicidin. Three concentrations were tested over time: MIC, 2-fold MIC and 5-fold MIC. Lichenicidin was incubated with 5×10^5 CFU/mL of *S. aureus* and the reduction of the colony forming units (CFU) was monitored as follows: over 24h, at different time intervals, aliquots were taken from the growing cultures, diluted and plated. Colonies were allowed to grow for 16 to 20h, at 37°C, and CFU were counted.

5.4.5 Hemolysis assay

Venous blood samples from healthy patients were collected in K₃EDTA tubes. The blood was centrifuged at $1000 \times g$ for 15 min, the supernatant was discarded and the remaining cells were washed three times with HEPES buffer pH 7.4. After the last centrifugation, the supernatant was discarded and the erythrocyte pellet was diluted to a final concentration of 1% (v/v), according to the provided hematocrit. This erythrocyte suspension was divided into aliquots, mixed with different concentrations of lichenicidin (ranging from 0.5 to 25 μ M) and then incubated at 37°C for 1 and 24h. 10% Triton X-100 was used as hemolytic agent for the positive control. Ampicillin, at different concentrations, was used as non-hemolytic control. After the incubation periods, the samples were centrifuged at $3000 \times g$ for 5 min. The hemolytic activity was calculated taking into account that the increase of Abs_{540nm} is proportional to the hemolysed

erythrocytes, due to the release of intracellular hemoglobin. Hemolysis was calculated as follows:

$$\% \text{ Hemolysis} = \frac{\text{Abs sample}}{\text{Abs positive control}} \times 100$$

The positive control was defined as 100% hemolysis. This procedure was performed in triplicate.

5.4.6 Cell viability assays

HFF-1 human fibroblasts cell line (ATCC SCRC-1041) was used to test lichenicidin cytotoxic effect. The cells were propagated in Dulbecco's modified Eagle medium (DMEM), previously warmed to 37°C, which was supplemented with 15% foetal bovine serum (FBS) and 1% penicillin/streptomycin (Pen/Strep). Cultures were allowed to grow at 37°C with 5% CO₂ until they reach ~80% confluence, with medium renewal every 2 to 3 days. For the cell viability assays, cells were detached from the flasks with a trypsin-EDTA solution (0.25% (w/v) trypsin/0.53 mM EDTA) and counted to adjust cell concentration to 1 × 10⁶ cells/mL.

For XTT (tetrazolium salt) assay, fibroblasts were seeded in 96-wells plates (TPP plate, treated, flat) and allowed to attach overnight at 37°C in 5% CO₂, until they reach around 95% confluence. Medium was replaced by fresh supplemented DMEM before testing the different lichenicidin concentrations (from 0.5 to 25 µM). Cells were then incubated with lichenicidin for 2 and 24h, at 37°C, in 5% CO₂. After incubation, 50 µL of Cell Proliferation kit II working solution (Roche, Switzerland), composed by XTT and PMS (N-methyl dibenzopyrazine methyl sulfate; intermediate electron carrier), at a final concentration of 0.128 mg/mL and 3.2 µM, respectively, were added to the cultures and incubated for 4h, as recommended by the manufacturer. Absorbance was measured at 454 and 650 nm.

Viability was calculated as follows:

$$\% \text{ viable cells} = \frac{\text{Abs sample 454nm} - \text{Abs sample 650nm}}{\text{Abs control 454nm} - \text{Abs control 650nm}} \times 100$$

For flow cytometry analysis, cells were incubated on ice for 2h, with the same range of lichenicidin concentrations previously tested. After that, calcein AM and ethidium homodimer-1 (EthD-1) were added and incubated for 30 min, according to the manufacturer's instructions (LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells, Molecular Probes, Invitrogen, UK). The non-fluorescent calcein AM is converted by the ubiquitous intracellular esterase activity in the polyanionic dye calcein (ex/em 494/517

nm), which has a high quantum yield and is well retained by living cells. EthD-1 enters cells with damaged membranes, producing a bright red fluorescence when bound to nucleic acids (ex/em 528/617 nm). Experiments were done in a BD LSRFortessa Flow Cytometer (BD Biosciences, San Jose, CA, USA), using a blue laser (488 nm) to excite the cells. Green and red fluorescence emissions were detected with 530/30 and 610/20 bandpass filters, respectively. Fluorescence emission was acquired in biexponential scale and data were collected for 10 000 cells using BD FACSDiva v.6.2 (BD Biosciences). All flow cytometer results were analysed using FlowJo Software v. 10 (Tree Star Inc., Ashland, OR, USA). Cells were gated to exclude cell debris (forward scatter (FSC-A) vs. side scatter (SSC-A)) and doublets (FSC-A vs. FSC-H). The percentage of cells in each group was determined after plotting the events regarding their fluorescence. The full gating strategy for each experiment is described in the results section and in the supplementary information. Single colour stained live and dead cells (heated at 60°C for 15 min) were used as standards for compensation and live/dead groups delimitation: green fluorescent live cells and red fluorescent dead cells.

5.4.7 Statistical analysis

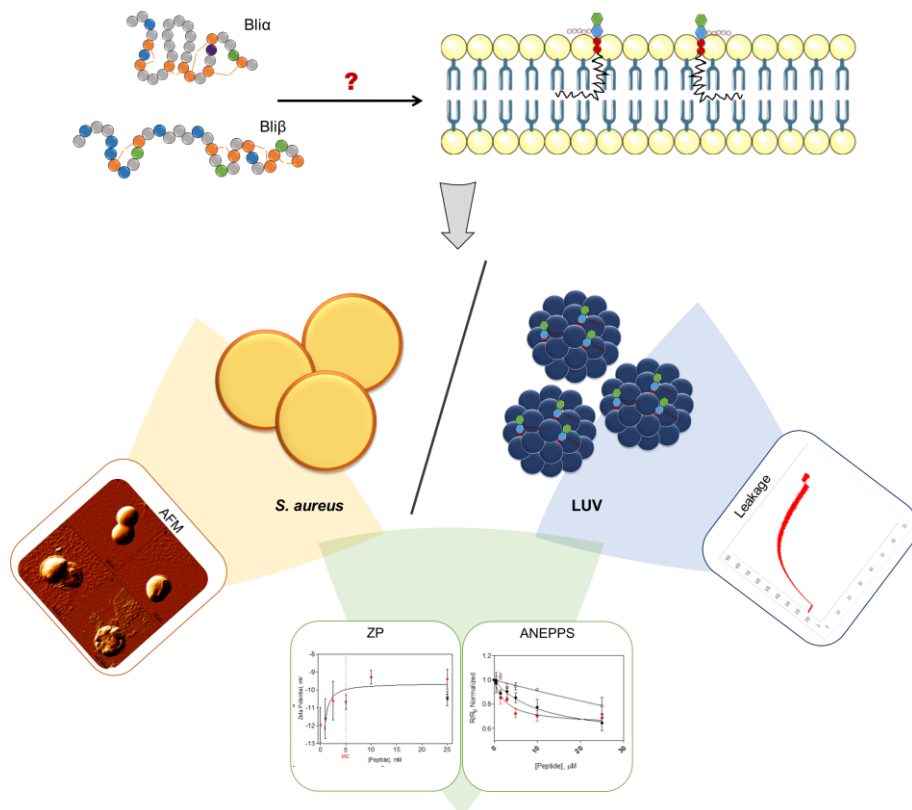
Two-way ANOVA was used for the statistical analysis of intergroup comparison followed for Bonferroni post-tests. Differences were considered statistically significant for $p < 0.05$. Statistical analysis was performed using GraphPad Prism 5.

5.4.8 Ethical approval

Blood from healthy blood donors was obtained with their previous written informed consent, following a protocol with the Portuguese Blood Institute (Lisbon, Portugal), approved by the joint Ethics Committee of Faculdade de Medicina da Universidade de Lisboa and Centro Hospitalar Lisboa Norte (Lisbon, Portugal).

CHAPTER VI

INSIGHTS INTO THE MODE OF ACTION OF THE TWO-PEPTIDE LANTIBIOTIC LICHENICIDIN



6.1 INTRODUCTION

Resistance development is part of the evolutionary process of microorganisms. However, this process has been accelerated due to selective pressure resulting from widespread and abusive use of antibacterial drugs, mainly the misuse and overuse for medical and veterinary applications. This problem becomes even more worrying when it leads to the development of multi-drug resistant bacteria, increasing the number of infections without effective therapeutic options [1,2]. Alternative strategies to overcome this global problem are being investigated, and include phage therapy and immune system modulation, along with further attempts to modify and improve existing and currently used bioactive molecules, while looking for novel antimicrobials, with improved bioactivities or with new modes of action [3]. Nature remains the best source for finding new compounds with novel and diverse chemical structures, whose chemical synthesis is sometimes difficult to achieve. Lantibiotics are promising molecules that have been reported as having high antibacterial activity against clinically relevant strains, including methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant Enterococci (VRE) [23]. Additionally, they have low toxicity towards mammals and reduced ability to develop resistance due to their mode of action [42]. A large number of the lantibiotics so far described target lipid II, a precursor of cell wall peptidoglycan, which is found on the outer layer of the bacterial membrane. Lipid II is the target binding molecule of several lantibiotics, mediating two different modes of action: i) causing physical sequestration of lipid II molecules, thus inhibiting peptidoglycan synthesis by preventing the catalysis of important steps of the cell wall assembly, such as the polymerization of lipid II and the crosslinking of the glycan chains; and ii) serving as docking molecule to induce de formation of defined and stable pores that cause membrane damage and depolarization, leading to the leakage of the intracellular content and ultimately to cell death [90,91]. As regards nisin, the same peptide performs both activities to induce bacterial death. In the cases of mersacidin, actagardine, and cinnamycin the peptides block only cell wall synthesis. For the two-peptide lantibiotics, such as lacticin 3147, haloduracin and lichenicidin, the presence of two mature peptides acting synergistically is required to achieve full activity [93]. The general mechanism of action of the two-peptide lantibiotics has been proposed based on the mode of action of lacticin 3147 and haloduracin. In these cases, each peptide performs one of the roles described above, so that the combined activity of the two peptides resembles that of nisin [91,92,94,95]. Briefly, the α -peptide was shown to bind to the peptidoglycan precursor lipid II with a 2:1 stoichiometry, thus inhibiting cell wall biosynthesis [94,95]. This complex then

recruits the β -peptide that inserts within the membrane leading to pore formation. The synergistic activity between Lan α and Lan β occurs at equimolar concentrations, forming a 2:2:1 Lan α :Lan β :lipid II complex [91,94].

This study aims to contribute to a better understanding of the mode of action of the two-peptide lantibiotics, taking as example the two-peptide lantibiotic lichenicidin. In lichenicidin the synergistic activity of the two mature peptides, Bli α and Bli β , is required for full activity against Gram-positive target strains, which include methicillin-resistant *Staphylococcus aureus* (MRSA), *Enterococcus faecium*, *Haemophilus influenza* and *Listeria monocytogenes* [54,194]. The independent activity of the Bli α and Bli β peptides was also detected for the most sensitive strains although less effective than the synergistic activity [101,261]. In addition, the optimal activity of lichenicidin has been established to occur at equimolar concentrations of each peptide (Chapter V; [101]). Investigating the mode of action of lichenicidin aims to understand the interaction of each peptide with the target membranes thus contributing with new and relevant information to the models described on the mode of action of the two-peptide lantibiotics. These studies are of great importance to better understand the possible mechanisms of development of resistance to lantibiotics that have been proposed, and are currently restricted to nisin-induced resistance and appear to result from the change in the total surface charge of the bacterial membrane [93,262].

6.2 RESULTS

6.2.1 Zeta-potential measurements

Zeta-potential (ζ) allows testing the interaction of lichenicidin with *S. aureus* with respect to changes in the bacterial cell surface charge. As shown in Figure 6.1, *S. aureus* membrane has a charge of approximately -11.9 ± 0.98 mV. The addition of lichenicidin, leads to a concentration-dependent increase in ζ . Despite the ζ stabilization (around -9.5 mV) at higher concentrations, electroneutrality was not achieved at the range of concentrations tested. The influence of each lichenicidin peptide was independently tested only at the highest concentration, 25 μ M. For both Bli α and Bli β , the observed zeta-potential was similar: -10.4 ± 0.11 mV for Bli α and -10.5 ± 0.29 mV for Bli β .

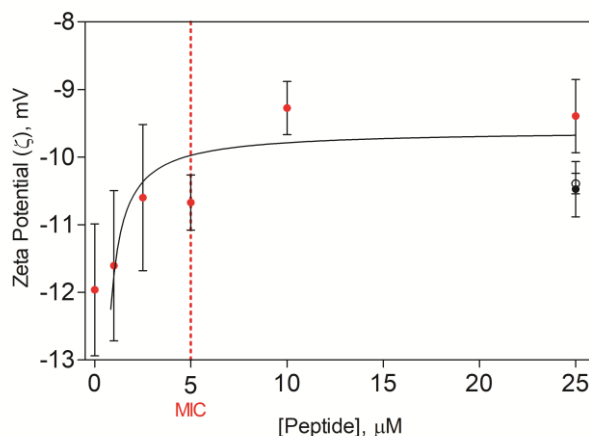


Figure 6.1. Zeta potential (ζ) changes of *S. aureus* induced after incubation with different concentrations of lichenicidin (red dots). ζ changes induced by higher concentration of Bli α (white dot) and Bli β (black dot) alone are also presented for comparison. Error bars represent the standard deviation of three independent replicates.

6.2.2 Lichenicidin-induced transmembrane voltage changes in *S. aureus*

Many antimicrobial peptides exert their activity by altering the membrane potential of the target cells, causing leakage of the intracellular content and, consequently, cell death. To assess whether or not lichenicidin causes changes in membrane potential, di-8-ANEPPS probe, which is strongly fluorescent when bound to the lipid bilayer, was used. The analysis of the obtained spectra and the calculation of the respective ratio revealed that Bli α does not have high affinity for *S. aureus* membrane, but contributes to the increase of Bli β affinity when in synergy with the latter. (Figure 6.2). In fact, the dissociation constant calculated for each condition was 83.3 μ M for Bli α peptide and it was only 2.5 μ M and 6.4 μ M for lichenicidin and Bli β , respectively.

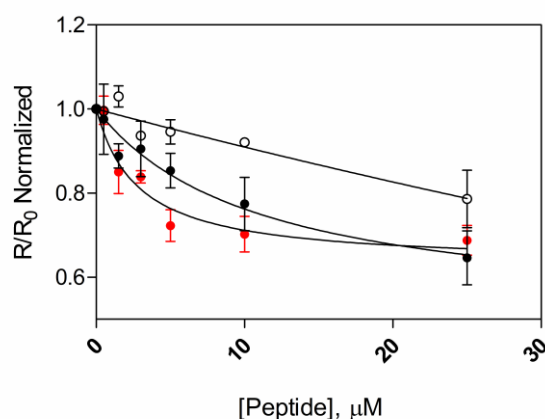


Figure 6.2. Membrane dipolar potential of *S. aureus* in the presence of different concentrations of lichenicidin (red circles), Bli α (white circles) and Bli β (black circles). Binding profiles of lichenicidin was assessed by calculating the excitation ratio R (I455/I525), normalizing for the value obtained in the absence of peptide. Experimental results fitted using equation (1). Error bars represent the standard deviation of three independent replicates.

6.2.3 Lichenicidin interaction with *S. aureus* imaged by AFM

Atomic force microscopy (AFM) is the ideal methodology for obtaining information on membrane surface properties and changes caused by the action of peptide(s) [263,264], which is extremely useful when evaluating their mode(s) of action. In order to evaluate these changes in response to the effect of lichenicidin, images of *S. aureus* at different incubation times (1, 5 and 24h) in the absence and presence of the lantibiotic were obtained. Images clearly show surface changes due to the action of lichenicidin (Figure 6.3). Without lichenicidin, *S. aureus* appears with a smooth surface and with the typical spherical shape (Figure 6.3A; [263,265]). It is worth mentioning that single cells are sometimes hard to spot, as *S. aureus* cells tend to agglomerate [265,266]. When compared to control cells, after 1 h incubation, an increase in bacterial surface roughness is observed (Figure 6.3A and B) and becomes more evident after 5 h of treatment. Bacterial cells start to lose their typical structure, and this is accompanied by the leakage of internal content (Figure 6.3C). When the cells were incubated for 24h with a lichenicidin concentration 5-fold the MIC, the membrane surface was severely damaged and the cellular structure was lost, culminating in cell lysis and, consequently, release of internal cellular content (Figure 6.3D).

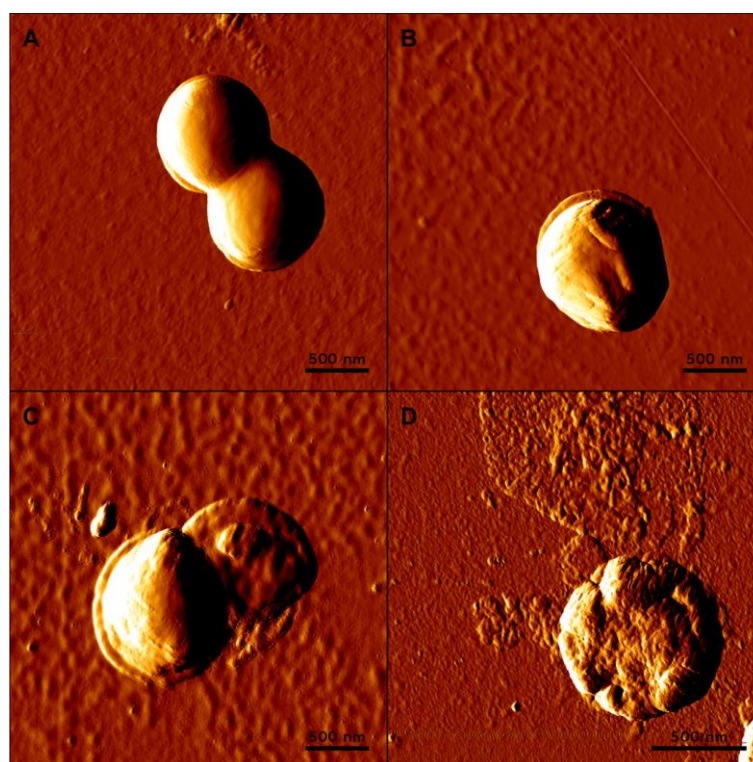


Figure 6.3. Effect of lichenicidin on *S. aureus*, imaged by AFM: (A) control, *S. aureus* in the absence of lichenicidin; (B) incubation for 1 h with 5 μ M lichenicidin (MIC); (C) incubation for 5 h with MIC; and (D) incubation for 24 h with 5 x MIC (25 μ M).

6.2.4 Interaction studies using LUV

To assess the ability of lichenicidin to form pores in the membranes or, at least, destabilize the membranes to cause leakage, some bacteria-like membranes have been tested. Accordingly, lipid compositions 1) and 2) were based in a previous study of Al-Kaddah and co-workers; the authors used DOPC (100) and DOPC plus 0.1 mol% lipid II vesicles to test the lantibiotic gallidermin [267]. Conditions 3), 4) and 5) were based on 1) and 2) and took into account the reported lipid percentages for the natural composition of *S. aureus* membranes [268–270].

At first, the ζ of each type of LUV was determined, to give insights not only on the overall charge of the LUV but also, on lipid II incorporation. The increase in negative charges of the LUV was consistent for the two membrane types to which lipid II was added (Figure 6.4A). As expected, the presence of 30% of negatively charged lipids (DPPG or DPPG:CL) strongly contributes to the negative charge of the LUV compared to the LUV containing the neutral DOPC lipid (Figure 6.4A).

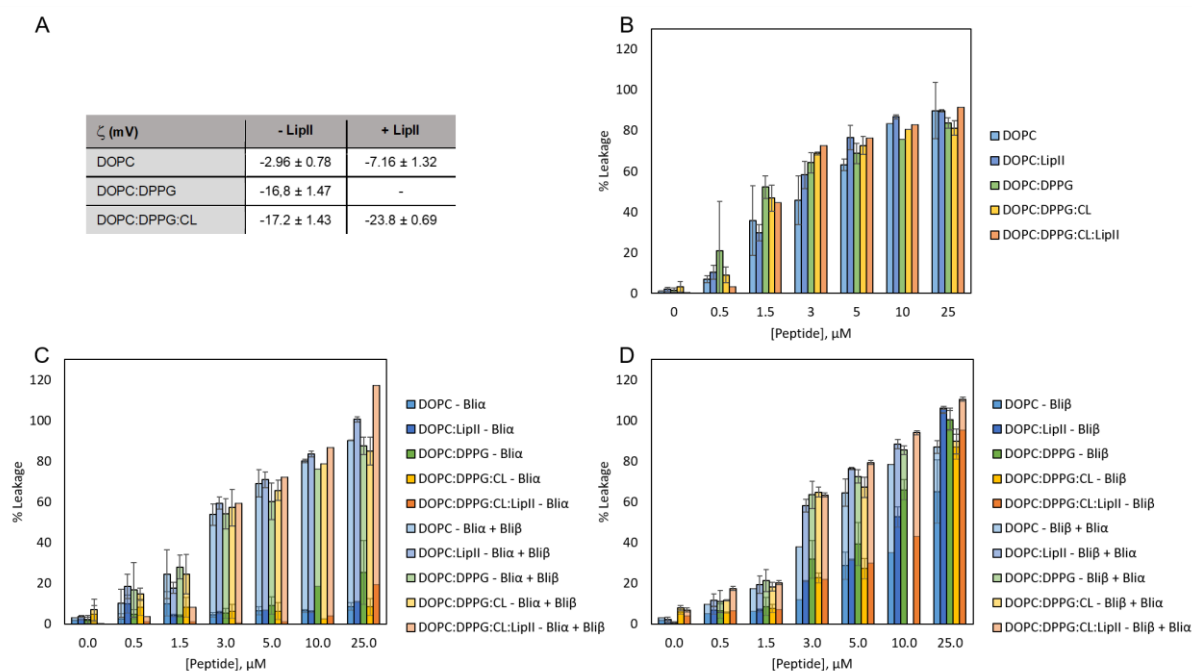


Figure 6.4. Zeta potential (ζ) of the lipid vesicles (A); percentages of leakage induced by lichenicidin (B) and by the sequential addition of Bli α followed by Bli β (C) and Bli β followed by Bli α (D). Error bars represent the standard deviation of three independent replicates; when these are not visible, standard deviation is too low to be noticed.

Addition of 5 μ M of lichenicidin, results in about 70% of leakage in any of the membrane compositions tested (Figure 6.4B). Increasing the lichenicidin concentration above this value does not result in a corresponding increase in the percentage of leakage,

as 5-fold increase in lichenicidin concentration leads only to a 10 to 20% increase in the percentage of leakage (Figure 6.4B). Regarding the sequential addition of each individual peptide, Bli α alone does not induce a high percentage of leakage (Figure 6.4C) with the maximum observed around 25% for DOPC:DPPG LUV against the highest concentration of Bli α ; however, after equimolar addition of Bli β , the percentage of leakage increased to about 100%, which was particular evident for both lipid II containing LUV (Figure 6.4C). In contrast, the addition of Bli β causes between 30 and 40% leakage for all the tested compositions at the MIC value; this value goes up to 87-100% when the concentration of Bli β is increased 5 times (Figure 6.4D). In this case, the negative charge of the LUV seems to be more important for this effect than the presence of lipid II itself, since the more neutral DOPC is the less it is affected by Bli β . The addition of Bli α , increases the leakage to the values obtained when low concentrations of lichenicidin are added. No effect is observed for the conditions in which Bli β has already induced 100% leakage (Figure 6.4D).

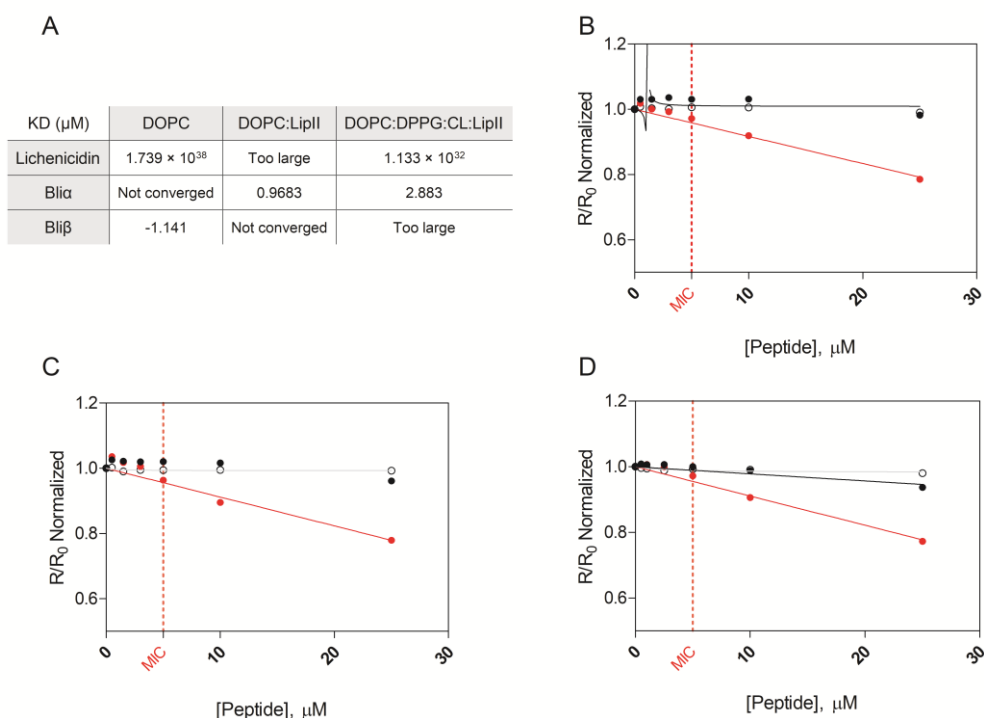


Figure 6.5. Lichenicidin affinity to LUV: dissociation constants (A) of lichenicidin (red), Bli α (white) and Bli β (black dots) calculated for the interaction with DOPC 100% (B), DOPC:LipII 99.9:0.1% (C) and DOPC:DPPG:CL:LipII 70:20:9.9:0.1% (D). The MIC value is represented by the vertical red line.

In general, the presence of lipid II does not seem to affect leakage. However, if we consider a reaction kinetic approach, it is possible to draw some conclusions about the speed of the reaction shortly after the addition of the peptide. For this, we considered the

rate of leakage increase, over 1 min after the addition of each peptide, of DOPC and DOPC:DPPG:CL vesicles with and without lipid II (Table S6.1). This analysis was performed at concentrations of peptide of 1.5, 5 and 25 μM . The slopes, corresponding to the leakage rate, were correlated as a ratio $(\text{LUV}+\text{LipII})/(\text{LUV})$. As far as a “reaction rate” is concerned, the interaction of lichenicidin with LUV containing lipid II is, generally, favoured in all the tested conditions, although not at all exceptional (Table S6.1). A more detailed analysis of the results for the sequential addition of each peptide shows that at the lower concentration (1.5 μM), the presence of lipid II increases the leakage rate, especially in the more complex composition DOPC:DPPG:CL, where the increase is of 2-3 fold. At higher concentrations, it appears that Bli α interacts with lipid II 2 to 4 times faster than in its absence, sequestering it and promoting some membrane destabilization; the subsequent interaction of Bli β remains identical in the presence and absence of lipid II (approx. 1), suggesting that its interaction may be independent of the presence of lipid II. For the initial addition of Bli β and at the lowest concentration, the leakage rate increases in the presence of lipid II for both membrane compositions; this effect is exacerbated upon addition of Bli α , and is most evident in vesicles that mimic the original target. We highlight the results obtained at high concentrations, where Bli β alone induces almost 100% leakage, which makes the results inconclusive as it is difficult to observe the effect of Bli α . Overall, the results show that Bli α has a higher affinity for lipid II than Bli β , although this effect is not so clear in the percentage of leakage itself, but in the interaction rate.

Regarding the affinity values obtained in the ANEPPS studies, it was possible to calculate Bli α dissociation constant only for Lipid II containing LUV (Figure 6.5); for both cases, the dissociation constant is low, which means a high affinity of Bli α as opposed to the absence of lipid II. For Bli β , the constant is too large or couldn't be calculated, which also influences the synergistic affinity of both peptides.

6.3 DISCUSSION

Given their effectiveness, mode of action and difficulty in developing resistance, lantibiotics are considered as attractive alternatives to combat infections caused by some clinically relevant Gram-positive bacteria. [42]. Nisin was the first lantibiotic to be identified and which has since been applied as a food preservative and only low levels of acquired resistance have been observed to date. Some Gram-positive bacteria are intrinsically resistant to nisin due to the presence of specialized proteins that can degrade or modify the peptide. In other cases though, sensitive strains can acquire resistance after exposure to increasing concentrations of the peptide – this mechanism is also termed physiological

adaptation, since this feature is not stable and is lost after removal of nisin pressure [49]. Acquired resistance to nisin has been reported as a protective mechanism, through the incorporation of positive charges in the cell wall that repel the positively charged peptide, preventing it from reaching lipid II. This can be achieved either by the accumulation of divalent cations or by altering the composition of the cell wall [49]. In Gram-positive bacteria, the outermost layer is composed of peptidoglycan and small amounts of teichoic acid, rendering the bacterial surface negative [262,271]. Bacteria can regulate its cell wall charge by attaching a D-alanine to teichoic acid, thereby adding a positive charge to it. This can be an important bacterial defence mechanism against the action of several positively charged antimicrobial peptides that target their membranes [49,262].

Lantibiotics usually use lipid II as docking molecule to exert their activity, either by blocking peptidoglycan biosynthesis or leading to pore formation. Nisin combines both activities in a single peptide. Similarly, two-peptide lantibiotics have one peptide dedicated to each function. Lichenicidin is a cationic peptide with an overall net charge of +3, which results from the presence of two positively charged amino acids in each peptide (Lys12 and Lys25 in Bli α ; Lys27 and Arg31 in Bli β) and a negatively charged Glu26 residue in Bli α . Therefore, it is reasonable to conclude that electrostatic interactions are important for the interaction of peptides with the bacterial cell surface. And this is true, for example, for lichenicidin which, when present, increases the surface charge of *S. aureus* without, however, achieving neutralization. Both peptides seem to contribute equally to the change in surface since their effect is similar and about half the neutralizing power of both. This was expected since the peptides do not differ much in terms of overall charge. In fact, although lichenicidin is positively charged, it is not highly charged, which explains why high concentrations of lichenicidin are not sufficient for electroneutralization, although stabilization of the bacterial surface charge occurs. Thus, lichenicidin mechanism of action does not appear to depend on membrane depolarization since peptides do not induce a significant increase in the membrane surface charge or transmembrane potential. Even so, Bli β appear to have a high affinity for the bacterial membrane and this can be correlated with the proposed role of pore formation across the cell membrane for other β -peptides. Similar studies revealed that Hal β is able to bind to anionic lipids of the cell membrane causing the transient formation of pore-like structures even in the absence of lipid II [49,91]. The same was also observed for nisin when tested against membranes without lipid II [272]. Lichenicidin has an even higher affinity to the cell membrane than Bli β alone. As such, it seems that Bli α and Bli β stabilize each other during the interaction with the target cell. We hypothesize that a mechanism similar to that observed for lactacin

3147 and haloduracin may occur [91,92]. Accordingly, Bli α binds to lipid II and, by changing its conformation, recruits Bli β to a newly available binding site; Bli α itself does not have high affinity for the membrane but this affinity increases after binding of Bli β . On the other hand, Bli β already has affinity for the membrane, but the presence of Bli α provides a desirable docking site, to assist the binding and insertion of Bli β , providing binding stability. A similar effect was reported for the dual mode of action of nisin, with the lipid II binding domain initiating membrane deformation followed by C-terminal insertion which strengthens this initial deformation and leads to pore formation [272].

Concerning the vesicle models, Bli β seems to have a high effect on inducing leakage in all the LUV compositions tested although its affinity is extremely low. On the other hand, Bli α seems to have a higher affinity with the LUV, particularly in the presence of lipid II; despite its role being not so clear in the leakage assay, its presence enhances the interaction of Bli β with the model membranes, and the interaction with lipid II containing vesicles seems to occur faster, although no significant difference were observed in the final leakage percentage. Interestingly, Wiedemann and co-workers also reported that none of the lacticin 3147 peptide could induce leakage alone, not even in the presence of lipid II [92]. However, when applied together to lipid II containing LUV, about 80% of leakage was achieved, which is in accordance with the results of our study. This ability to induce pore formation and leakage was observed in *S. aureus* cells using AFM. Lichenicidin (5 μ M) effects seem to be time-dependent. Structural changes are more evident after 5h of incubation than after only 1h. However, the most dramatic effects were observed after 24h of incubation with 5-fold the MIC (25 μ M). Such conditions induce severe cell surface damage along with lysis and consequent loss of intracellular content, thus, leading to loss of viability. Such changes in bacteria morphology have been previously assessed using AFM and observed in response to other antimicrobial agents [263,265].

Altogether, our results support the model proposed for other two-peptide lantibiotics and nisin. A model for lichenicidin activity is shown in Figure 6.6. As proposed, lichenicidin acts through a dual mode of action that involve Bli α recognition of lipid II, providing specificity and stability for the interaction of Bli β . The ability of Bli β to form pores and induce leakage of the intracellular contents was also demonstrated. This, along with Bli β higher affinity to the hydrophobic membrane compartment, explains why Bli β alone has a stronger antimicrobial effect than Bli α . However, given the high affinity between Bli α and lipid II we hypothesize that, if extremely high amounts of Bli α are to be applied to the

bacterial cells and for longer incubation periods, inhibition of the cell wall synthesis could possibly be observed, leading to a bacteriostatic effect, or even, cell death.

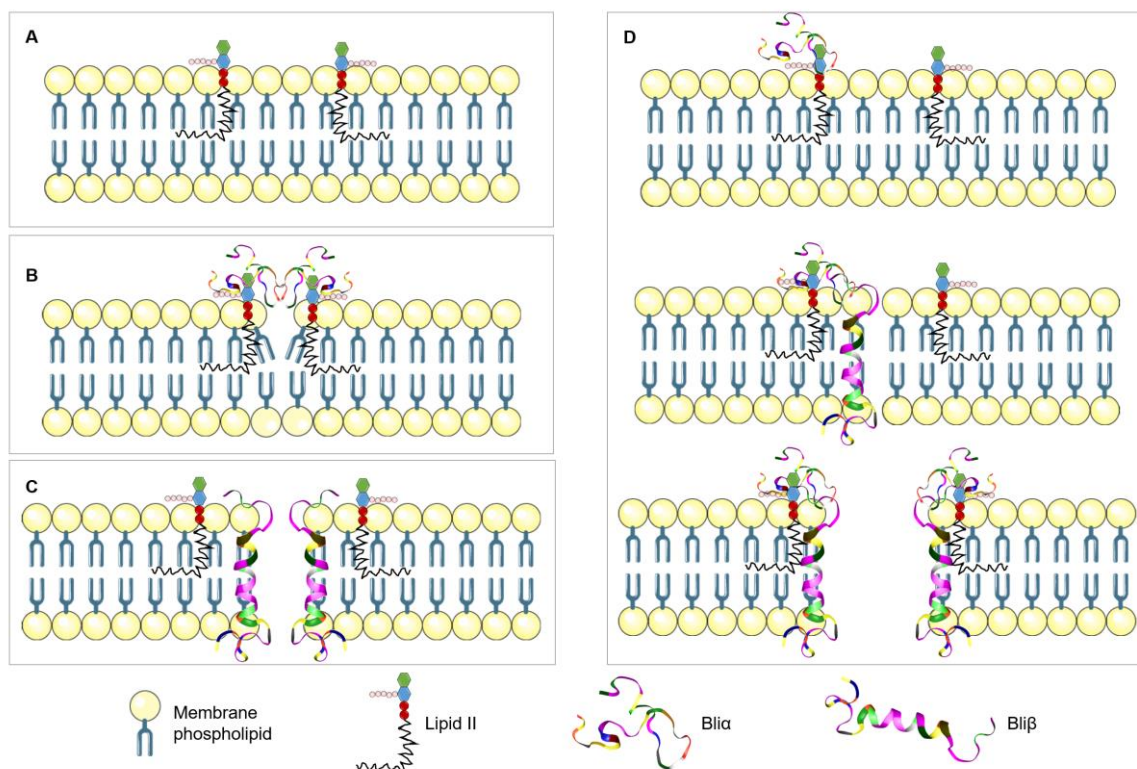


Figure 6.6. Proposed mode of action of lichenicidin. (A) target membrane containing lipid II; (B) possible mode of action of Bli α by sequestering lipid II and destabilizing the membrane without pore formation; (C) mode of action of Bli β with transient pore formation without using lipid II as anchoring molecule; (D) dual mode of action of lichenicidin: Bli α recognizes and binds lipid II, recruiting Bli β that then inserts within the target membrane, forming a stable pore.

6.4 MATERIAL AND METHODS

6.4.1 Peptide production and purification

Lichenicidin peptides were produced by *E. coli* strains that were optimized for the independent expression of each peptide (Chapter V). Briefly, batches of 6 L of the *E. coli* culture supernatant were prepurified by extraction with 1/4 volume of ethyl acetate to remove byproducts followed by extraction with 1/5 volumes of 1-butanol. The organic phase was vacuum dried. The dried extracts were then dissolved in 70% ACN and loaded onto a RepoSil XR120 C8 (150x25 mm, 10 μ m) column from Dr. Maisch (Ammerbuch-Entringen, Germany). The sample was eluted with the following gradient: 5%-25% ACN in 1 min, increasing to 42% ACN in 13 min and finally increasing to 100% ACN in 2 min, with a flowrate of 50 mL/min. Peptides eluted between 32 and 42% ACN. The respective

fractions were collected and vacuum dried. In the last purification step, samples were diluted in as little as possible of 70% ACN and precipitated in ice-cold acetone. The precipitate was centrifuged and acetone was removed and the sample was lyophilised. Purity of the samples was confirmed by ESI-Orbitrap-MS Exactive, 1200 Series HPLC (Thermo Scientific) coupled to an analytical HPLC.

6.4.2 Bacteria preparation

S. aureus ATCC 25293 strain was plated on tryptic soy agar (TSA) overnight at 37°C. A single colony was used to inoculate tryptic soy broth (TSB) and the culture was allowed to grow overnight at 37°C. 100 µL of this culture were used to inoculate 5 mL of TSB and the new suspension was incubated at 37°C, 180 rpm for ~1.5h, until it reached a concentration of 3×10^8 CFU/mL. The cells were centrifuged at $4000 \times g$, 10°C, 25 min and washed three times with TSB or HEPES buffer, according to the protocol to be used. The remaining cells were suspended in 5 mL of TSB or HEPES and OD_{600nm} was measured to adjust cell concentration.

6.4.3 Preparation of lipid vesicles

1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DPPG) and cardiolipin (Heart, Bovine, sodium salt) were purchased from Avanti Polar Lipids (Alabaster, AL, USA); lipid II was kindly provided by Prof. Dr. Tanja Schneider, (Institute for Pharmaceutical Microbiology, University of Bonn) and extracted as described elsewhere [267]. Large unilamellar vesicles (LUV) with 100 nm diameter, with different compositions were obtained by extrusion of multilamellar vesicles. The compositions tested were: 1) DOPC (100%); 2) DOPC:LipII (99.9:0.1); 3) DOPC:DPPG (70:30); 4) DOPC:DPPG:CL (70:20:10); and 5) DOPC:DPPG:CL:LipII (70:20:9.9:0.1) Lipids were dissolved in chloroform and dried in round-bottom flasks to obtain a thin film. Multilamellar vesicles were obtained by dissolving the film in the working buffer and subjected to 8 freeze-thaw cycles. This suspension was extruded through a 100 nm pore filter in an Avanti extruder and reserved until use. Lipid mixtures with different proportions were chosen as models of Gram-positive bacteria membrane.

6.4.4 Membrane dipole potential studies with di-8-ANEPPS

The membrane probe di-8-ANEPPS assesses dipole potential by shifting its excitation spectrum [273]. Briefly, for di-8-ANEPPS studies, HEPES buffer (pH 7.4) was used for

hydration of lipid film. 500mM of extruded lipid vesicles were incubated with di-8-ANEPPS 10mM for 1h with stirring and light protected. Excitation was measured at 455 and 525 nm ($R=I_{455}/I_{525}$), with emission set to 670 nm. R variation with peptide concentration was analysed using the following model [274]:

$$\frac{R}{R_0} = 1 + \frac{R_{min}/R_0 \times [Peptide]}{KD + [Peptide]}$$

in which R_0 is the value in the absence of peptide, R_{min} defines the asymptotic minimum value of R and KD is the dissociation constant. The fitting of this equation to the experimental data was done by non-linear regression using GraphPad Prism 5.

For *S. aureus* testing with di-8-ANEPPS, the bacterial cell suspension was adjusted to a final concentration of 1×10^5 cells/mL and labelled with 100 μ M di-8-ANEPPS (final concentration) using HEPES buffer (pH 7.4) with 0.05% of Pluronic F-127. The probe was allowed to incorporate the membrane for 1h, stirring, protected from light. Unlabelled cells were used as the non-fluorescent control. For LUV labelling, a final concentration of 10 μ M of di-8-ANEPPS was used for incubation in HEPES buffer for 1h, stirring and protected from light. After labelling, a final concentration of 1×10^4 cells/mL or 200 μ M of labelled LUV were incubated with increasing peptide concentrations for 1h; Bli α and Bli β were tested either together or independently. Excitation spectra were recorded as described below using a spectrofluorimeter (Varian Cary Eclipse Fluorescence Spectrophotometer, Mulgrave, AU) in a 0.5 mm optical path quartz cuvette.

6.4.5 Zeta-potential measurements

Bacterial concentration was fixed at 1×10^7 CFU/mL in TSB, in order to acquire high enough count rates. The zeta-potential of LUVs used for the leakage assays was also assessed to determine the incorporation of the negatively charged lipids. The final lipid concentration used was 200 μ M. *S. aureus* or LUV solutions were prepared in HEPES buffer (pH 7.4). Zeta-potential was determined at 25°C from the mean of 15 measurements (100 runs each). Initial equilibration time was set 15 minutes. *S. aureus* was tested in the absence and in the presence of different concentrations of lichenicidin. The results for each condition are represented as the mean of three independent measurements. Zeta-potential experiments were carried out on a Malvern Zetasizer Nano ZS (Malvern Instruments, Malvern, UK), using disposable zeta cells DTS 1060 (Malvern, UK) with gold electrodes. All the results were processed using the instrumental Malvern's DTS software, after three independent experiments.

6.4.6 Atomic force microscopy imaging of *S. aureus*

S. aureus cells were prepared as described above with the cell number adjusted to 5×10^5 CFU/mL. Cells were incubated with 5 μ M of lichenicidin (MIC determined for this strain (Chapter V)) at 37°C, for 1h and 5h, and 5 times the MIC (25 μ M) for 24h. As control, cells were incubated without lichenicidin. A 25 μ L droplet of each test sample was applied onto a poly-L-lysine (PLL)-coated glass slide and left to stand at room temperature for 1h. After deposition, the sample was rinsed 10 times with sterile Milli-Q water and air-dried at room temperature. On average, five individual bacterial cells per sample were imaged at high resolution (512 \times 512 pixels) for each peptide concentration. AFM studies were conducted using a JPK NanoWizard IV (Berlin, Germany) mounted on a Zeiss Axiovert 200 inverted microscope (Jena, Germany). Measurements were carried out in intermittent contact mode using uncoated silicon ACL cantilevers (Applied NanoStructure, Santa Clara, CA). These cantilevers have typical resonance frequencies of 145-230 kHz and spring constants of 20-90 N/m. Height, error and phase-shift images were recorded and line-fitted as required. All images were obtained with similar AFM parameters (setpoint, scan rate and gain values). The scan rate was set between 0.3 and 0.6 Hz and the setpoint was close to 0.3 V. Height and error signals were collected and images were analysed with the JPK image processing software v. 6.0.55.

6.4.7 Leakage assays with membrane models

Peptide-induced lipid vesicle leakage was measured by fluorescence spectroscopy. In this assay, we monitored the release of 5,(6)-carboxyfluorescein (CF; Sigma, St. Louis, MO), trapped in the LUV. LUVs were prepared as described above, with dried film hydration in phosphate buffered saline (PBS) containing 100 mM CF (pH was adjusted to 7.4). Free CF was removed by passing the suspension through an EconoPac 10 DG column from Bio-Rad (Richmond, CA), where the vesicles are eluted with the void volume. A final concentration of approximately 10 μ M of LUV was used to test different concentrations of lichenicidin. Fluorescence was recorded continuously for 1h at 25°C, with excitation at 492 nm and emission at 517 nm (5 and 10 nm excitation and emission slits, respectively). Kinetics was followed for all the three tested combinations: i) addition of both peptides together, ii) addition of Bli α followed by the addition of equimolar amounts of Bli β and iii) the opposite, Bli β followed by the addition of Bli α . LUV were added first and the fluorescence signal was allowed to stabilize for 5 min, followed by the addition of the corresponding volume of peptide to be tested; when required, the second peptide was

added at the intermediate time point (30 min). After 55 min, Triton X-100 10% was added to a final concentration of 1% to ensure the total release of CF from the vesicles, corresponding this value to the highest fluorescence intensity. Leakage percentage was calculated as follows:

$$\% \text{ leakage} = \frac{F - F_0}{F_{100} - F_0} \times 100$$

where F_0 is the fluorescence of the LUV only, F is the fluorescence intensity of the sample containing LUV and peptide and F_{100} is the intensity of the sample after the addition of Triton X-100, which corresponds to 100% of leakage. The fluorescence intensities were corrected for the dilution introduced by the addition of peptide and Triton X-100 [275]. Leakage assays were conducted in triplicate.

CHAPTER VII

SYNOPSIS AND FUTURE PERSPECTIVES

7.1 SYNOPSIS

This thesis focused on a group of natural antimicrobial peptides, the so-called lantibiotics, which have been viewed with increasing interest as a new alternative to the more traditional antibiotics. Although nisin was described at about the same time as penicillin, the “golden era” of lantibiotics has begun in last decades, with an exponentially increasing number of compounds being discovered over the last 20 years. Lantibiotics attract attention due to their effectiveness against relevant strains, mainly Gram-positive, and also due to their low mammalian toxicity. Fortunately for the human health, we are convinced that this group of compounds is full of promising candidates that can help overcome the worldwide problem of multidrug resistance. The misuse and overuse of antibiotics has been pointed out as the main cause of the antibiotic resistance. However, resistance has always existed in nature, as an ecological function of survival, and genes conferring resistance to various groups of compounds are widely distributed, even in places where anthropogenic activity is reduced.

Novel antibacterials with new targets, more specific mechanisms of action and less subject to resistance development are urgently needed. The unique mode of action of lantibiotics and specially the combination of more than one mode of action in a single compound and also the low concentrations required, make these peptides more effective against a wide range of bacteria. Additionally, acquiring resistance is more difficult. This was demonstrated for nisin that, despite being a intensively and widely used lantibiotic for over five decades, low incidence of resistance has been detected so far, and studies involving the development of resistant mutants have revealed that once the selective pressure is removed, bacteria resume their sensitive phenotype.

Nature is still the main source of novel bioactive molecules and in particular extreme environments have always been considered reservoirs of unknown compounds and enzymes with peculiar chemical structures and various applications. Thus, these environments are conducive to the discovery of new enzyme/antimicrobial-producing organisms with new activities, targets and modes of action. Once identified, bioengineering approaches can be employed to enhance these products for their biotechnological application. *Bacillus* spp. are a prolific source of valuable enzymes and secondary metabolites. Among these are several antimicrobial peptides, including lantibiotics. So, in **Chapter I** of this thesis, a review of 17 different lantibiotics produced by various species of *Bacillus* is presented. These included seven class I and ten class II lanthipeptides; within the latter class, five two-peptide lantibiotics are described. So far,

only class I and class II lantibiotics were identified in *Bacillus* spp., i.e., only lanthipeptides exhibiting antimicrobial activity, which clearly shows the relevance of *Bacillus* spp. as a source of new antimicrobials. As more and more genomes are being sequenced and the molecular, genetic and bioinformatics tools are evolving, the number of discovered and characterized lantibiotics is increasing and is expected to increase even more in the coming years. Although none of the *Bacillus* lantibiotics described to date have entered clinical trials, they have been shown to be effective against clinically relevant and foodborne pathogens. Despite the advances already made, further studies are still required envisaging the characterization and application of lantibiotics in food and pharmaceutical industries. One of the major problems that has to be overcome for industrial use of such compounds is the optimization of their production and purification methods to obtain high yields of pure peptides.

With this in mind, our study focused on lichenicidin, a class II two-peptide lanthipeptide. It was described in 2009 for the first time by two independent groups, through genome mining of *B. licheniformis* strains. Nowadays, it is known that almost all (if not all) *B. licheniformis* encode the biosynthesis of lichenicidin in their genomes. It was the first lantibiotic to be heterologously expressed, completely *in vivo*, in the Gram-negative host *E. coli*, which facilitated its manipulation and characterization. Lichenicidin has activity against several Gram-positive bacteria, including *B. subtilis*, *B. pumilus*, *B. megaterium*, *S. aureus* (both MSSA and MRSA), and *L. lactis* strains.

In **Chapter II**, a site-directed mutagenesis approach was used to generate lichenicidin variants. These studies aimed to generate new variants of lichenicidin with increased bioactivity. In total, 57 lichenicidin variants were designed, 29 for Bli α and 28 for Bli β . The variants were expressed in *E. coli* and their bioactivity was screened using a methodology, developed in our group that is specially tuned to screen two-peptide libraries. Biologically active variants were obtained and their bioactivity and structure were evaluated. The mutants obtained allowed us to better understand the relationship between the structure and activity of lichenicidin. Our results showed that some variants showed slightly improved bioactivity, although their relative production yields, determined by MS, seem to be higher than the control. Several factors may explain these results, such as: i) differences in peptide solubility and diffusion and ii) since bioactivity requires the presence of the complementary peptide, the bioactivity levels observed can be limited by the amount of complementary peptide that is fed in the bioassay. Although some aspects of the procedure still need to be improved, given its simplicity, it can be used for a first

screening of large libraries of two-peptide lantibiotic mutants, provided it is complemented by MS analysis. In general, the results were consistent with those already reported for other lantibiotics, which suggests that the Gram-negative heterologous host does not influence the results obtained. The negatively charged residue Glu26, which is conserved in various lantibiotics, particularly in α -peptides, does not tolerate mutations unless the negative charge is maintained. Furthermore, the insertion of other negatively charged residues is not tolerated as it reduces or impairs the bioactivity of such variants. Interestingly, in most cases, Ser and Thr residues could substitute each other in ring formation without affecting bioactivity. However, the specificity of the post-translational machinery was affected by such alterations, as the dehydration of some of those residues was not complete or ring formation was blocked. Additionally, it was hypothesized that the dehydration of Ser and Thr residues, does not depend on the residue itself, but on the position of the amino acid and/or its vicinity. Nevertheless, our results demonstrate that the dehydration “code”, previously proposed for nisin, is not universal in that the residues proposed to promote dehydration that are surrounded by hydrophobic residues, escape dehydration by their dedicated enzyme. Unlike Bli β A-ring, Bli α A-ring tolerates mutations. Further, B-ring is fundamental for Bli α activity.

This work contributed to deepen the knowledge on the flexibility of lantibiotic biosynthetic enzymes, opening perspectives for future applications in the production and improvement of peptides. Nonetheless, further studies are needed to improve the screening method with regard to its accuracy and sensitivity. In addition, mutants with potentially increased activity and/or productivity should be further studied in the future, as they may represent improved variants of lichenicidin with medical application. As for the Bli α and Bli β , variants their bioactivity and physico-chemical properties should be evaluated and the best performing variants should be tested synergistically.

In order to understand the importance of the hexapeptide and of the double Gly motif in the proteolytic activity of LicP and LicT enzymes, an approach similar to that adopted in the previous chapter was used. Thus, in **Chapter III**, several Bli β ’mutants were made, directed to the hexapeptide and double Gly motif. LicP and LicT enzymes are involved in lichenicidin processing: LicP cleaves the remaining hexapeptide in Bli β , after LicT cleavage. Lantibiotic leader peptides provide the recognition and anchoring for post-translational modifications enzymes. For lichenicidin, it was established that, *in vitro*, the LicA2 hexapeptide is important for the recognition and activity of LicP. The generated

mutants were evaluated for their bioactivity using the bioassay method described above followed by MS analysis.

The mutants obtained confirmed that, *in vivo*, and using *E. coli* as the heterologous expression host, the hexapeptide is important for the maturation of Bli β . The presence of a negatively charged residue at position -1, preferably Glu, which is conserved among β -peptides and other “hexapeptide-containing” lantibiotics, directly influences the cleavage rate and also the dehydration/cyclization processes. In addition, alterations in the length of the hexapeptide had a deleterious effect on the enzyme processing rate and/or specificity, decreasing the production yield of the mature Bli β or originating unexpected peptide variants. Again, those mutants should be further investigated as they can provide valuable information regarding the exact nature of the interaction between the post-translational modification machinery and their recognition sequences, with respect to specificity and catalytic activity.

More importantly, two variants, Val-4Ala and Asp-5Ala, were obtained in which the yield of mature Bli β was significantly increased compared to the native sequence. Furthermore, the optimization of the double-Gly motif, where LicT cleavage takes place, to GlyAla produced higher amounts of fully mature Bli β . These mutants will also be studied in the future to improve the yield of lichenicidin production.

Proteases are widely used in various industrial applications. Therefore, and considering a possible application of LicT and LicP enzymes in industry, we have investigated the ability of these enzymes to cleave and secrete other peptides of interest, using *E. coli* as host. Bioengineering approaches were used to produce enzymes with improved specificity, efficiency and stability. However, achieving all of these improved features together is challenging and, most of the time, efficiency and stability are lost when gaining specificity and vice-versa. As such, nature still remains the main source of new proteases with novel specificities and stability, and this depends on the environment from which the producers are isolated. The vast majority of lantibiotic gene clusters encoded, at least, one protease. Thus, a wide range of highly specific putative recognition and cleavage sequences are closely encoded in the respective structural genes.

The hypothesis underlying the second part of this work is to understand whether lantibiotic trimming enzymes are capable of cleaving fusion peptides into which the leader peptide “tag” is fused upstream of the peptide of interest. Using lichenicidin as proof-of-concept, LicA2 leader peptide was fused to several peptides, some of them, with interest for human medicine, such as insulin chain A and somatostatin. It was found that LicA2 leader peptide can direct the cleavage of insulin A, amylin and epidermin. For amylin and

lunasin, it seems to work also as a solubilisation tag. Although the process requires optimization, since some of the peptides could not be detected at all, it may be suggested that lantibiotic enzymes could be directed to cleave other peptides which have been fused downstream of their cognate leader sequence.

Still, the expression systems employed require further optimization to increase the yield of the recovered peptide. However, the possibility of expressing these high-value peptides in *E. coli* by harnessing heterologous microbial enzymes for biotechnological applications, opens new perspectives for the use of microorganisms as “*in vivo* microbial cell factories”. Furthermore, the approach employed constitutes an easier way to produce pure, safe and biocompatible compounds.

Following previous studies, it became clear that improving the yields of lichenicidin by heterologous expression is a priority. Thus, **Chapter IV**, aimed at developing an expression system that will give high yields of pure lichenicidin, while reducing the handling and purification steps. Heterologous expression of lichenicidin in *E. coli* was achieved by inserting a fosmid containing the entire *lic* gene cluster in this host. At the time this was done, the expression yields of lichenicidin in *E. coli* were not compared, by bioassay nor MS quantification, with those of the original producer, *B. licheniformis* I89. Here, that study was completed, and we found that the expression in *E. coli* is lower than in *B. licheniformis*. However, the expression of each peptide in a separate system seems to facilitate the purification process by simplifying the subsequent application of the peptides at defined concentrations and enabling the various combinations. Furthermore, by directing the cell resources to the production of only one peptide can increase, *per se*, the peptide yields. The first attempt to produce each peptide separately was accomplished by knocking out each structural gene, to express only the remaining peptide, under the expression of *Bacillus* regulatory determinants. Optimized vectors, containing *E. coli* promoters and RBS, were developed in this work and also by another group. The comparison of all these expression vectors revealed that lichenicidin production is favoured by the inclusion of optimized *E. coli* transcriptional regulatory elements, as previously reported. Also, as expected, the separate production of each peptide is advantageous for peptide purification. The best systems to produce Bli α and Bli β are Gold pA1M1T and Gold HP β , respectively. Clearly, the requirements for the production of each peptide are distinct, since the higher production levels were achieved with different optimized vectors. However, for Bli β , the additional trimming step required for its maturation is a limiting factor for its high yield production. Even in the best Bli β expression

system high levels of hexapeptide-containing LicA2 were detected. Thus, a possible way of increasing Bli β yields may be the further cleavage of the hexapeptide after simultaneous purification of the hexa-LicA2 variant.

It is clear to us that alternatives other than those applied in this study may be tested, such as, the insertion of the mutations described in the previous chapters, which may contribute to increase the processing of the enzymes LicT and LicP, thus contributing to the increase of peptide production. In addition, manipulation of culture conditions can also be tested. It is worth mentioning that, once again, the bioactivity and the quantification results show that there is a good correspondence. This confirms the robustness of the bioassay method for a first screening to distinguish between interesting variants in a large number of samples, before undertaking further time consuming and costly analyses like MS, for example. This approach is particularly useful for the screening of two-peptide lantibiotics, whose inhibition phenotype requires the presence of both functional peptides.

Once the best expression system for each lichenicidin peptide was selected, the production and purification process was optimized. In **Chapter V**, purified lichenicidin was used to investigate its mode of action and toxicity.

The optimized purification procedure yielded 1 mg/L and 0.4 mg/L of highly pure Bli α and Bli β , respectively, which was lower than expected. Therefore, it is clear that further optimization is required to increase yields and improve possible losses that occur during purification with organic solvents. Purified lichenicidin was used to determine the MIC for a range of various Gram-negative and Gram-positive bacterial strains. Lichenicidin bioactivity spectrum was previously assessed by another group; however, the variant tested (VK21) presents a slightly different structure that might have impact on lichenicidin effectiveness. As expected, no inhibition was detected against the Gram-negative bacteria tested. As with most lantibiotics, lichenicidin has activity against all the Gram-positive strains tested, including methicillin sensitive and resistant *S. aureus*. When a strain has a resistance phenotype against a given antimicrobial, it has, in general, higher resistance to other antibiotics. And this was also the case of lichenicidin, whose MIC for MSSA was half (or less) than that for the MRSA.

Lichenicidin was also found to be a strong bactericide, completely eliminating MSSA in less than 3 h. At higher concentrations, this time is greatly reduced, suggesting that the mode of action of lichenicidin involves cell lysis. These results are in agreement with the proposed mechanism of action for the β -peptide of two-peptide lantibiotics, which results from the formation of pores in the cell membrane. Even so, this assay still needs to be

performed for the MRSA strain. Although we had tried to study the effect of lichenicidin on growing and already established *S. aureus* biofilms, the results were unsatisfactory as we had some difficulties establishing the biofilms. This study is in perspective considering the relevance of these structures in the clinical context.

Regarding assessment of lichenicidin toxicity, we performed human cell assays using erythrocytes from healthy donors and a fibroblast cell line. No toxic effect was observed under the tested conditions. Lichenicidin is non-hemolytic and does not affect the viability of human fibroblasts. Further tests should be performed using other cell lines, or even 3D-cell cultures, along with stability tests to assess an approximate half-life of lichenicidin in the body. However, this is the first study done with two-peptide lantibiotics and, therefore, is a good starting point for a possible application of lichenicidin, highlighting the potential of this class of natural compounds as promising for therapeutic application in human medicine.

As mentioned in Chapter I, the low ability to induce resistance phenotypes is another important feature of lantibiotics which makes them good candidates for human health applications. This is due to its mode of action, which generally targets lipid II, a peptidoglycan cell wall precursor. A general mechanism of action has been proposed for the two-peptide lantibiotics: the α -peptide binds to the peptidoglycan precursor lipid II, inhibiting cell wall biosynthesis and recruiting the β -peptide that inserts within the membrane leading to pore formation. **Chapter VI** presents the results of the lichenicidin studies, which corroborate the model already proposed for other lantibiotics. The studies were performed with *S. aureus*, using the conditions set out in chapter V, and using membrane models, which include the putative target molecule, lipid II, in its composition. The tests performed aimed to evaluate membrane changes after incubation with lichenicidin by i) the ability of the peptides to alter surface charges and membrane potential, and /or ii) to affect membrane integrity.

It was observed that the cationic lichenicidin increases the surface charge of *S. aureus* cells, with both peptides contributing equally to these changes. This increase in charge tends to stabilize at higher concentrations of lichenicidin, but unsurprisingly, complete electroneutralization has not been achieved. Additionally, lichenicidin does not substantially affect the transmembrane potential, which also indicates that its mode of action does not rely on membrane depolarization. Bli β appears to have a higher affinity for the bacterial cell membrane than Bli α , which may be related to the pore formation role that is proposed for β peptides. Furthermore, lichenicidin was shown to have a higher affinity

for bacterial membranes than Bli β . Although Bli α does not have a high affinity to the membrane, this affinity increases after binding of Bli β . On the other hand, Bli β has higher affinity to the transmembrane environment, the presence of Bli α provides a docking site, to assist binding and insertion of Bli β , and providing additional binding stability. The effect of lichenicidin can be clearly visualized by AFM imaging. The integrity of *S. aureus* cells is severely affected at high concentrations and/or long incubation periods, leading to the loss of the typical cell morphology and ultimately to the leakage of internal content and cell death.

Opposite affinity results were observed when lichenicidin peptides were incubated with the LUV models. Bli α showed to have higher affinity for the LUV, particularly in the presence of lipid II. Bli β 's affinity is extremely low, but the presence of Bli α increases the speed of interaction, even if the leakage percentage does not increase. Bli β alone is capable of inducing leakage, which, once again is in agreement with the mode of action proposed for β -peptides.

In total, the results are in agreement with the general mechanism of action proposed for the two-peptide lantibiotics: Bli α interacts with lipid II at the surface of the target membranes, although with low affinity. This event can prevent peptidoglycan formation to some extent. However, this low affinity explains why the activity of Bli α alone requires much higher concentrations than those of Bli β or lichenicidin to exert an inhibitory effect even against highly sensitive strains. Bli α does have a significant role in recruiting and stabilizing Bli β . Even though Bli β can present a considerable level of bioactivity against some target strains, its effect is enhanced by anchoring with Bli α and lipid II. Our studies contributed to further clarify and confirm what is known about the activity of lantibiotics of two peptides, reinforcing that they share a common mechanism of action. Despite the differences in their sequences, the conserved regions seem to be enough to guarantee a similar effect. Also, these studies contribute to a better understanding of the exact mode of action of lantibiotics by providing tips for predicting possible mechanisms of lantibiotic resistance development and finding ways to suppress them.

Although much remains to be done to make know the true potential of lantibiotics, this work has helped to clarify several aspects, namely: structure-activity relationship, production, toxicity and mode of action, using lichenicidin, a two-peptide lantibiotic, as a case-study. Significant steps have also been taken to apply lantibiotic modifying enzymes, particularly in engineering proteolytic enzymes. As far as the two-peptide lantibiotic subgroup is concerned, only a few members have been described so far. The fact that

they are composed of two structural peptides, makes their research more laborious and time consuming. So far, there are a limited number of studies focused on these peptides.

The present study contributed with relevant information that can place lantibiotics of two peptides as strong alternatives for application in human health.

7.2 HIGHLIGHTS

The present work focused on the characterization of lantibiotics of two peptides, taking lichenicidin as a model compound. The results contributed to a better understanding of aspects of its structure, bioactivity, production and mode of action, as well as the regulation of the mechanisms of post-translational modifications and the flexibility and applicability of its modification enzymes. More specifically:

- the residue Glu26 is extremely important for lichenicidin bioactivity;
- in general, Ser and Thr residues can be inter-substituted without major impact on the structure or bioactivity of lichenicidin;
- the insertion of negatively charged residues in the lichenicidin sequence is detrimental to its bioactivity;
- *in vivo*, the LicA2 leader peptide, particularly the hexapeptide, is essential to direct LicM2 and LicT and LicP trimming activities;
- a negatively charged residue, preferably Glu, at position -1 (LicP cleavage site) is essential for the correct maturation of Bli β , *in vivo*;
- LicT and LicP trimming activity can be directed to cleave non-lantibiotic peptides, *in vivo*, if the leader sequence is fused upstream of the peptide of interest;
- in *E. coli*, lichenicidin production is favoured by the separate expression of each peptide and when promoters and RBS of the original producer *B. licheniformis* are optimized for *E. coli*;
- the immunity genes, *licFEGHI*, do not affect lichenicidin expression in *E. coli*;
- lichenicidin I89 is active against several Gram-positive bacteria including MSSA and MRSA, yet the MIC is at least two-fold higher against MRSA; no activity was detected against Gram-negative bacteria.
- at the MIC, lichenicidin I89 kills methicillin-sensitive *S. aureus* cells in less than 3 h; at higher concentrations rapid cell death occurs, which suggests a mode of action by pore formation as reported for β -peptides of other lantibiotics;
- lichenicidin is non-hemolytic nor cytotoxic towards human fibroblasts;

- each peptide has some activity against the most sensitive target strains although Bli β is more potent than Bli α ; the synergistic effect is always greater than the isolated activity of each peptide;
- Bli β has strong affinity for *S. aureus* membranes but not to LUV; however, when alone it can induce LUV leakage, indicating a pore formation mechanism;
- Bli α does not have high affinity to *S. aureus* membranes, but when combined with Bli β it strongly binds lipid II-containing LUV, contributing to speed up the leakage process;
- Bli α and Bli β stabilize each other when binding to *S. aureus* target cells: Bli α serves as anchoring molecule for Bli β , which then stabilizes lichenicidin interaction.

7.3 FUTURE PERSPECTIVES

At the end of this work, more questions arose than answers. Several aspects need to be further clarified and/or investigated, and include:

- refine the bioactivity screening methods for two-peptide lantibiotics, using well-defined peptide concentrations and optimizing their solubility and diffusion conditions;
- further investigate and characterize possible improved mutants obtained by SDM;
- improve the expression systems by combining the highest yielding mutants, using the chosen vectors and optimized culture conditions;
- further investigate the application of the modification enzymes to other relevant peptides.

In addition, and as regards the activity of lichenicidin, in view of its possible application in human medicine, it will be important to:

- understand the mechanism(s) underlying the susceptibility differences between sensitive and resistant strains of the same species;
- test lichenicidin on other cells lines and/or models, including, for instance, 3D-cell culture;
- merge the two peptides to originate a single nisin-like chimeric peptide, followed by activity testing, to understand if it is possible to combine both activities in one single peptide.

As with other works, the work developed and the results obtained opened perspectives for further studies. However, in addition to the studies carried out and the results that compose this document, other studies have lagged behind. Many of these studies are relevant and will be the basis for future studies, namely:

- Heterologous expression of other lantibiotics totally *in vivo*: during this work preliminary tests were performed regarding the expression of mersacidin and haloduracin in *E. coli*; technical issues impaired the insertion of the fully functional gene cluster within the expression vector. However, given that new cloning methods are being developed, it would be important to try again in the future;
- Evaluate the effect of lichenicidin on growing and established *S. aureus* biofilms: considering biofilms as a defence-mechanism developed by some clinically relevant strains, it is extremely important to understand whether lichenicidin is capable of killing or, at least, inhibit sessile bacterial cells. The establishment of the MSSA biofilms was not successful and therefore, for the sake of not spending peptide that would be required for further assays, these assays were not performed.
- Develop *K. rhizophila* mutants resistant to lichenicidin: as this strain is highly sensitive to lichenicidin, it is expected that relatively low amounts of lichenicidin would be required to induce lichenicidin resistant mutants. The main idea is to compare the genome/transcriptome of the resistant mutant with that of the original lantibiotic-sensitive strain. Other parameters, such as the evaluation of surface cell charge and transmembrane potential, or membrane lipid content, could also be analysed. At the end of this work, no mutant with acquired-resistance was obtained. Other bacterial species may also be tested.

The results presented and discussed here contributed to advances in the knowledge and understanding of lantibiotics, especially the two-peptide lantibiotics. However, and while lantibiotics are promising for application in human medicine, it is clear that there is still a long way to go. Yet, it has been shown that enzymes involved in the biosynthesis of these peptides can be used as viable "enzymatic tools" for industrial application.

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SUPPLEMENTARY INFORMATION

CHAPTER II – SUPPLEMENTARY INFORMATION

Supplementary Figures

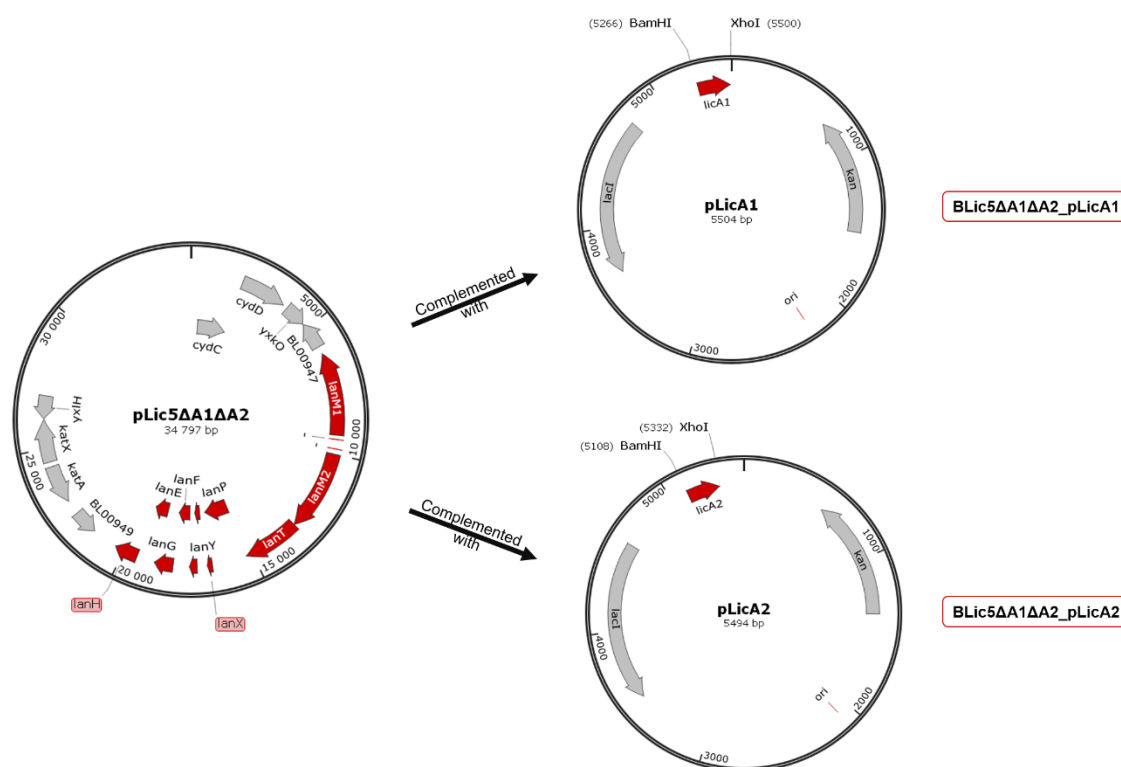


Figure S2.1. *trans* complementation system constructed for the expression of lichenicidin variants.

A two plasmid system was developed to express each peptide separately, and to allow an easier comparison of the variants. The first vector, pLic5ΔA1ΔA2, is a fosmid that contains the entire lichenicidin gene cluster except the structural genes, *licA1* and *licA2*. Each structural gene was independently amplified from *B. licheniformis* I89 genomic DNA and cloned into a pET-24a+ vector, as described by Caetano *et al.* (2011). Both fosmid and vector were transformed into *E. coli* BL21(DE3) Gold competent cells, originating BLic5ΔA1ΔA2_pLicA1 and BLic5ΔA1ΔA2_pLicA2 strains, producing Bliα and Bliβ, respectively. To generate lichenicidin variants, the vector containing the structural gene was used for the SDM protocol. The resulting plasmid was inserted into *E. coli* BL21(DE3) Gold along with the pLic5ΔA1ΔA2 fosmid and further expressed under the same conditions as the control.

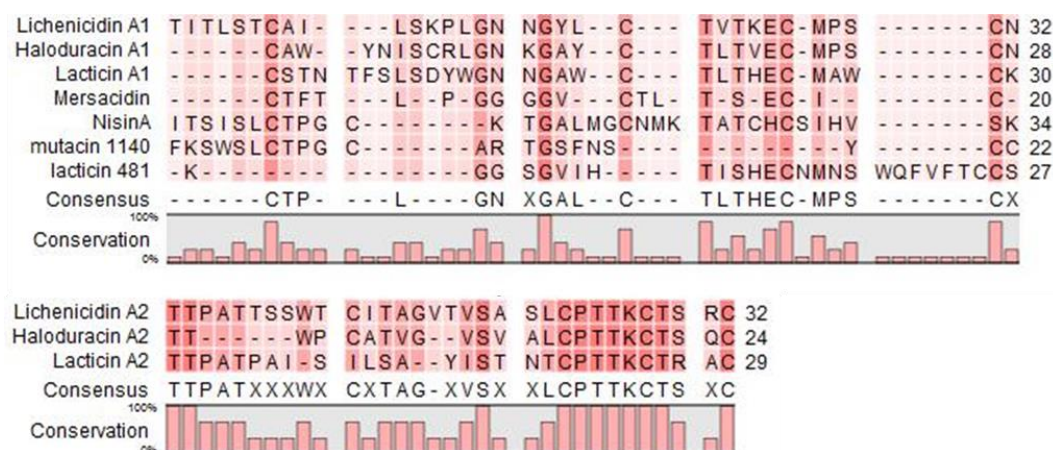


Figure S2.2. Alignment of lichenicidin amino acid sequence with similar lanthipeptides.

Alignments were performed for structural comparison purposes. Top: lichenicidin Bli α sequence compared with mersacidin-like peptides and other two-component peptides: haloduracin α , lacticin 4137 α , mersacidin, nisin A, mutacin 1140 and lacticin 418. Bottom: lichenicidin Bli β sequence compared with other β -peptides from two-component lanthipeptides: haloduracin β and lacticin 4137 β . Colored amino acids represent conserved residues.

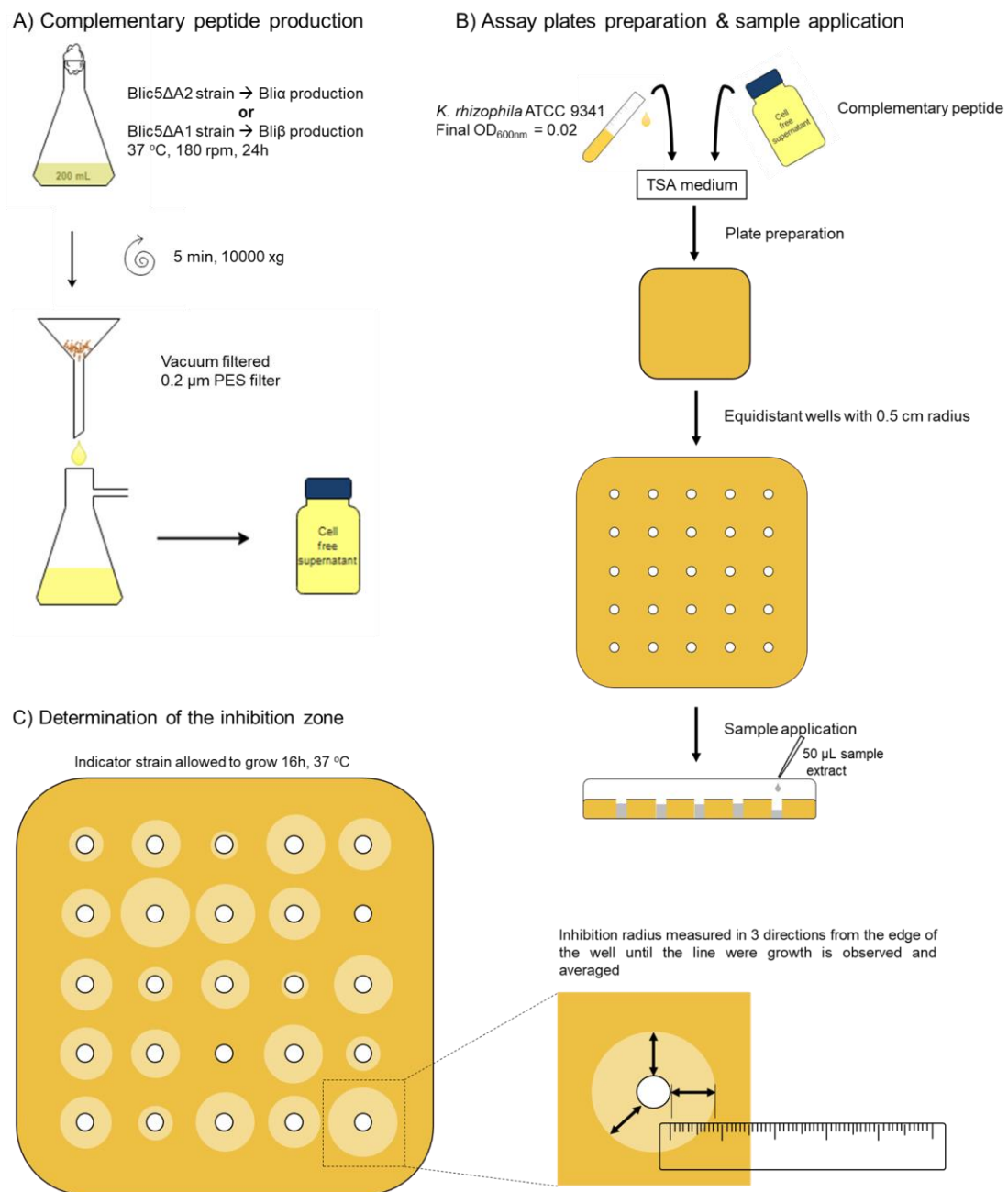


Figure S2.3. Antagonistic deferred bioassay procedure. A) Preparation of cell free supernatant containing the complementary peptide required for synergistic bioactivity of the mutant peptides. B) Preparation of bioassay agar plates containing the indicator strain as well as the complementary peptide; sample application. C) Analysis and measurement of the resulting inhibition zones.

Supplementary Information

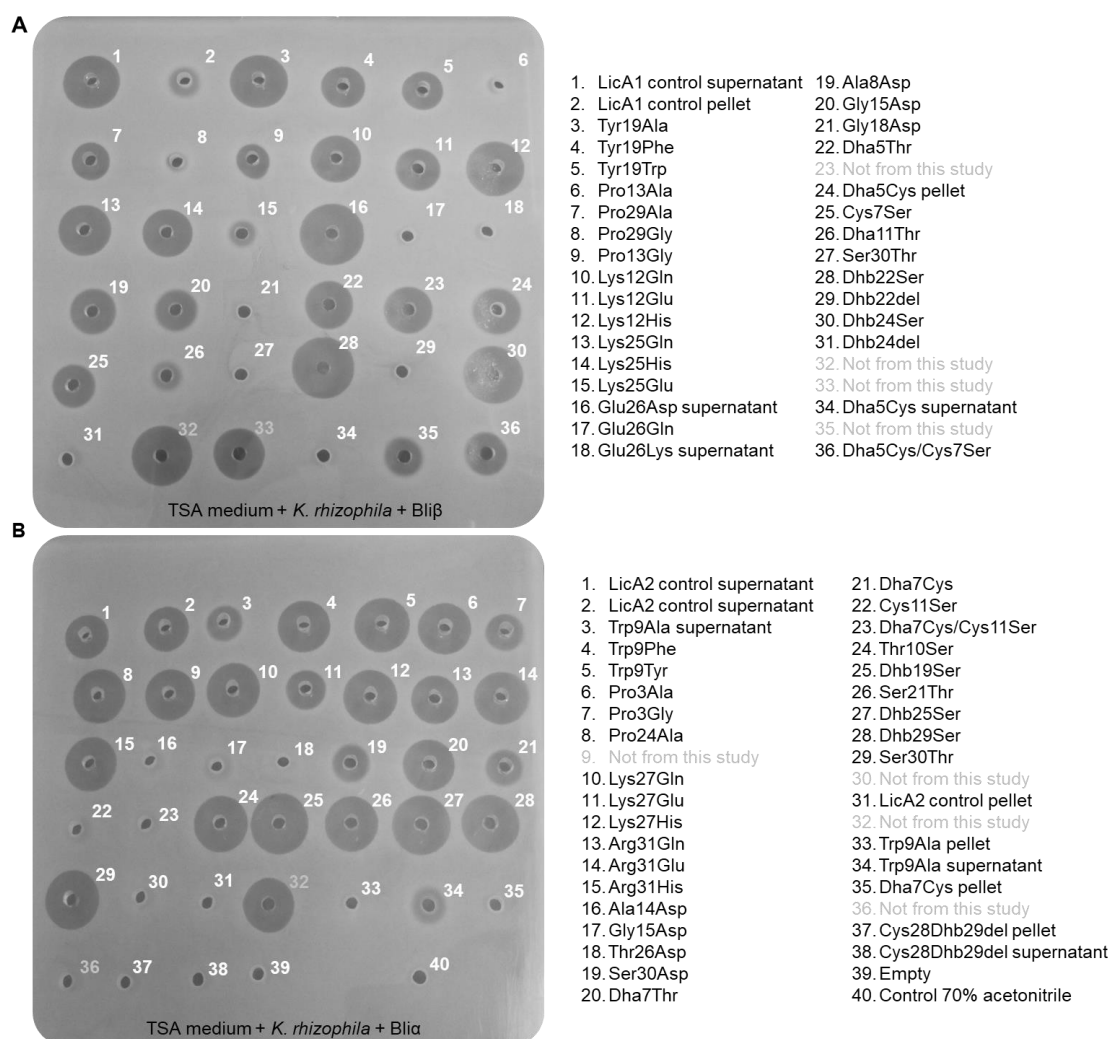


Figure S2.4. Antagonistic deferred bioassay plates of Bliα (A) and Bliβ (B) mutants. Plates were prepared as indicated in Material and Methods section. Extracts were tested in replicates against *K. rhizophila* ATCC 9341. Only one example plate (one replicate) is presented. Native peptides are on the right bottom corner of each plate. The inhibition radius was measured in three directions starting from the edge of the well until the line where no growth could be observed

Supplementary Tables

Table S2.1. List of primers, and respective annealing temperatures, used in the present work.

Primers were designed using PrimerX to generate lichenicidin variants. The mutated codons are underlined. ^ indicates a deletion position.

Mutation	Primer sequence (5' → 3')	Annealing temperature (°C)
BliA		
<i>Aromatic aa</i>		
Tyr19Trp	Fw: ACGGATGGCTGTGTACAG Rv: CTGTACACAGCCATCCGT	50
Tyr19Phe	Fw: ACGGATTTCTGTGTACAGTG Rv: CACTGTACACAGAAATCCGT	48
Tyr19Ala	Fw: GAAATAACGGAGCGCTGTGTACAG Rv: CTGTACACAGCGCTCCGTTATTTCC	53
<i>Proline</i>		
Pro13Ala	Fw: CCATCTTGAGCAAGGCGTTAGGAAATAACG Rv: CGTTATTTTCCTAACGCCTTGCTCAAGATGG	54
Pro13Gly	Fw: CTTGAGCAAGGGCTTAGGAAAT Rv: ATTTTCCTAAGCCCTTGCTCAAG	56
Pro29Ala	Fw: GTGACAAAAGAATGCATGGCGAGCTGTAAGTTCTCG Rv: CGAGAACTTAGTTACAGCTCGCCATGCATTCTTTTGTCAC	56
Pro29Gly	Fw: GAATGCATGGGCAGCTGTAAC Rv: GTTACAGCTGCCCATGCATTC	60
<i>Charged aa</i>		
Lys12His	Fw: ATCTTGAGCCATCCGTTAGGA Rv: TCCTAACGGATGGCTCAAGAT	50
Lys12Glu	Fw: ATCTTGAGCGAACCCTTAGGA Rv: TCCTAACGGTTGCTCAAGAT	50
Lys12Gln	Fw: TCTTGAGCCAGCCGTTA Rv: TAACGGCTGGCTCAAGA	48
Lys25His	Fw: ACAGTGACACATGAATGCATGC Rv: GCATGCATTGATGTCACTGT	51
Lys25Glu	Fw: CAGTGACAGAAGAATGC Rv: GCATTCTTCTGTCACTG	42
Lys25Gln	Fw: ACAGTGACACAGGAATGCATG Rv: CATGCATTCCTGTGTCACTGT	50
Glu26Asp	Fw: GACAAAAGATTGCATGCC Rv: GGCATGCAATCTTTTGTC	44
Glu26Lys	Fw: GTGACAAAAAATGCATGC Rv: GCATGCATTITTTTGTCAC	44
Glu26Gln	Fw: GTGACAAAACAGTGCATGCCA Rv: TGGCATGCACTGTTTGTGTCAC	52
Ala8Asp	Fw: GCACTTGTGATATCTTGAGC Rv: GCTCAAGATATCACAAAGTGC	46
Gly15Asp	Fw: AAGCCGTTAGATAATAACGGAT Rv: ATCCGTTATTATCTAACGGCTT	46
Gly18Asp	Fw: GGAAATAACGACTACCTGTGT Rv: ACACAGGTAGTCGTTATTTCC	46
<i>Ser/Thr/Cys</i>		
Ser5Thr	Fw: ACGCTCACCACTTGTGC Rv: GCACAAGTGGTGAGCGT	50

Supplementary Information

Mutation	Primer sequence (5' → 3')	Annealing temperature (°C)
Ser5Cys	Fw: ACGCTCTGCACTTGTGC Rv: GCACAAGTGCAGAGCGT	52
Cys7Ser	Fw: AGCACTAGCGCCATCTTGAG RV: CTCAAGATGGCGCTAGTGCT	52
Ser5Cys-Cys7Ser	Fw: CAATCACGCTCTGCACCAGCGCCATCTTGAG Rv: CTCAAGATGGCGCTGGTGCAGAGCGTGATTG	60
Ser11Thr	Fw: ATCTTGACCAAGCCGTT Rv: AACGGCTTGGTCAAGAT	46
Thr22Ser	Fw: GGATACCTGTGTAGCGTGACAAAAGAATG Rv: CATTCTTTTGTACACGCTACACAGGTATCC	54
Thr24Ser	Fw: AGTGAGCAAAGAATGCATGC Rv: GCATGCATTCTTTGCTCACT	50
Thr22del	Fw: CGGATACCTGT^GTGTGACAAAAGAATG Rv: CATTCTTTTGTACAC^ACAGGTATCCG	42
Thr24del	Fw: TACAGTGA^AAGAATGCAT Rv: ATGCATTCTT^TCACTGTA	42
Ser30Thr	Fw: CATGCCAACCTGTA ACT Rv: AGTTACAGGTTGGCATG	44
Bliβ		
<i>Aromatic aa</i>		
Trp9Tyr	Fw: CAACCTCTTCTTATACTTGCATCAC Rv: GTGATGCAAGTATAAGAAGAGGTTG	48
Trp9Phe	Fw: CAACCTCTTCTTTTACTTGCATCAC Rv: GTGATGCAAGTAAAAGAAGAGGTTG	50
Trp9Ala	Fw: CAACCTCTTCTGCGACTTGCATC Rv: GATGCAAGTCGCAGAAGAGGTTG	60
<i>Proline</i>		
Pro3Ala	Fw: GTCAATCCTGAAACA ACTGCGGCTACAACCTCTTCTTG Rv: CAAGAAGAGGTTGTAGCCGCA GTGTTTCAGGATTGAC	62
Pro3Gly	Fw: GAAACA ACTGGCGCTACAACC Rv: GGTTGTAGCGCCAGTTGTTTC	56
Pro24Ala	Fw: GGTTTCTGCTTCATTATGCGCGACA ACTAAGTGTACAAGC Rv: GCTTGTACACTTAGTTGTGCGCGCATAATGAAGCAGAAACC	62
Pro24Gly	Fw: CTTCATTATGCGGCACA ACTAAGTG Rv: CACTTAGTTGTGCGCGCATAATGAAG	60
<i>Charged aa</i>		
Lys27His	Fw: CCAACA ACTCATTGTACAAGCC Rv: GGCTTGTACAATGAGTTGTTGG	50
Lys27Glu	Fw: CCAACA ACTGAATGTACAAGCC Rv: GGCTTGTACATTCAGTTGTTGG	50
Lys27Gln	Fw: CCAACA ACTCAGTGTACA Rv: TGTACACTGAGTTGTTGG	44
Arg31His	Fw: GTACAAGCCATTGCTAGGG Rv: CCCTAGCAATGGCTTGCTAC	48
Arg31Glu	Fw: GTACAAGCGAATGCTAGGG Rv: CCCTAGCATTGCTTGCTAC	48
Arg31Gln	Fw: TACAAGCCAGTGCTAGGG Rv: CCCTAGCACTGGCTTGTA	50

Mutation	Primer sequence (5' → 3')	Annealing temperature (°C)
Ala14Asp	Fw: GCATCACAGATGGTGTAAACG Rv: CGTTACACCATCTGTGATGC	48
Gly15Asp	Fw: CACAGCCGATGTAACGGT Rv: ACCGTTACATCGGCTGTG	50
Thr26Asp	Fw: TGCCCAACAGATAAGTGTAC Rv: GTACACTTATCTGTTGGGCA	48
Ser30Asp	Fw: AAGTGTACAGATCGATGCTAGG Rv: CCTAGCATCGATCTGTACACTT	48
<i>Ser/Thr/Cys</i>		
Thr10Ser	Fw: CTTGGAGCTGCATCACAG Rv: CTGTGATGCAGCTCCAAG	48
Ser21Thr	Fw: CTGCTACCTTATGCCCAACAA Rv: TTGTTGGGCATAAGGTAGCAG	50
Ser30Thr	Fw: GTACAACCCGATGCTAG Rv: CTAGCATCGGGTTGTAC	44
Ser7Thr	Fw: CAACCACCTCTTGGACTTGC Rv: GCAAGTCCAAGAGGTGGTTG	50
Ser7Cys	Fw: ACAACCTGTTCTTGGAC Rv: GTCCAAGAACAGGTTGT	44
Cys11Ser	Fw: CTTGGACTAGCATCACA Rv: TGTGATGCTAGTCCAAG	44
Ser19Thr	Fw: ACGGTTACCGCTTCATTATGC Rv: GCATAATGAAGCGGTAACCGT	50
Thr25Ser	Fw: GCCCAAGCACTAAGTGTAC Rv: GTACACTTAGTGCTTGGGC	48
Thr29Ser	Fw: AAGTGTAGCAGCCGATGC Rv: GCATCGGCTGCTACACTT	50
Cys28Thr29del	Fw: CAACAACCTAAG [^] AGCCGATGCTAG Rv: CTAGCATCGGCT [^] CTTAGTTGTTG	50

Supplementary Information

Table S2.2. Predicted and observed masses of the mutants obtained during this study. “n” indicates the charge state of the peptide. The observed masses are also indicated in **bold** for an easier identification of the ionization state. Extracts were run in an ESI-Triple-Quadrupole-MS.

Mutant	Predicted mass				Observed mass
	n	H+	Na+	K+	
Bliα					
Bliα	1	3249.538	3271.521	3287.495	1625.3 1636.3 1083.9 1091.2
	2	1625.273	1636.264	1644.251	
	3	1083.852	1091.179	1096.504	
Aromatic aa					
Tyr19Trp	1	3272.555	3294.537	3310.511	1091.5
	2	1636.781	1647.772	1655.759	
	3	1091.524	1098.851	1104.176	
Tyr19Phe	1	3233.544	3255.526	3271.500	1078.5
	2	1617.276	1628.267	1636.254	
	3	1078.520	1085.847	1091.172	
Tyr19Ala	1	3157.513	3179.495	3195.469	1053.2
	2	1579.260	1590.251	1598.238	
	3	1053.176	1060.503	1065.828	
Proline					
Pro13Ala	1	3223.523	3245.505	3261.479	1075.2
	2	1612.266	1623.257	1631.244	
	3	1075.180	1082.507	1087.832	
Pro13Gly	1	3209.508	3231.490	3247.464	1070.5
	2	1605.258	1616.249	1624.236	
	3	1070.508	1077.835	1083.160	
Pro29Ala	1	3223.523	3245.505	3261.479	1075.2
	2	1612.266	1623.257	1631.244	
	3	1075.180	1082.507	1087.832	
Pro29Gly	1	3209.508	3231.48963	3247.464	1070.5
	2	1605.258	1616.24873	1624.236	
	3	1070.508	1077.83509	1083.160	
Charged aa					
Lys12His	1	3258.503	3280.485	3296.459	1086.8
	2	1629.755	1640.746	1648.733	
	3	1086.840	1094.167	1099.491	
Lys12Glu	1	3250.487	3272.469	3288.443	1084.2
	2	1625.747	1636.738	1644.725	
	3	1084.167	1091.495	1096.819	
Lys12Gln	1	3249.503	3271.485	3287.458	1083.8
	2	1625.255	1636.246	1644.233	
	3	1083.839	1091.167	1096.491	
Lys25His	1	3258.503	3280.485	3296.459	1086.8
	2	1629.755	1640.746	1648.733	
	3	1086.840	1094.167	1099.491	
Lys25Glu	1	3250.487	3272.469	3288.443	1084.2
	2	1625.747	1636.738	1644.725	
	3	1084.167	1091.495	1096.819	

Mutant	Predicted mass				Observed mass
	n	H+	Na+	K+	
Lys25Gln	1	3249.503	3271.485	3287.458	1083.8
	2	1625.255	1636.246	1644.233	
	3	1083.839	1091.167	1096.491	
Glu26Asp	1	3235.523	3257.505	3273.479	1079.2
	2	1618.266	1629.257	1637.244	
	3	1079.180	1086.507	1091.832	
Glu26Lys	1	3248.591	3270.573	3286.547	1083.5
	2	1624.800	1635.791	1643.778	
	3	1083.536	1090.863	1096.188	
Glu26Gln	1	3248.555	3270.537	3286.511	1083.5
	2	1624.781	1635.772	1643.759	
	3	1083.524	1090.851	1096.176	
Ala8Asp	1	3293.529	3315.511	3331.485	1098.5
	2	1647.268	1658.259	1666.246	
	3	1098.515	1105.842	1111.167	
Gly15Asp	1	3307.544	3329.526	3345.500	1103.2
	2	1654.276	1665.267	1673.254	
	3	1103.187	1110.514	1115.839	
Gly18Asp	1	3307.544	3329.526	3345.500	1103.2
	2	1654.276	1665.267	1673.254	
	3	1103.187	1110.514	1115.839	
Ser/Thr/Cys					
Dha5Thr (if Thr)	1	3281.565	3303.547	3319.521	--
	2	1641.287	1652.278	1660.264	
	3	1094.527	1101.854	1107.179	
Dha5Thr (if Dhb)	1	3263.555	3285.537	3301.511	1088.5
	2	1632.281	1643.272	1651.259	
	3	1088.523	1095.851	1101.175	
Dha5Cys (only pellet extract)	1	3283.527	3305.509	3321.483	1095.2
	2	1642.267	1653.259	1661.245	
	3	1095.181	1102.508	1107.833	
Cys7Ser (if Ser)	1	3233.562	3255.543	3271.518	--
	2	1617.285	1628.276	1636.263	
	3	1078.526	1085.853	1091.178	
Cys7Ser (if Dha)	1	3215.551	3237.533	3253.507	1072.5
	2	1608.280	1619.271	1627.258	
	3	1072.522	1079.850	1085.174	
Cys7Ser (if both Ser)	1	3251.572	3273.554	3289.528	--
	2	1626.290	1637.281	1645.268	
	3	1084.529	1091.857	1097.181	
Dha5Cys-Cys7Ser (if Ser)	1	3267.550	3289.532	3305.505	--
	2	1634.279	1645.270	1653.257	
	3	1089.855	1097.182	1102.507	
Dha5Cys-Cys7Ser (if Dha)	1	3249.539	3271.521	3287.495	1625.3 1083.9
	2	1625.273	1636.264	1644.251	
	3	1083.852	1091.179	1096.504	
Dha11Thr (if Thr)	1	3281.565	3303.547	3319.521	1641.3 1652.3 1094.5 1101.9
	2	1641.287	1652.278	1660.264	
	3	1094.527	1101.854	1107.179	

Supplementary Information

Mutant	Predicted mass				Observed mass
	n	H+	Na+	K+	
Dha11Thr (if Dhb)	1	3263.555	3285.537	3301.511	1632.3 1643.3 1088.5 1095.9 1101.2
	2	1632.281	1643.272	1651.259	
	3	1088.523	1095.851	1101.175	
Dhb22Ser (if Ser)	1	3253.533	3275.516	3291.490	1627.3 1085.2 1092.5
	2	1627.271	1638.262	1646.249	
	3	1085.183	1092.511	1097.835	
Dhb22Ser (if Dha)	1	3235.523	3257.505	3273.479	1618.3 1629.3 1079.2 1086.5
	2	1618.266	1629.257	1637.244	
	3	1079.180	1086.507	1091.832	
Dhb22del	1	A wide range of molecular masses do occur; several masses scanned; no related mass identified			- -
	2				
	3				
Dhb24Ser (if Ser)	1	3253.534	3275.516	3291.490	1627.3 1638.3 1085.2 1092.5
	2	1627.271	1638.262	1646.249	
	3	1085.183	1092.511	1097.835	
Dhb24Ser (if Dha)	1	3235.523	3257.505	3273.479	1618.3 1629.3 1079.2 1086.5
	2	1618.266	1629.257	1637.244	
	3	1079.180	1086.507	1091.832	
Dhb24del	1	A wide range of masses do occur; several masses scanned; no related mass identified			- -
	2				
	3				
Ser30Thr (if Thr)	1	3263.555	3285.537	3301.511	- -
	2	1632.281	1643.272	1651.259	
	3	1088.523	1095.851	1101.175	
Ser30Thr (if Dhb)	1	3245.544	3267.526	3283.500	- -
	2	1623.276	1634.267	1642.254	
	3	1082.520	1089.847	1095.172	
Bliß					
Bliß	1	3019.384	3041.366	3057.340	1510.2 1521.2 1007.1 1014.5
	2	1510.196	1521.187	1529.174	
	3	1007.133	1014.460	1019.785	
Aromatic aa					
Trp9Tyr	1	2996.368	3018.350	3034.324	1498.7 999.5
	2	1498.688	1509.679	1517.666	
	3	999.461	1006.788	1012.113	
Trp9Phe	1	2980.373	3002.355	3018.329	1490.7 994.1
	2	1490.690	1501.681	1509.668	
	3	994.130	1001.457	1006.782	

Mutant	Predicted mass				Observed mass
	n	H+	Na+	K+	
Trp9Ala	1	2904.342	2926.323	2942.298	1452.7 968.8
	2	1452.675	1463.666	1471.653	
	3	968.786	976.113	981.438	
Proline					
Pro3Ala	1	2993.368	3015.350	3031.324	1497.2 998.5
	2	1497.188	1508.179	1516.166	
	3	998.462	1005.789	1011.113	
Pro3Gly	1	2979.352	3001.334	3017.308	1490.2 993.8
	2	1490.180	1501.171	1509.158	
	3	993.789	1001.117	1006.441	
Pro24Ala	1	2993.368	3015.350	3031.324	1497.2 998.5
	2	1497.188	1508.179	1516.166	
	3	998.461	1005.789	1011.113	
Pro24Gly	1	2979.352	3001.334	3017.308	1490.2 993.8
	2	1490.180	1501.171	1509.158	
	3	993.789	1001.117	1006.441	
Charged aa					
Lys27His	1	3028.348	3050.330	3066.304	1514.7 1010.1
	2	1514.678	1525.669	1533.656	
	3	1010.121	1017.448	1022.773	
Lys27Glu	1	3020.331	3042.313	3058.287	1510.7 1007.4
	2	1510.670	1521.661	1529.648	
	3	1007.449	1014.776	1020.101	
Lys27Gln	1	3019.347	3041.329	3057.303	1510.2 1007.1
	2	1510.178	1521.169	1529.156	
	3	1007.121	1014.448	1019.773	
Arg31His	1	3000.342	3022.324	3038.297	1500.7 1000.8
	2	1500.675	1511.666	1519.653	
	3	1000.786	1008.113	1013.438	
Arg31Glu	1	2992.325	3014.307	3030.281	1496.7 998.1
	2	1496.667	1507.658	1515.645	
	3	998.114	1005.441	1010.766	
Arg31Gln	1	2991.341	3013.323	3029.297	1496.2 997.8
	2	1496.175	1507.166	1515.152	
	3	997.786	1005.113	1010.438	
Ala14Asp	1	3063.374	3085.356	3101.330	1021.8
	2	1532.191	1543.182	1551.169	
	3	1021.796	1029.124	1034.448	
Gly15Asp	1	3077.389	3099.371	3115.345	1539.2 1026.5
	2	1539.199	1550.190	1558.176	
	3	1026.468	1033.796	1039.120	
Dhb26Asp	1	3051.373	3073.356	3089.330	- -
	2	1526.191	1537.182	1545.169	
	3	1017.796	1025.124	1030.448	
Ser30Asp	1	3047.379	3069.361	3085.335	1524.2 1016.5
	2	1524.193	1535.184	1543.171	
	3	1016.465	1023.792	1029.117	
Ser/Thr/Cys					
Thr10Ser (if Ser)	1	3005.368	3027.350	3043.324	1503.2 1514.2

Supplementary Information

Mutant	Predicted mass				Observed mass
	n	H+	Na+	K+	
	2	1503.188	1514.179	1522.166	1002.5 1009.8 1015.1
	3	1002.461	1009.789	1015.113	
Thr10Ser (if Dha)	1	2987.356	3009.340	3025.313	--
	2	1494.182	1505.174	1513.161	
	3	996.458	1003.785	1009.110	
Ser21Thr (if Thr)	1	3033.399	3055.381	3071.355	1517.2 1528.2 1011.8 1019.1 1024.5
	2	1517.204	1528.195	1536.182	
	3	1011.805	1019.132	1024.457	
Ser21Thr (if Dhb)	1	3015.389	3037.371	3053.345	--
	2	1508.198	1519.189	1527.176	
	3	1005.802	1013.129	1018.453	
Ser30Thr (if Thr)	1	3033.399	3055.381	3071.355	1517.2 1528.2 1011.8 1019.1 1024.5
	2	1517.204	1528.195	1536.182	
	3	1011.805	1019.132	1024.457	
Ser30Thr (if Dhb)	1	3015.389	3037.371	3053.345	--
	2	1508.198	1519.189	1527.177	
	3	1005.802	1013.129	1018.453	
Dha7Thr (if Thr)	1	3051.410	3073.392	3089.366	--
	2	1526.209	1537.200	1545.187	
	3	1017.809	1025.136	1030.461	
Dha7Thr (if Dhb)	1	3033.399	3055.381	3071.355	1517.2 1528.2 1011.8 1019.1 1024.5
	2	1517.204	1528.195	1536.182	
	3	1011.805	1019.132	1024.457	
Dha7Cys	1	3053.372	3075.353	3091.327	--
	2	1527.190	1538.181	1546.168	
	3	1018.462	1025.790	1031.114	
Cys11Ser (if Ser)	1	3003.407	3025.389	3041.363	--
	2	1502.207	1513.198	1521.185	
	3	1001.807	1009.135	1014.459	
Cys11Ser (if Dha)	1	2985.396	3007.378	3023.352	--
	2	1493.202	1504.193	1512.180	
	3	995.804	1003.131	1008.456	
Cys11Ser (if both Ser)	1	3021.417	3043.399	3059.373	--
	2	1511.213	1522.204	1530.190	
	3	1007.811	1015.138	1020.463	
Dha7Cys-Cys11Ser (if Ser)	1	3037.394	3059.376	3075.350	1519.2 1530.2

Mutant	Predicted mass				Observed mass
	n	H+	Na+	K+	
	2	1519.201	1530.192	1538.179	1013.1 1020.5 1025.8
	3	1013.137	1020.464	1025.789	
Dha7Cys-Cys11Ser (if Dha)	1	3019.384	3041.366	3057.340	--
	2	1510.196	1521.187	1529.174	
	3	1007.133	1014.460	1019.785	
Dha19Thr (if Thr)	1	3051.410	3073.392	3089.366	--
	2	1526.209	1537.200	1545.187	
	3	1017.809	1025.136	1030.461	
Dha19Thr (if Dhb)	1	3033.399	3055.382	3071.355	1517.2 1528.2 1536.2 1011.8 1019.1 1024.5
	2	1517.204	1528.195	1536.182	
	3	1011.805	1019.132	1024.457	
Dhb25Ser (if Ser)	1	3023.379	3045.361	3061.335	1512.2 1008.5 1015.8 1021.1
	2	1512.193	1523.184	1531.171	
	3	1008.465	1015.792	1021.117	
Dhb25Ser (if Dha)	1	3005.368	3027.350	3043.324	1503.2 1514.2 1522.2 1002.5 1009.8 1015.1
	2	1503.188	1514.179	1522.166	
	3	1002.461	1009.789	1015.113	
Dhb29Ser	1	3023.379	3045.361	3061.335	1512.2 1008.5 1015.8 1021.1
	2	1512.193	1523.184	1531.171	
	3	1008.465	1015.792	1021.117	
Dhb29Ser	1	3005.368	3027.350	3043.324	1503.2 1514.2 1002.5 1009.8 1015.1
	2	1503.188	1514.179	1522.166	
	3	1002.461	1009.789	1015.113	
Cys28Dhb29del	1	A wide range of masses do occur; several masses scanned; no related mass identified			--
	2				
	3				

Table S2.3. MSMS fragmentation pattern for mutants in Ser, Thr and Cys residues, with potential impact on ring formation. The middle column contains a schematic representation of the mutated peptide, with the mutation site indicated with a grey circle; dehydrated Ser and Thr residues are shown in blue, the non-dehydrated are colored green; red letters represent Ser, Thr and Cys residues involved in ring formation; the first Thr is shown in purple and undergoes an additional modification (Obu) after dehydration. The last column shows a representative spectra from the fragmentation pattern; four different spectra were obtained for each variant, which varied in the fragmentation energy voltage used; the fragmentation pattern in the middle column results from the combination of fragments of all four spectra which may not all be represented in the presented spectrum.

Mutant	MSMS profile	Spectra
Blia		
licA1		
Dha5Thr (not dehydrated)		

Mutant	MSMS profile	Spectra
Dha5Thr (dehydrated)		
Dha5Cys		
Cys7Ser		

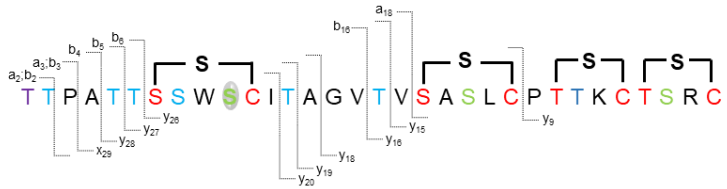
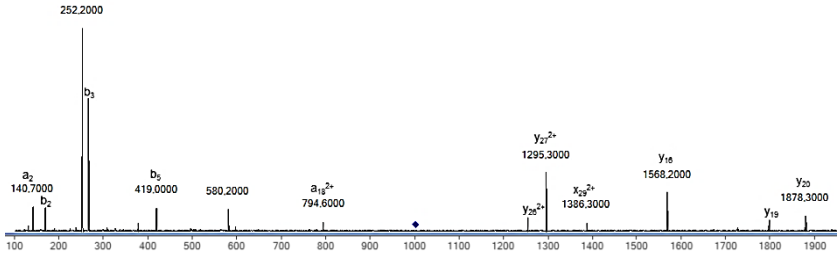
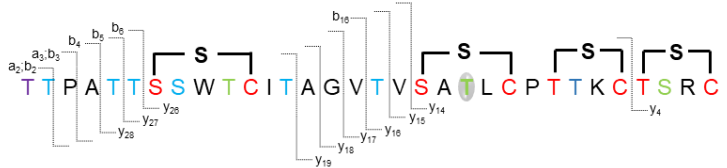
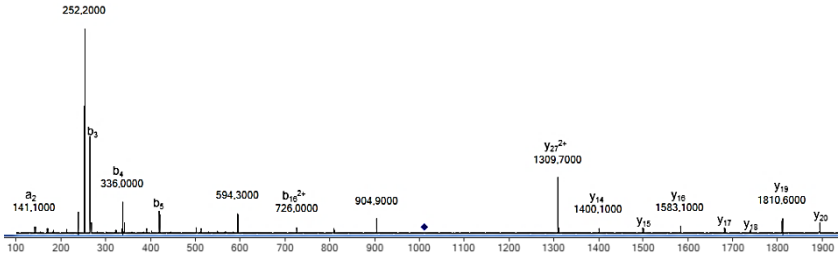
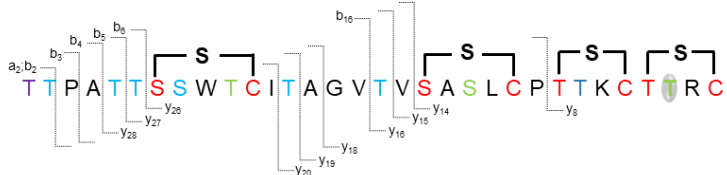
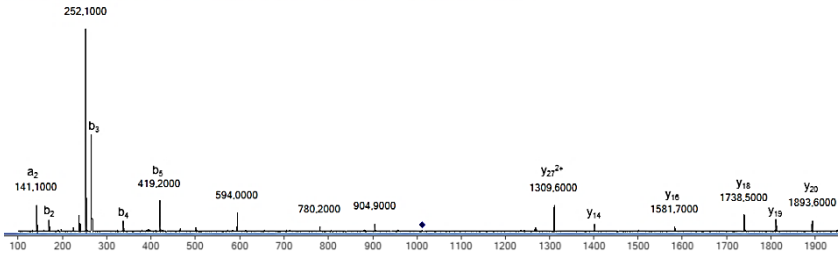
Supplementary Information

Mutant	MSMS profile	Spectra
Dha5Cys/Cys7Ser		
Dha11Thr (not dehydrated)		
Dha11Thr (dehydrated)		

Mutant	MSMS profile	Spectra
Dhb22Ser (not dehydrated)		
Dhb22Ser (dehydrated)		
Dhb24Ser (not dehydrated)		

Mutant	MSMS profile	Spectra
Dhb24Ser (dehydrated)		
Bliβ		
licA2		
Dha7Thr		

Mutant	MSMS profile	Spectra
Dha19Thr		
Dhb25Ser		
Dhb29Ser		

Mutant	MSMS profile	Spectra
Thr10Ser		
Ser21Thr		
Ser30Thr		

CHAPTER III – SUPPLEMENTARY INFORMATION

Supplementary Figures

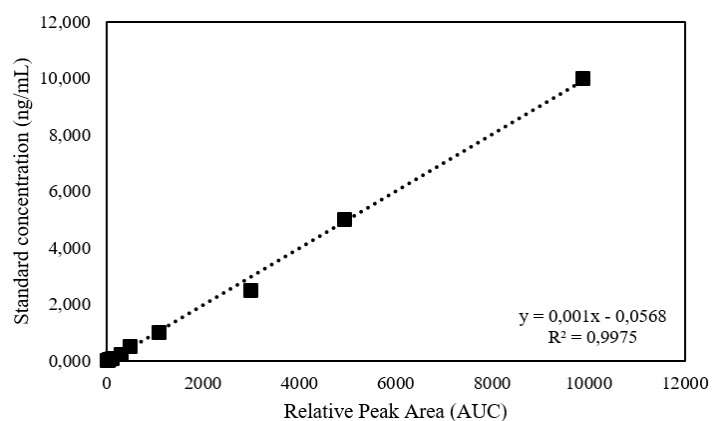


Figure S3.1. Lichenicidin standard calibration curve for Bli β . Liquid stocks of purified Bli β peptide were prepared in 70% ACN. Concentrations applied for MS measurements were 0.01, 0.025, 0.05, 0.1, 0.25 0.5, 1, 2.5, 5 and 10 ng mL⁻¹. The concentration was plotted against the resulting peak area in MRM measurements. Best fit line and coefficient of determination R^2 were calculated as indicated.

Supplementary Tables

Table S3.1. List of primers, and respective annealing temperatures, used in the SDM of Bli β hexapeptide and GG-motif. Primers were designed using PrimerX to generate lichenicidin variants. The mutated codons are underlined. ^ indicates a deletion position.

Mutation	Primer sequence (5' → 3')	Annealing temperature (°C)
Glu-1Ala	Fw: TGACGTCAATCCT <u>GCG</u> ACAACCTCTGCTA Rv: TAGCAGGAGTTGT <u>GCG</u> CAGGATTGACGTCA	55
Glu-1Asp	Fw: GTCAATCCTGATACAACCTCTGC Rv: GCAGGAGTTGTATCAGGATTGAC	50
Glu-1Gln	Fw: CGTCAATCCT <u>CAG</u> ACAACCTCTG Rv: CAGGAGTTGTCTGAGGATTGACG	52
Pro-2Ala	Fw: AAATGACGTCAAT <u>GCG</u> GAAACAACCTCTG Rv: CAGGAGTTGTTT <u>CCG</u> CATTGACGTCATT	55
Asn-3Ala	Fw: AGGAAATGACGT <u>C</u> GCGCTGAAACAACCTCC Rv: GGAGTTGTTTCAGGCGCGACGTCATTTCT	55
Asn-3His	Fw: AATGACGTCCATC <u>T</u> GAAAC Rv: GTTTCAGGATGACGTCATT	48
Asn-3Gln	Fw: GAAATGACGTCCAGCCTGAAACAAC Rv: GTTGTTTCAGGCTGGACGTCATTTCT	54
Val-4Ala	Fw: AGGAGGAAATGAC <u>G</u> CGAATCCTGAAACAA Rv: TTGTTTCAGGATT <u>GCG</u> TCATTTCTCTCT	55
Asp-5Ala	Fw: GGTAGGAGGAAATG <u>CCG</u> TCAATCCTGAAAC Rv: GTTTCAGGATTGACGGCATTTCCTCTACC	55
Asn-6Ala	Fw: CTTTGGTAGGAGGAG <u>GCG</u> ACGTCAATCCTG Rv: CAGGATTGACGT <u>CCG</u> CTCCTCTACCAAAG	55
Asn-6Gly	Fw: GGTAGGAGGAGGCGACGTCAAT Rv: ATTGACGT <u>CGC</u> CTCTCTCTACC	56
Asn-6Ser	Fw: GTAGGAGGAAGCGACGTCAAT Rv: ATTGACGT <u>CGC</u> TTCTCTCTAC	52
no_hexa	Fw: TGAAAGCTTTGGTAGGAGGAACAACCTCTGCTACAAC Rv: GTTGTAGCAGGAGTTGTTCTCTCTACCAAAGCTTTCA	55
HexaAla (two sequential PCR reactions)	Fw1: CTTTGGTAGGAGGAGCAGCAGCCAATCCTGAAACAAC Rv1: GTTGTTTCAGGATTGGCTGCTGCTCCTCTACCAAAG Fw2: GTAGGAGGAGCAGCAGCCGCAGCAGCAACAACCTCTGCTACAAC Rv2: GTTGTAGCAGGAGTTGTTGCTGCTGCGGCTGCTGCTCTCTCTAC	55
2*hexa	Fw: CGTCAATCCTGAAAACGACGTAAACCCAGAAACAACCTCTGCTAC Rv: GTAGCAGGAGTTGTTTCTGGGTTTACGTCGTTTTTCAGGATTGACG	55
5Ala_Glu-1	Fw: GCCGCTGCTGAAACAACCTCC Rv: GGAGTTGTTTCAGCAGCGGC	62
Glu-1Thr1- AlaAla	Fw: GAAATGACGTCAATCCTGCAGCAACTCCTGCTACAACC Rv: GGTTGTAGCAGGAGTTGCTGCAGGATTGACGTCATTTCT	55
Thr1Ala	Fw: GACGTCAATCCTGAAGCAACTCCTGCTACAAC Rv: GTTGTAGCAGGAGTTGCTTCAGGATTGACGTC	55 [54]
GG-AA	Fw: GGAATTGAAAGCTTTGGTAGCAGCAATGACGTCAATCCTGAAA Rv: TTTCAGGATTGACGTCATTTGCTGCTACCAAAGCTTTCAATTCC	55
GG-GA	Fw: TTTGGTAGGAGCGAATGACGTCA Rv: TGACGTCATTGCTCTCTACCAA	53
GG-GS	Fw: CTTTGGTAGGATCAAATGACGTC Rv: GACGTCATTTGATCCTACCAAAG	50

Table S3.2. List of primers used for amplification of the chimeric genes. The annealing temperatures, size of the amplicons and restriction sites (bold) of each primer are also indicated. In the last column the restriction enzymes employed in each case is indicated; when shown in brackets, the restriction site is encoded within the amplicon and not in the primer itself.

Amplicon	Primer sequence (5' → 3')	Annealing temperature (°C)	Size (bp)	Restriction enzyme
licT.P	Fw: AGGTGAC CCATGG GCTTGTTTTTTCATAAGACACCG Rv: TACA GTCGACT CACTCCTTGTTTCATCATTTTC	51	3623	Sall NcoI
Amy	Fw: AGGGTTTTCCAGTCACGAC Rv: CATATGCTCGAGATAGGTATT	48	231	(NdeI) (XhoI)
Luna	Fw: CAGGAAACAGCTATGAC Rv: CAGGAAACAGCTATGAC	45	249	(NdeI) (XhoI)
InsA	Fw: ACCAGCTGCCTCGAGCATATG Rv: CAGGAAACAGCTATGAC	50	183	(NdeI) (XhoI)
Soma	Fw: AGCATTGCCTCGAGCATATG Rv: CATATGCTCGAGGCAGCTGGT	58	162	(NdeI) (XhoI)
PlnE	Fw: AGGGTTTTCCAGTCACGAC Rv: CATATGCTCGAGGCGAATGCT	53	219	(NdeI) (XhoI)
Epi	Fw: CATATGAAAACCATGAAAAA Rv: TGCTCGAGACAACAATAACT	50	198	(NdeI) (XhoI)
Mrs	Fw: AACAGGTAGTTTTAACAGTTATTGTT Rv: CTCGAGACAAATACATTCAG	50	192	(NdeI) (XhoI)

Supplementary Information

Table S3.3. Predicted peptide molecular masses of the chimeric genes obtained in this study. “n” indicates the charge state of the peptide. The observed mass peaks are also indicated in **bold** to facilitate identification of the ionization state. The extracts were run in an ESI-qTOF mass spectrometer and the masses, except for the monoisotopic, correspond to the H⁺ adducts.

Amylin			
N	A2_Leader+Amylin	A2_hexa+Amylin	Amylin
Monoisotopic mass	9278.43145	5737.66755	5069.39099
1	9279.439275	5738.675375	5070.398815
2	4640.22355	2869.8416	2535.70332
3	3093.818308	1913.563675	1690.804822
4	2320.615688	1435.424713	1268.355573
5	1856.694115	1148.541335	1014.886023
6	1547.413067	957.28575	845.9063233
7	1326.498032	820.6746179	725.2065379
8	1160.811756	718.2162688	634.6816988
9	1031.944653	638.5264417	564.2734906
10	928.85097	574.77458	507.946924
11	844.5015932		
12	774.2104458		
13	714.7333212		
14	663.7529286		
15	619.5699217		
16	580.9097906		
17	546.7979103		
18	516.4762389		
19	489.3463224		
20	464.9293975		
Lunasin			
N	A2_Leader+Lunasin	A2_hexa+Lunasin	Lunasin
Monoisotopic mass	10341.79392	6801.03002	6132.75346
1	10342.80175	6802.037845	6133.761285
2	5171.904785	3401.522835	3067.384555
3	3448.272465	2268.017832	2045.258978
4	2586.456305	1701.26533	1534.19619
5	2069.366609	1361.213829	1227.558517
6	1724.640145	1134.512828	1023.133402
7	1478.406956	972.5835421	877.1154621
8	1293.732065	851.1365775	767.6020075
9	1150.096038	756.6778272	682.4248761
10	1035.187217	681.110827	614.283171
11	941.1709086		
12	862.823985		
13	796.5304342		

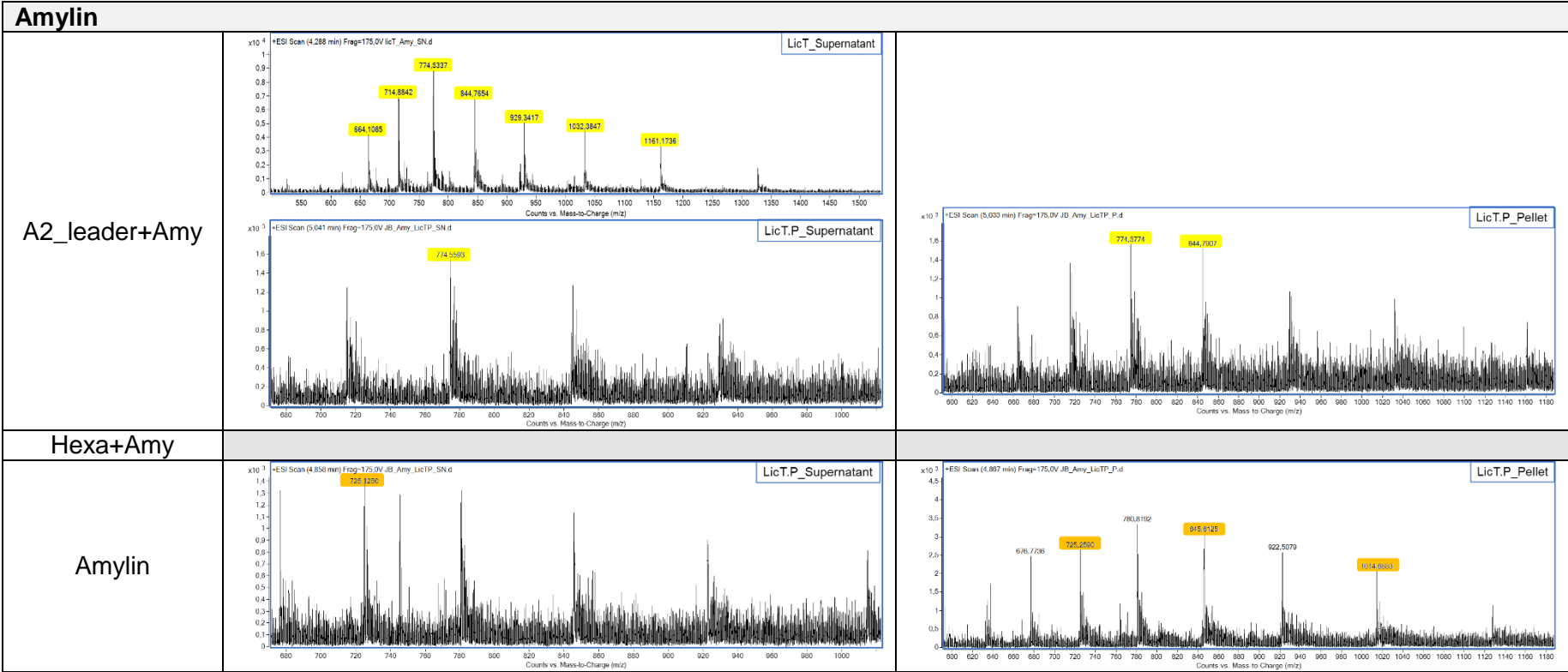
14	739.7073907		
15	690.460753		
16	647.369945		
17	609.3486438		
18	575.5519317		
19	545.3127682		
20	518.097521		
Plantaricin E			
N	A2_Leader+PlnE	A2_hexa+PlnE	PlnE
Monoisotopic mass	8917.53804	5376.77414	4708.49758
1	8918.545865	5377.781965	4709.505405
2	4459.776845	2689.394895	2355.256615
3	2973.520505	1793.265872	1570.507018
4	2230.392335	1345.20136	1178.13222
5	1784.515433	1076.362653	942.707341
6	1487.264165	897.1368483	785.7574217
7	1274.941831	769.1184164	673.6503364
8	1115.70008	673.1045925	589.5700225
9	991.845385	598.4271739	524.1742228
10	892.761629	538.685239	471.857583
11	811.6931014		
12	744.135995		
13	686.9722896		
14	637.9748279		
15	595.510361		
16	558.3539525		
17	525.5688862		
18	496.426605		
19	470.3519324		
20	446.884727		
Somatostatin			
N	A2_Leader+Soma	A2_hexa+Soma	Soma
Monoisotopic mass	7013.30054	3472.53664	2804.26008
1	7014.308365	3473.544465	2805.267905
2	3507.658095	1737.276145	1403.137865
3	2338.774672	1158.520038	935.761185
4	1754.33296	869.141985	702.072845
5	1403.667933	695.515153	561.859841
6	1169.891248	579.7639317	468.384505
7	1002.907902	497.0844879	401.6164079
8	877.6703925	435.074905	351.540335
9	780.2634406	386.8452294	312.5922783
10	702.337879	348.261489	281.433833

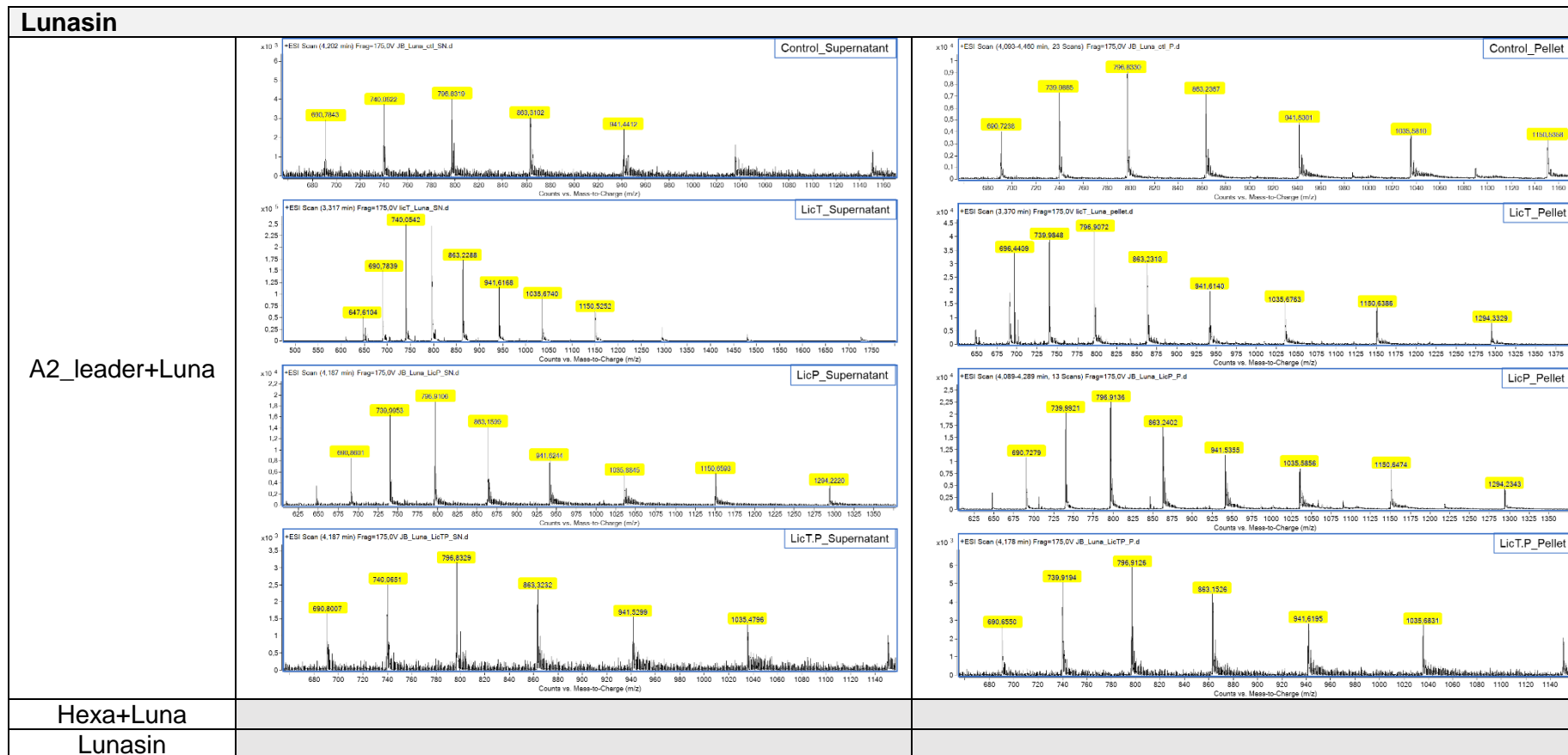
Supplementary Information

11	638.5806014		
12	585.4495367		
13	540.4924819		
14	501.9578636		
15	468.5611943		
16	439.3391088		
17	413.5549156		
18	390.6356328		
19	370.1289061		
20	351.672852		
Insulin A			
N	A2_Leader+InsA	A2_hexa+InsA	InsA
Monoisotopic mass	7756.56828	4215.80438	3547.52782
1	7757.576105	4216.812205	3548.535645
2	3879.291965	2108.910015	1774.771735
3	2586.530585	1406.275952	1183.517098
4	1940.149895	1054.95892	887.88978
5	1552.321481	844.168701	710.513389
6	1293.769205	703.6418883	592.2624617
7	1109.089008	603.2655936	507.7975136
8	970.57886	527.9833725	444.4488025
9	862.848745	469.4305339	395.1775828
10	776.664653	422.588263	355.760607
11	706.1503959		
12	647.388515		
13	597.6669235		
14	555.0484164		
15	518.112377		
16	485.7933425		
17	457.2765474		
18	431.928285		
19	409.2482608		
20	388.836239		
Epidermin			
N	A2_Leader+Epi	A2_hexa+Epi	Epi
Monoisotopic mass	7674.57804	4133.81414	3465.53758
1	7675.585865	4134.821965	3466.545405
2	3838.296845	2067.914895	1733.776615
3	2559.200505	1378.945872	1156.187018
4	1919.652335	1034.46136	867.39222
5	1535.923433	827.770653	694.115341
6	1280.104165	689.9768483	578.5974217
7	1097.376116	591.5527021	496.0846221

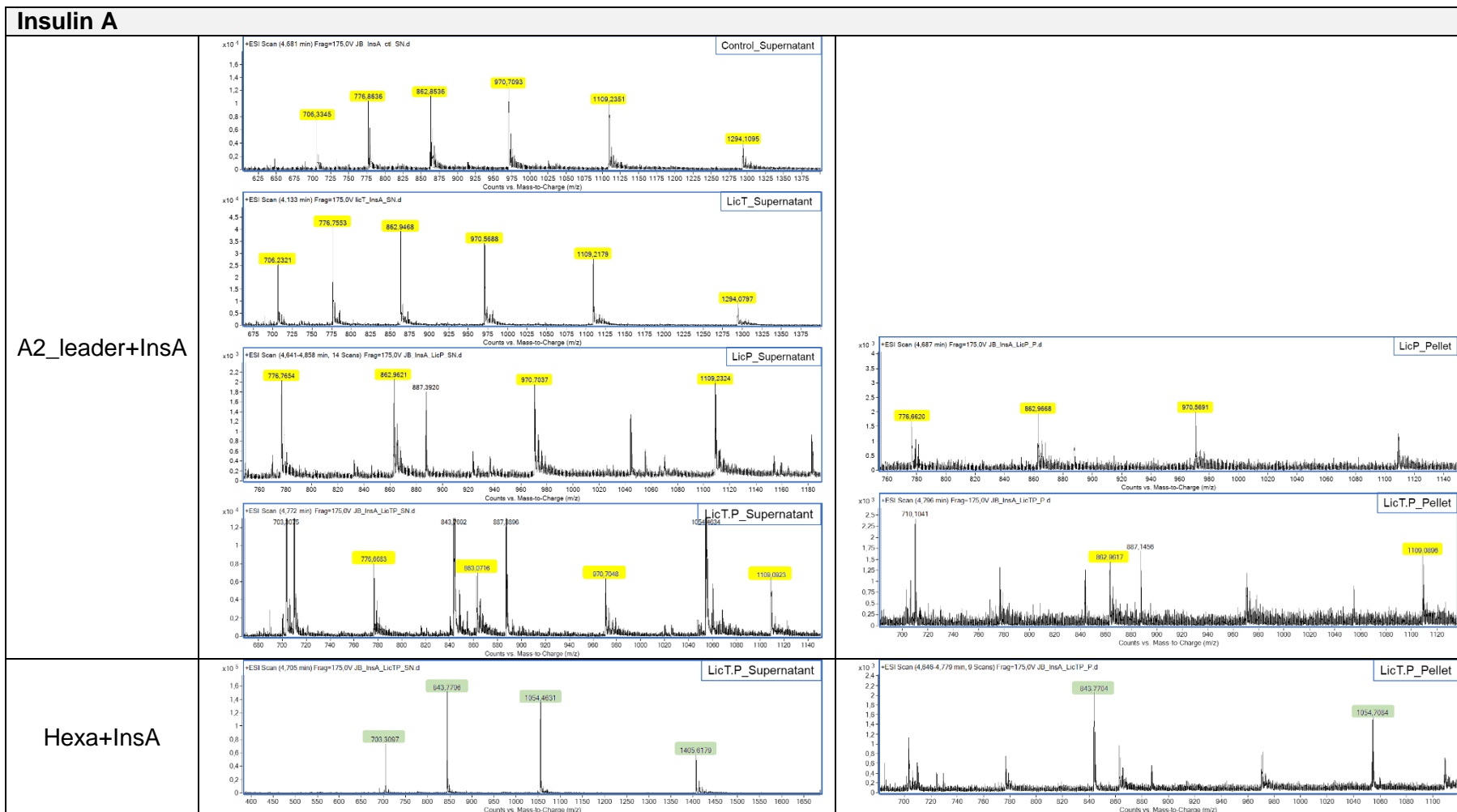
8	960.33008	517.7345925	434.2000225
9	853.7387183	460.3205072	386.0675561
10	768.465629	414.389239	347.561583
11	698.6967377		
12	640.555995		
13	591.3599819		
14	549.1919707		
15	512.646361		
16	480.6689525		
17	452.4535921		
18	427.3732717		
19	404.932985		
20	384.736727		
Mersacidin			
N	A2_Leader+Mrs	A2_hexa+Mrs	Mrs
Monoisotopic mass	7335.40851	3794.64461	3126.36805
1	7336.416335	3795.652435	3127.375875
2	3668.71208	1898.33013	1564.19185
3	2446.143995	1265.889362	1043.130508
4	1834.859953	949.6689775	782.5998375
5	1468.089527	759.936747	626.281435
6	1223.57591	633.4485933	522.0691667
7	1048.923326	543.0999121	447.6318321
8	917.9338888	475.3384013	391.8038313
9	816.053215	422.6350039	348.3820528
10	734.548676	380.472286	313.64463
11	667.8631441		
12	612.2918675		
13	565.2700181		
14	524.9655757		
15	490.035059		
16	459.4708569		
17	432.5024432		
18	408.53052		
19	387.0819571		
20	367.7782505		

Table S3.4. MS scan spectra of each of the extracts, highlighting the molecular mass signals corresponding to the individual peptides.

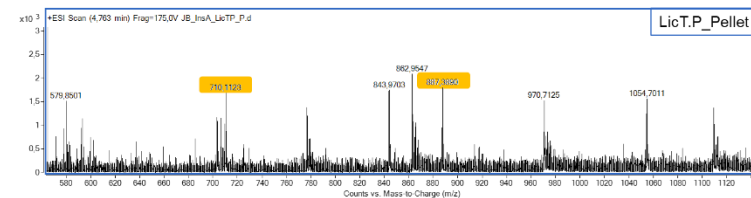
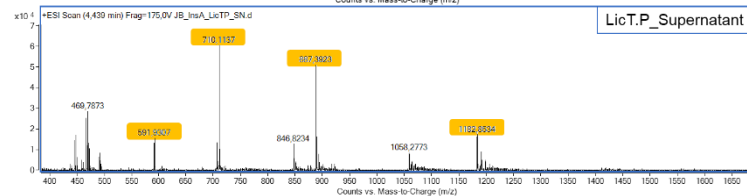
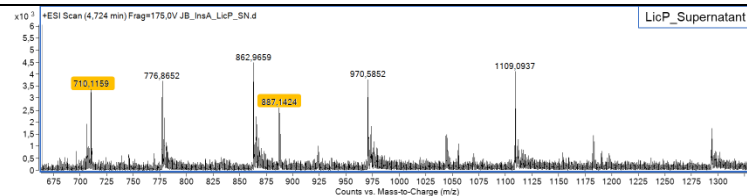


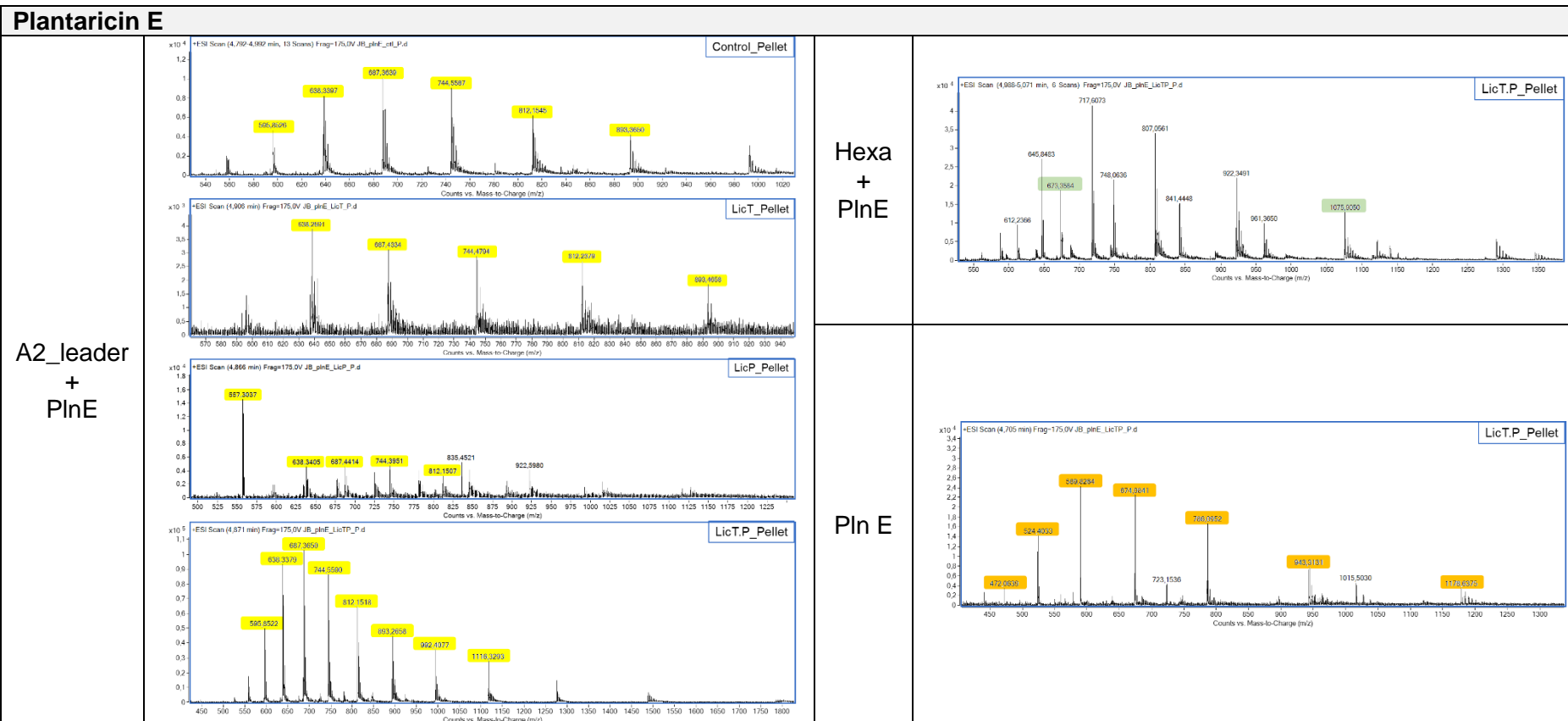


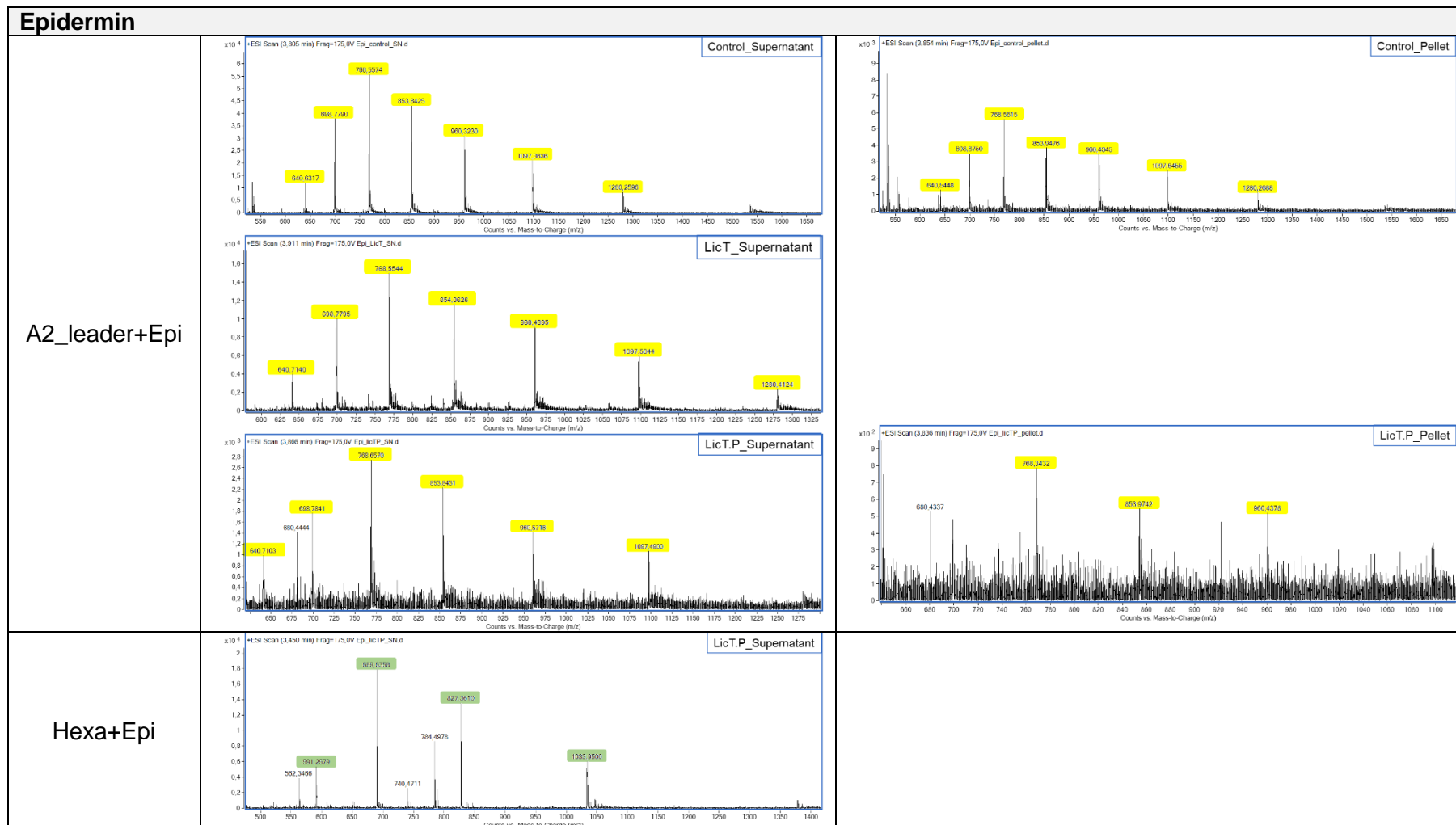
Supplementary Information



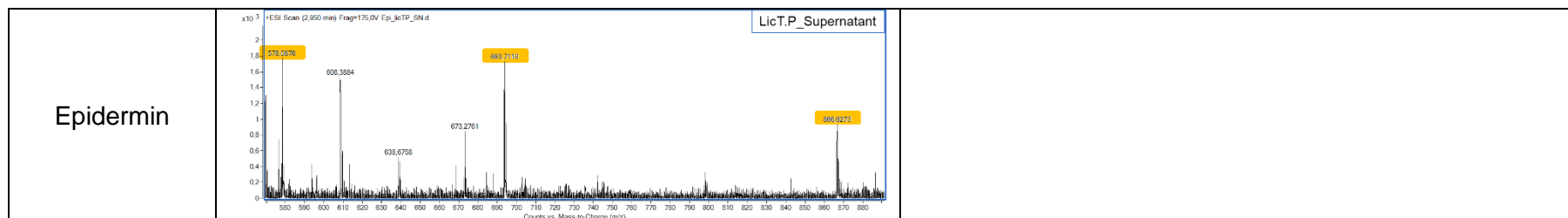
Insulin A







Supplementary Information



CHAPTER IV – SUPPLEMENTARY INFORMATION

Supplementary Figures

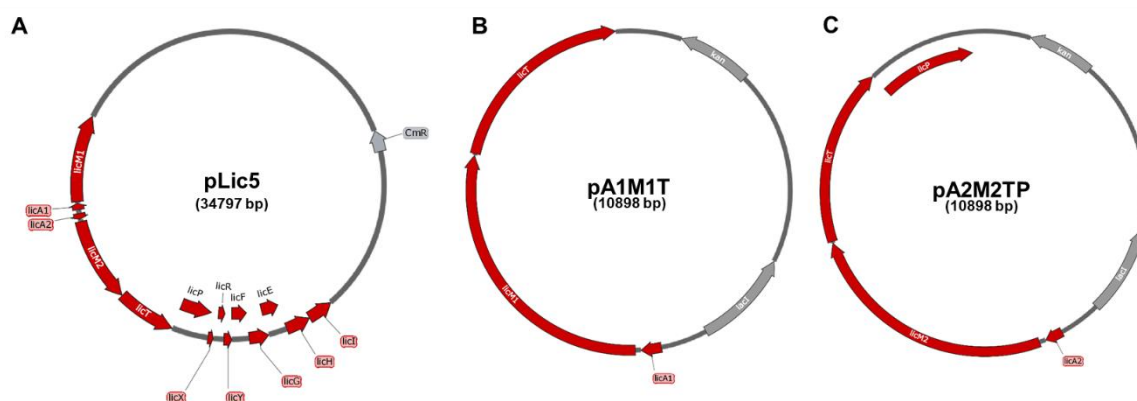


Figure S4.1. Schematic representation of the main plasmids used in this study. pLic5 (A) contains the entire lichenicidin gene cluster from the producer *B. licheniformis* I89. pA1M1T (B) and pA2M2TP (C) are based on the pET-24a+ vector and contain only the essential genes for the production of Bli α and Bli β in *E. coli*, respectively.

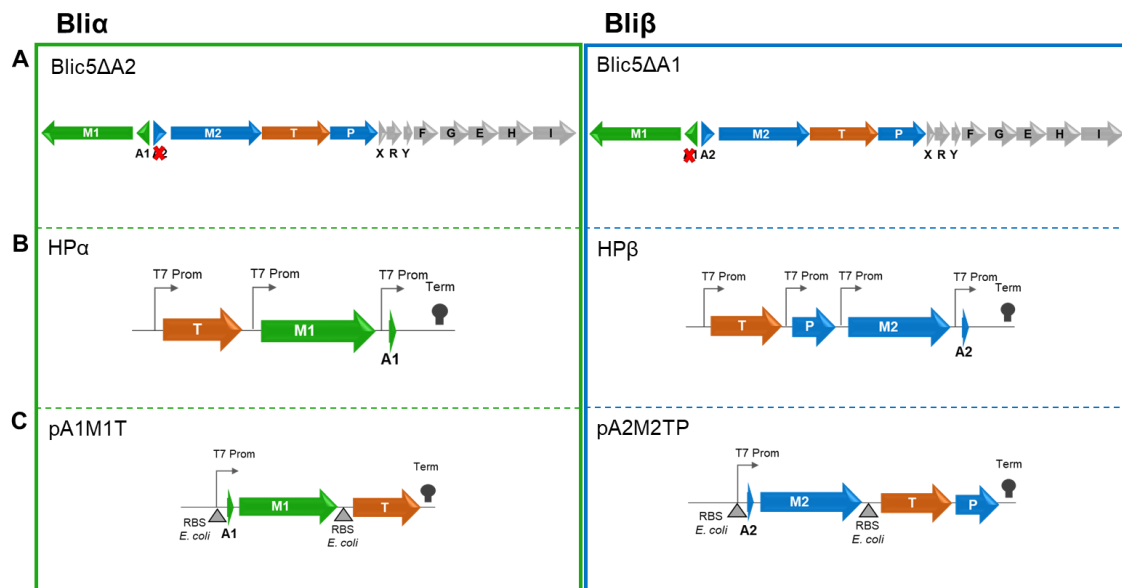


Figure S4.2. Design of the expression vectors employed in this study. (A) Initial vectors constructed for the independent expression of each peptide by [54], based on the original expression fosmid containing the whole *lic* gene cluster and the native *B. licheniformis* l89 regulatory determinants (promoters and ribosomal-binding sites (RBS)) with chloramphenicol as selective agent. (B) Optimized expression plasmids constructed by [175] using a multigene assembly strategy to clone each gene under the control of a strong inducible promoter, with kanamycin as selective agent. (C) Vectors constructed in the present study, containing only the genes essential for the production of each peptide, regulated by a single promoter and with *E. coli* optimized RBS, with kanamycin as selective agent. Each vector was cloned into *E. coli* BL21(DE3)Gold and *E. coli* BL21(DE3)Star strains.

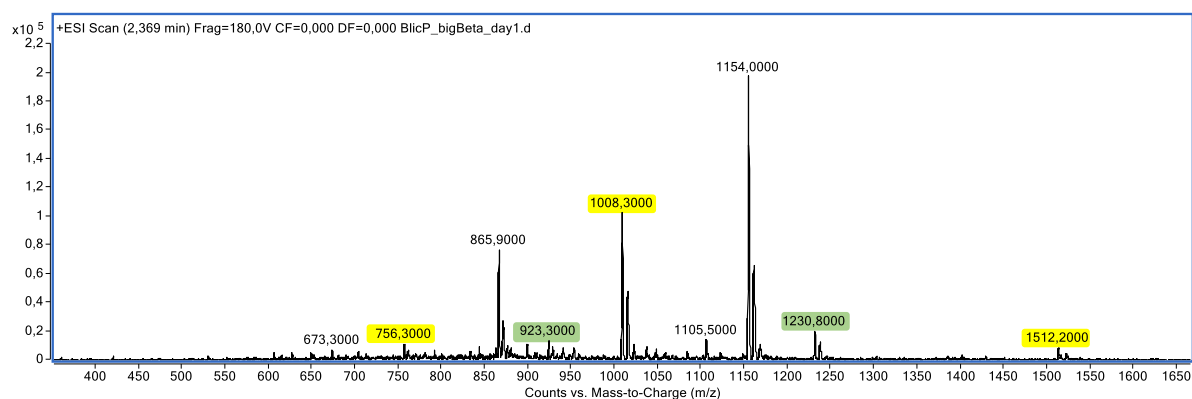


Figure S4.3. MS spectrum of Bli β obtained upon cleavage of hexa-LicA2 after 24h incubation with and LicP-producing strain. In yellow, masses corresponding to newly cleaved Bli β ; in green, masses corresponding to the remaining hexa-LicA2.

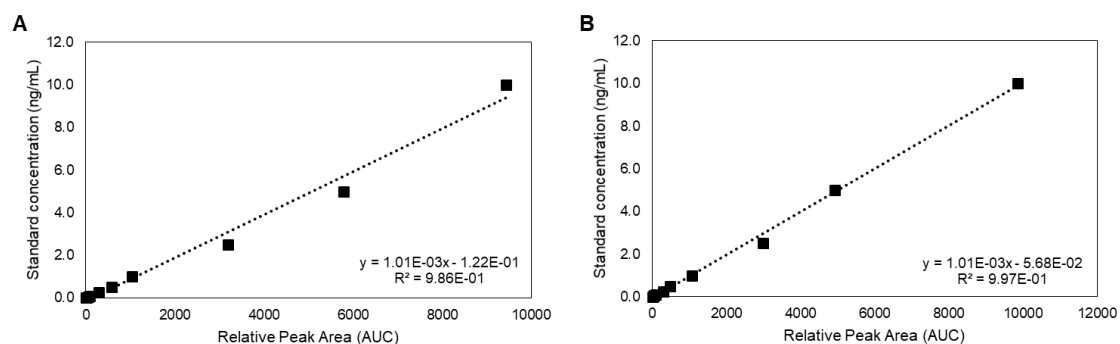


Figure S4.4. Lichenicidin standard calibration curve for (A) Bli α and (B) Bli β . Liquid stocks of purified Bli α and Bli β peptide were prepared in 70% ACN. Concentrations applied for MS measurements were 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5 and 10 mg/L. The concentration was plotted against the resulting peak area in MRM measurements. Best fit line and coefficient of determination R^2 were calculated as indicated.

Supplementary Tables

Table S4.1. Main characteristics of the plasmids employed in this study. Kan^R – encode kanamycin resistance; Amp^R – encode ampicillin resistance; Clo^R – encode chloramphenicol resistance.

Plasmids	Description	Reference
pLic5	pCC2FOS including the entire <i>lic</i> gene cluster; Clo ^R	[54]
pLic5ΔA1	pCC2FOS including the all <i>lic</i> gene cluster but the structural gene <i>licA1</i> ; Clo ^R	
pLic5ΔA2	pCC2FOS including all the <i>lic</i> gene cluster but the structural gene <i>licA2</i> ; Clo ^R	
ptolC	pET-15 with outer membrane encoding gene <i>tolC</i> ; Amp ^R	This work
pA1M1T	pET-24a+ with the essential genes for Bli α peptide production – <i>licA1</i> , <i>licM1</i> and <i>licT</i> ; Kan ^R	
pA2M2TP	pET-24a+ with the essential genes for Bli β peptide production – <i>licA2</i> , <i>licM2</i> , <i>licT</i> and <i>licP</i> ; Kan ^R	
pFGEHI	pUC19a with lichenicidin immunity genes <i>licFGEHI</i> ; Amp ^R	[175]
pHP α	pRSF-Duet with the genes for Bli α peptide production – <i>licA1</i> , <i>licM1</i> and <i>licT</i> ; Kan ^R	
pHP β	pRSF-Duet with the essential genes for Bli β peptide production – <i>licA2</i> , <i>licM2</i> , <i>licT</i> and <i>licP</i> ; Kan ^R	

Table S4.2. List of primers used to amplify *licA1M1*, *licA2M2*, *licT*, *licTP* and *licFGEHI* genes for the construction of the expression vectors. The enzymes restriction sites are represented in bold and the initiation codon is underlined (when applicable). Annealing temperatures and extension times for the required PCR reactions are also indicated.

Amplicon	Primers	Primer sequence (5'→3')	Restriction Enzyme	T _{annealing} (°C)	Extension time (min)
<i>licA1M1</i>	Comp_licA1_Fw	aggtg gggatcc <u>at</u> gtcaaaaaggaaatg	<i>Bam</i> HI	54	4
	Comp_licM1_Rv	catacat tctcgag ttaaacacgttttc	<i>Xho</i> I		
<i>licT</i>	licT_RBS_Fw	tag cgggccg caggaggtataaggcatg <u>tttttc</u> cataaga	<i>Not</i> I	57	3
	Comp_licT_Rv	ggtggtggtg ctcgag tcacatcatcacctctgcagatt	<i>Xho</i> I		
<i>licA2M2</i>	Comp_licA2_Fw	atcag ggatcc <u>at</u> gaaaacaatgaaaaattcag	<i>Bam</i> HI	58	4
	Comp_licM2_Rv	tagtg cgggccg ctcacctgcccgtcggaatc	<i>Not</i> I		
<i>licTP</i>	licT_RBS_Fw	tag cgggccg caggaggtataaggcatg <u>tttttc</u> cataaga	<i>Not</i> I	56	4
	Comp_licP_Rv	tttg cgggccg ctcactccttctcatcatttc	<i>Not</i> I		
<i>licFGEHI</i>	Comp_licF_Fw	cagt gctagc atggttgacgctccaattgtcatg	<i>Nhe</i> I	52	5
	Comp_licI_Rv	tat ggatcc ttatgacgtgacaatatcc	<i>Bam</i> HI		

CHAPTER V – SUPPLEMENTARY INFORMATION

Supplementary Procedures

Construction of expression plasmids. First, *licA1M1* and *licA2M2* genes were amplified and cloned into pET-24a+, originating the plasmids plicA1M1 and plicA2M2, respectively. Then, the *licT* and *licTP* genes were amplified and inserted in plicA1M1 and plicA2M2, to originate the plasmids pA1M1T and pA2M2TP, respectively. All the amplifications were performed in a 50 μ L reaction, containing 25 mM dNTPs, 5 \times Herculase II buffer, 1 μ L of DMSO, 10 pmol/ μ L each primer, 100-400 ng of *B. licheniformis* I89 total DNA and 5 U of Herculase II DNA polymerase. The primers, annealing temperature and extension time for each set of genes are listed in Table S1. All the digestion reactions were carried out in a final volume of 40 μ L, containing 1000 ng of the insert or 700 ng of plasmid DNA and the appropriate enzymes and reaction buffer (Thermo Scientific; Table S1). Digestions were performed at 37°C for 1h and purified with NZYGelpure kit (NZYtech), according to the manufacturer's instructions. Ligation reactions were performed in a total volume of 20 μ L, containing 50 ng of plasmid DNA, 150 ng of the DNA insert, 1 \times T4 DNA ligase buffer and 5 U of T4 DNA ligase (Thermo Scientific). The reactions were incubated at 22°C for 1 h and immediately used to transform chemically competent *E. coli* DH5 α cells. Once the final plasmids pA1M1T and pA2M2TP were obtained, they were purified and used to transform chemically competent *E. coli* BL21(DE3)Gold and BL21(DE3)Star cells. The transformants were selected on LA plates with 50 μ g/mL of kanamycin (Kan). Positive clones were screened by PCR following sequencing of the inserts using the T7 promoter and T7 terminator universal primers.

Supplementary Figures

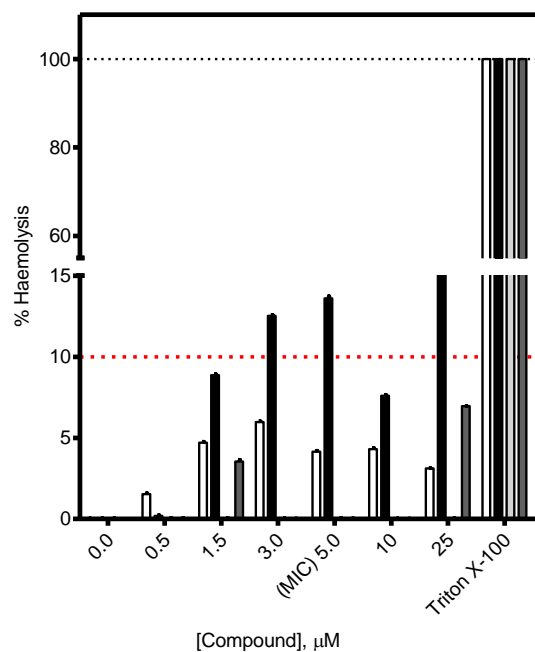


Figure S5.1. Percentage of hemolysis of the controls: ampicillin after 1 h (white) and 24 h (black) of incubation; methanol-equivalent concentration controls after 1 h (light grey) and 24 h (dark grey) of incubation. Error bars represent standard deviation of three independent replicates. Triton X-100 was used as positive hemolytic control. Ampicillin was used as non-hemolytic control. The MIC value is indicated in the xx axis. The 10% hemolysis threshold, above which a compound is considered to be hemolytic, is indicated by a red dotted line.

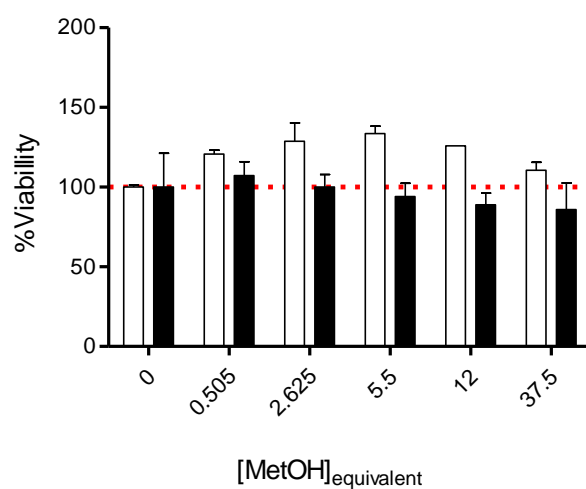


Figure S5.2. Percentage of viability of HFF-1 human fibroblasts after 2 h (white bars) and 24 h (black bars) of incubation with different methanol concentrations, using XTT cell proliferation assay. Samples contain the same percentage of methanol that was used to dissolve lichenicidin. Error bars represent standard deviation of three independent replicates; when these are not visible, standard deviation is too low to be noticed.

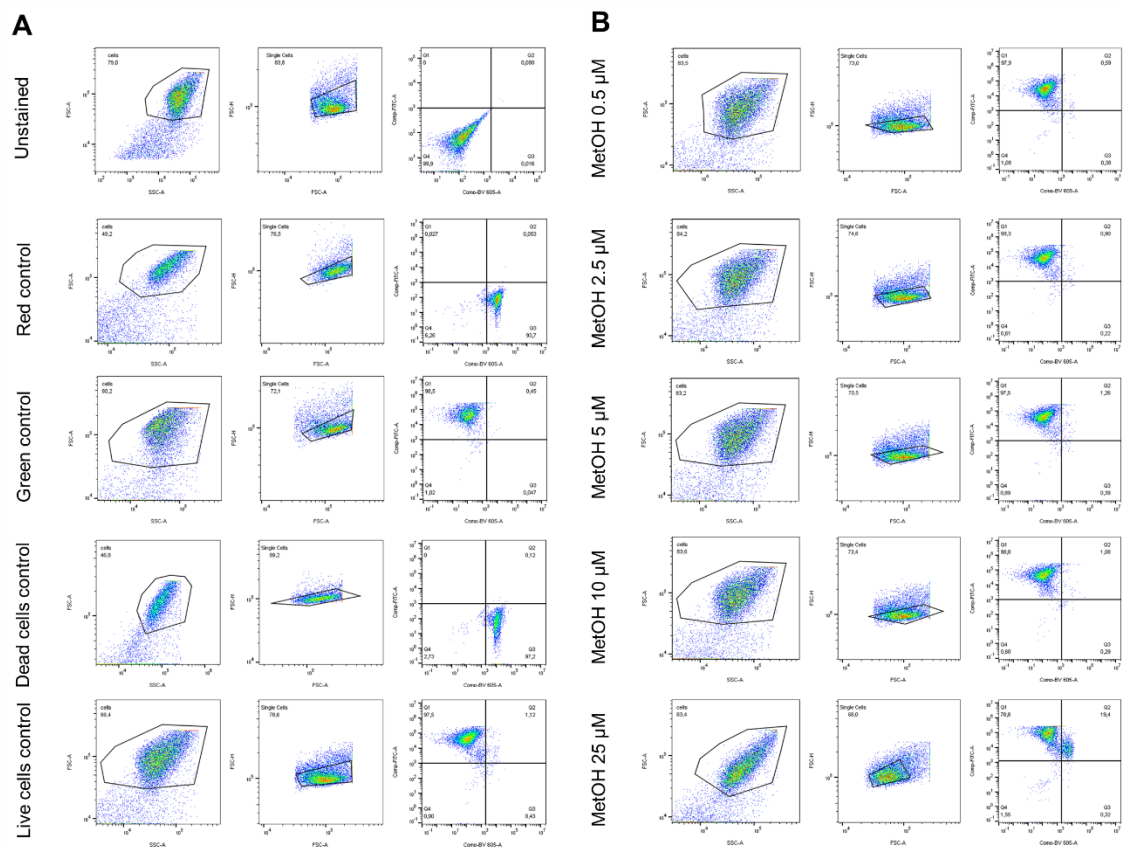


Figure S5.3. Flow cytometry analysis of (A) technical controls and (B) methanol controls of treated HFF-1 fibroblasts and respective gating strategy.

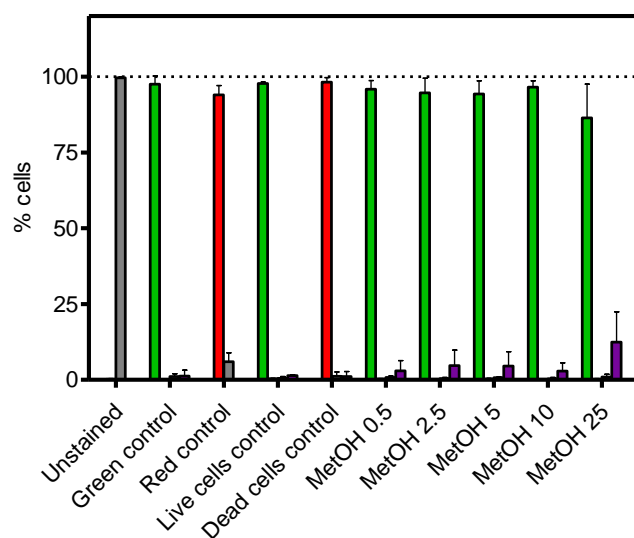


Figure S5.4. Controls of the flow cytometry viability experiments of HFF-1 human fibroblasts after 2 h of incubation, including different methanol concentrations. Unstained cells are represented in grey; live cells, marked with calcein, are shown in green; dead cells, marked with ethidium homodimer-1, are shown in red; double-stained cells are represented in purple. Error bars represent standard deviation of three independent replicates.

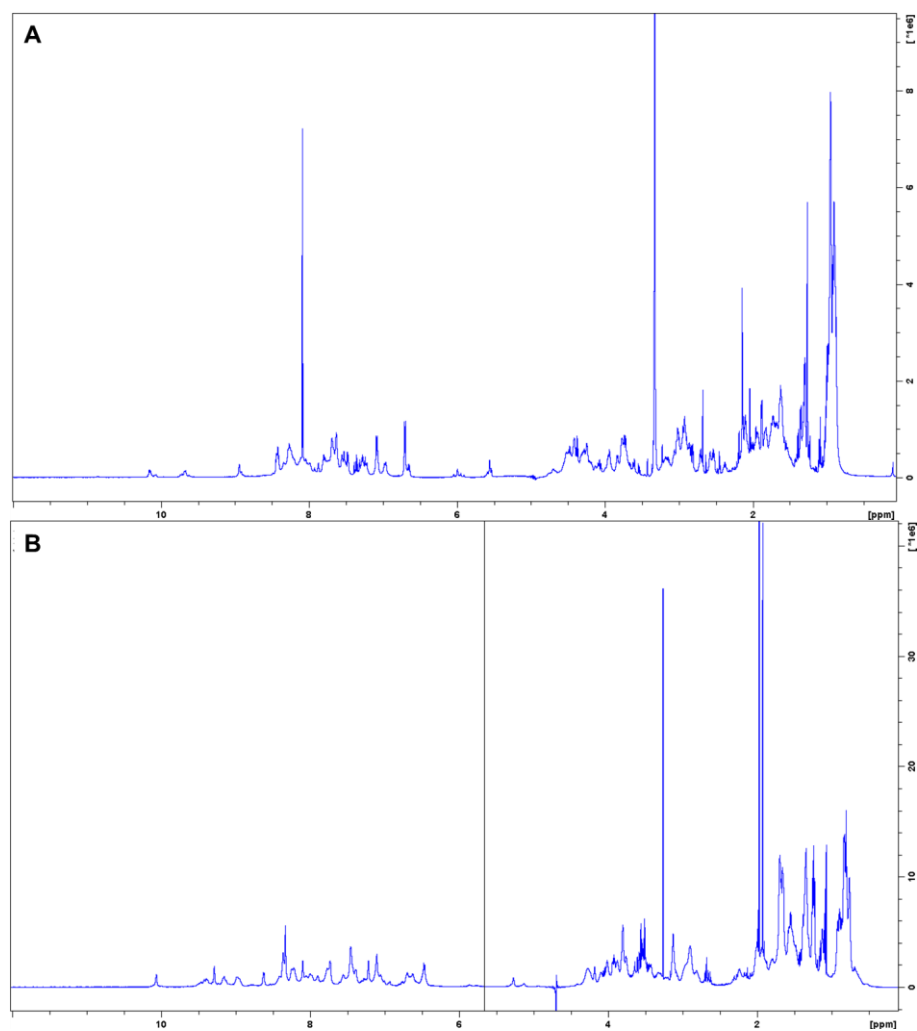


Figure S5.5. Proton NMR spectra with water suppression of pure Bli α (A) and Bli β (B).

CHAPTER VI – SUPPLEMENTARY INFORMATION

Supplementary Tables

Table S6.1. Comparison of the interaction rate of LUV with and without Lipid II with the lichenicidin peptides. The ratio (LUV+LipII)/(LUV) is presented for DOPC and DOPC:DPPG:CL vesicles for all the tested conditions used in the leakage assay; for that, 300 points were plotted, corresponding to 1 min after the addition of each peptide. The slope was taken as an indicator of the speed of immediate interaction between the peptide and the LUV and, thus, of the affinity between them. In green, values higher than 1 indicating higher speed of interaction with LUV containing Lipid II; in red, values lower than 1 representing less interaction in the presence of Lipid II; in grey, values close to 1, for non-significant interaction changes in the presence and absence of lipid II.

		DOPC	DOPC:DPPG:CL
1.5 μ M	Lichenicidin	1.73	1.26
	Bli α + Bli β	1.24	2.03
		1.36	3.83
	Bli β + Bli α	2.41 1.75	1.39 3.55
5 μ M	Lichenicidin	1.72	1.63
	Bli α + Bli β	2.11	4.47
		0.91	0.96
	Bli β + Bli α	1.92 2.18	0.78 0.47
25 μ M	Lichenicidin	1.44	1.78
	Bli α + Bli β	2.77	2.51
		1.07	1.09
	Bli β + Bli α	1.54 0.46	0.44 3.09