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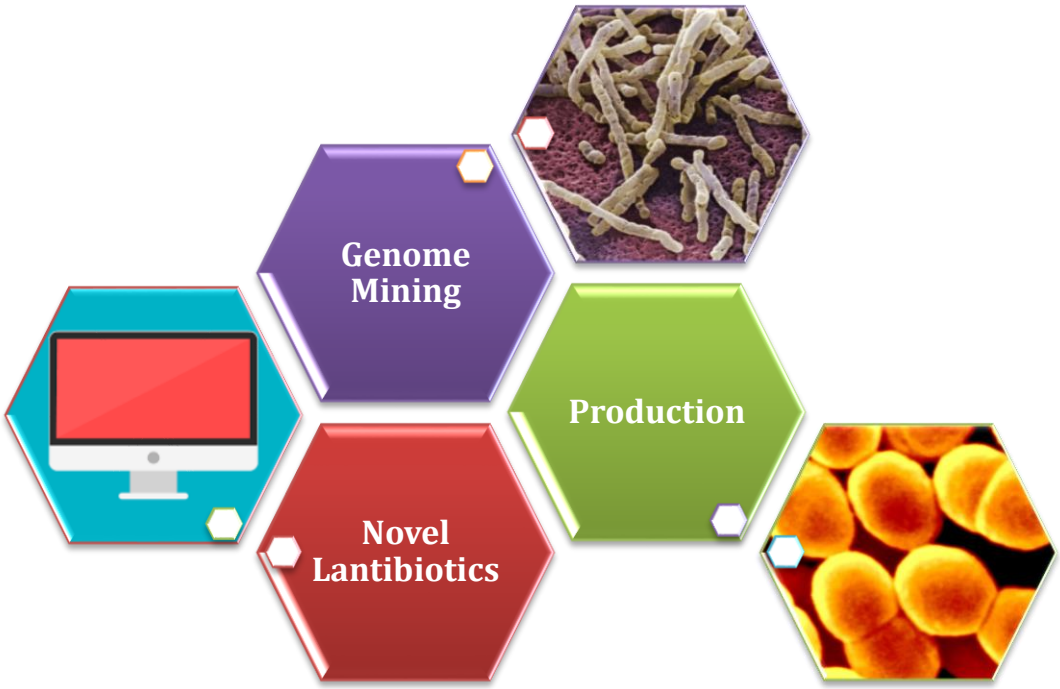
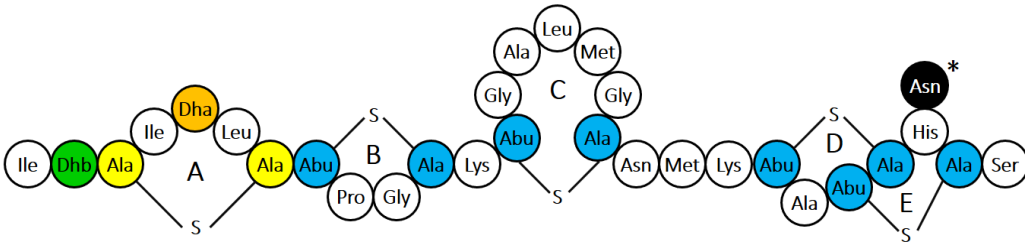
Identification And Production Of Novel Lantibiotics from *Clostridium* Species via Heterologous Expression in *Lactococcus lactis*





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Abstract

The capability of nisin induced gene expression system (NICE) in *Lactococcus lactis* to modify small peptides into lanthipeptides, provide an exciting opportunity for novel antimicrobials production to combat various pathogens. This system can be exploited to produce "new to nature" lantibiotics from various organisms using synthetic biology approach. This study aimed to identify and produce novel lantibiotics from the genus of *Clostridium* that had not been discovered.

As a result, 54 putative clostridia new lantibiotic genes were discovered after *in silico* analysis with BAGEL3 and Anti-SMASH. Subsequently, based on the novelty and the compatibility to expression system, twelve putative clostridia lantibiotic genes were selected as candidates and tested for production via heterologous expression in *Lactococcus lactis*. Interestingly, eleven peptides except Clos16 displayed antimicrobial activity against *L. lactis* NZ9000 as indicator strain. Moreover, *Micrococcus flavus* is susceptible to Clos4, Clos12, Clos22 and ClosDP. Additionally, Clos2, Clos14 and ClosDP lantibiotics showed antimicrobial activity against *Clostridium sporogenes* and *Micrococcus luteus*. Nevertheless, mass spectrometry analyses using MALDI-TOF and LC-MS/MS revealed multiple dehydrated serines and/or threonines in several putative lantibiotic candidates, confirming successful production and secretion of novel lantibiotics from the genus of *Clostridium* by nisin synthetic machinery.

Keywords: antimicrobial resistance, antimicrobial peptides, lantibiotics, *Clostridium*, synthetic biology, heterologous expression, *Lactococcus lactis*

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Chapter 1. Introduction

1.1 The Necessity Of Novel Antimicrobial Compounds

Nowadays and since some years, antimicrobial resistant bacteria are causing a significant problem for global health that encouraged development of new therapies or drugs. As reported in antimicrobial resistance review (O'Neill, 2014), this phenomenon estimated could kill 10 million people a year in 2050 and may cause some serious implications to the global economy and welfares. In 2015, *World Health Organization* (WHO) released "Global Action Plan on Antimicrobial Resistance" that encouraged more research and studies in development of novel antimicrobials, and also recommended global stakeholders to increase investments in new medicine production. In fact, last year the WHO published the list of bacteria for which new antimicrobial are urgently needed (http://www.who.int/medicines/publications/WHO-PPL-Short_Summary_25Feb-ET_NM_WHO.pdf).

The current situation of new antimicrobial discovery is at critical level. Since lipopeptides 30 years ago, none of new family antibiotics have been discovered, meanwhile the development of novel antibiotics is dwindling in recent years and become more expensive in the prices (Fig.1). On the other hand, the multidrug resistant pathogen strains in the world are increasing significantly (Gelband et al., 2015; Trimble & Hancock, 2017).

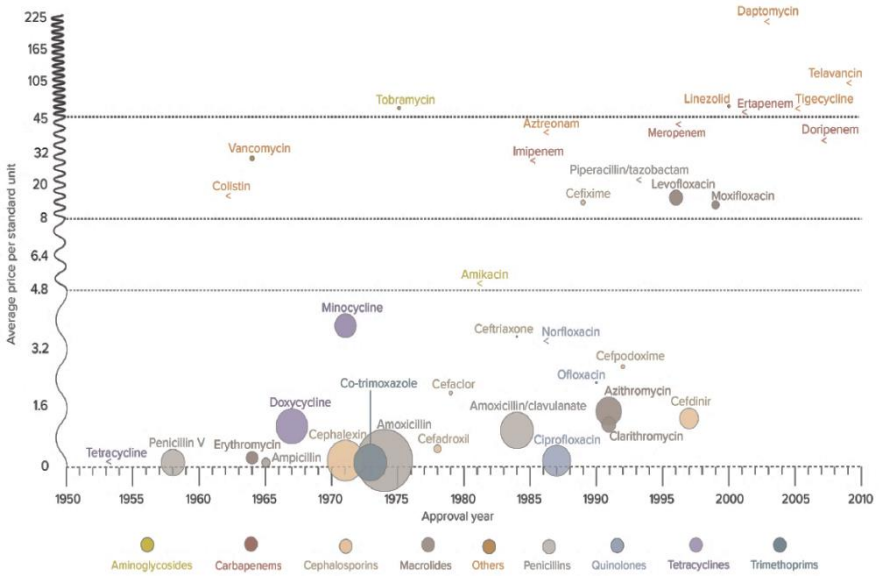

Fig. 1. The price and consumption of antibiotic in the United States by year of FDA approval (FDA, 2010)

This fact also triggered significant exodus of pharmaceutical industries for investing their research and development on production of novel antimicrobial compounds, due to the complexity and big production costs, resulting significant fewer incomes. Since the exodus of big pharma, now, discovery of novel antimicrobial compound becomes scientific challenge for academia (Banin et al., 2017; Trimble & Hancock, 2017). Recently, many researchers are focusing on how to produce new antimicrobials from common antibiotic derivatives or to find promising approaches to produce novel compounds in synthetic, natural, or in combination, by exploiting the existing biological system in nature. These approaches open up opportunity to produce novel antimicrobials in safe and well-standardized methods which are important for global antibiotic use.

1.2. Lantibiotics: Promising Candidate to Fight Against Various Pathogens

Recent studies showed promising potential of lantibiotics as alternative to prevalent antibiotics. Lantibiotics displayed broad-spectrum activity against Gram-positive pathogens including methicillin-resistance *Staphylococcus aureus* (MRSA) and vancomycin resistance *enterococci* (VRE) and other Gram-positive species such as *Micrococcus sp.* and *Listeria sp* (Montalban-Lopez et al., 2012). The term of lantibiotic itself referred to the *lanthionine* peptides containing antibiotic. They are ribosomally synthesized peptides which produced by Gram-positive and also Gram-negative bacteria. Lantibiotics are characterized by the presence of typical amino acids: dehydrobutirine and dehydroalanine (after dehydration of threonine and serine) that are able to react with the SH group of cysteine forming a thioether-linked amino acid lanthionine and methyl-lanthionine rings (Willey & van Der Donk, 2007).

Lanthipeptides are categorized into four classes based on posttranslational modification and maturation enzymes, also antimicrobial activity. Class I lanthipeptides need two distinct enzymes namely LanB for dehydrations of serine-threonine residues, and LanC enzyme to form the rings. For the secretion, they require LanT enzyme, carrying peptide outside membrane and finally LanP protease releasing the leader peptide (Repka et al., 2017). Class II lanthipeptides are processed by a bifunctional enzyme called LanM (Fig.2) which can do both modifications (dehydration and cyclation). Both classes I and II lanthipeptides shared *lan*T transporter gene, but in class II this gene is multifunctional which also involved in release of the leader by a N-terminal Cys protease domain (Willey & van Der Donk, 2007).

Moreover, Class II lanthipeptides have a unique group which composed by two distinct lantibiotic parts designated as lantibiotic alpha (LanA1) and beta (LanA2), both may have different activity each other, but work synergistically to perform antimicrobial activity (Martin et al., 2004). Class III lanthipeptides are a group which employ a versatile enzyme: LanL. LanL is a lyase/serine-threonine kinase/cyclase that can handle all modification steps. Furthermore, class IV lanthipeptides synthesized by a different multifunctional enzyme (RamC/LabRK) that can form labionine, a new modified structure. However, based on antimicrobial activity, only class I and class II lanthipeptides that noticed as lantibiotics (Montalban-Lopez et al., 2012).

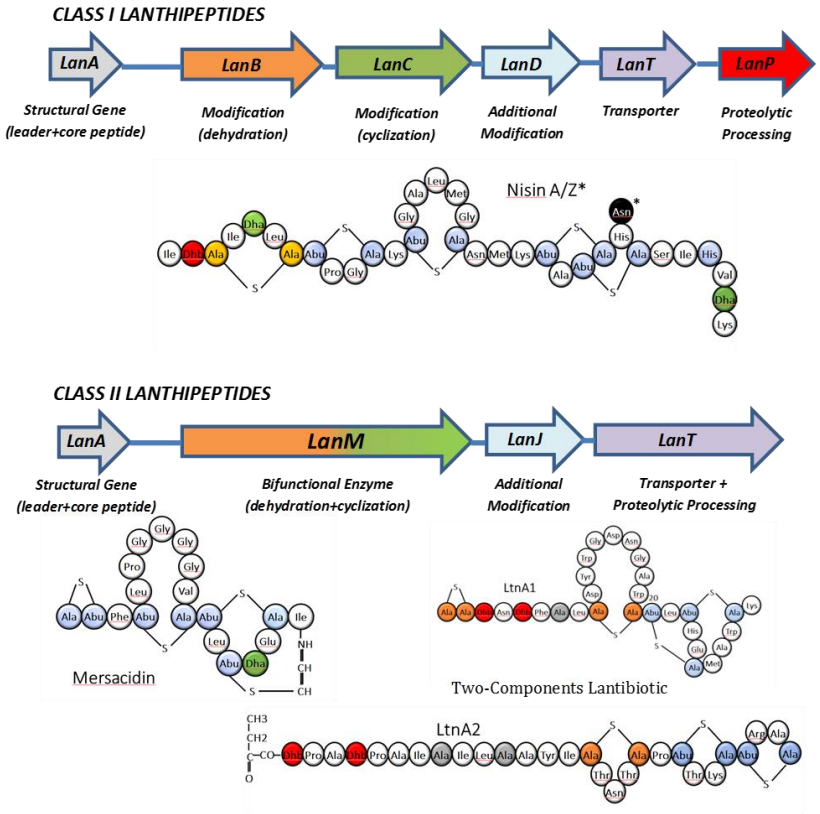

Fig.2: The scheme of typical gene operon encoding class I and class II lanthipeptides

(adapted from Sandiford, 2014).

Lantibiotics are also characterized by their low resistance level because they have multiple mode of actions like pore-forming on the cell walls causing ATP leakage or the sequestration of cell wall precursor, lipid II, that inhibit the cell wall synthesis and the replication. The low resistance level of lantibiotics uncovered from the study of nisin, a food grade lantibiotic that has been used as a food preservative for over 50 years with low resistance development (Weidemannn et al., 2001; Montalban-Lopez et al., 2012).

1.3. Synthetic Biology of Lantibiotics

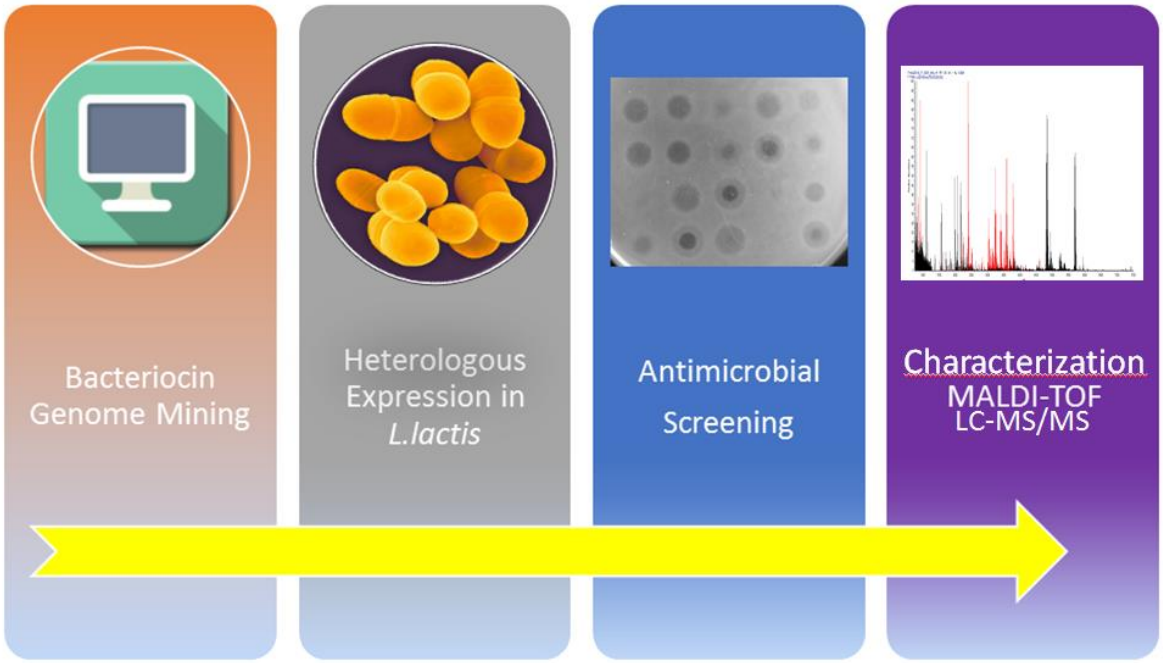

Fig.3: The strategy of novel lantibiotics production using synthetic biology

Synthetic biology approach could perform gene mining of novel lantibiotics by employing the modularity and the orthogonality of engineering into biology insights. This approach applies combination method between *in silico* analysis and heterologous expression for production system. *In silico* analysis of putative lanthipeptide genes via bioinformatic tools (BAGEL3 or Anti-Smash) provided an accurate prediction of putative novel lantibiotics. BAGEL3 software could analyze DNA sequences by two different approaches (van Heel et al., 2013). First, an indirect approach which is the context of bacteriocin-or RiPP gene-based mining and the direct approach which is structural gene-based mining directly via Glimmer, a software for finding genes in microbes (van Heel et al., 2013; Delcher et al., 2007). These approaches could improve the success rate by reducing false positive probability and minimize manual evaluation of results. In addition, Anti-SMASH is an application that could predict putative genes also their biochemical properties, and further details including gene cluster description, annotation and genomic loci for biosynthetic pathway (Weber et al., 2005; Zhao et al., 2016). Combination of BAGEL3 and Anti-SMASH for genome mining give accurate information for identification of unknown lanthipeptide genes in various organisms.

Afterwards, new DNA sequence encoding putative lanthipeptide gene fused to nisin leader sequence and subsequently introduced into the production host, for example, *Lactococcus lactis.* Naturally, *Lactococcus lactis* will run biosynthesis system and bring out a novel and mature peptide. Nonetheless, characterization of a novel compound accomplished using MALDI-TOF or LC-MS/MS mass spectra analysis (Majchrzykiewicz et al., 2010; Montalban-Lopez et al., 2012; van Heel et al., 2016).

1.4. Novel Lantibiotics Production via Nisin Controlled Expression System in *L. lactis*

Nisin controlled gene expression system (NICE) has been established as a powerful tool for production of new lantibiotics in *L. lactis*. To develop the system, the cells require some nisin genes. In case of wild-type nisin production, eleven genes are required. First, the regulation system *nis*KR genes, in which *nis*K is the receptor for the inducer (nisin) and *nis*R is the activator of nisin promoter to produce precursor of nisin, modification enzymes and transporter (NisBTC), the leader peptidase NisP, and additionally, immunity genes *nis*EFGI to protect cell from final product's cytotoxicity (Kuipers et al., 1995) (Fig.4A).

NICE system requires the combined activity of two plasmids (Fig. 4B). *Lactococcus* strains used for NICE induction system should harbor *nis*KR genes. The *nis*A gene that encoded nisin precursor should be cloned in expression vector together with the leader. This gene could be modified by inserting DNA sequence of a putative lantibiotic gene and integrate it to the conserved leader peptide (Majchrzykiewicz et al., 2010). Leader peptide will keep the peptide inactive, also lead it to promiscuous modification enzymes (NisBC). In another plasmid, *nis*BTC are required. The N-terminal region of NisB will bind to FNLD box motif in the leader peptide sequence and perform the glutamylation of serine-threonine using a cofactor called glutamyl-tRNA^{Glu}, therefore resulting dehydrated amino acids (Ortega et al., 2015; Zhou, 2016). Another modification enzyme, NisC, is responsible for performing cyclase reaction after serine-threonine dehydrations. Importantly, NisC enzyme binds to a conserved motif FxLx in the leader peptide to catalyze the reaction (Abts et al., 2013).

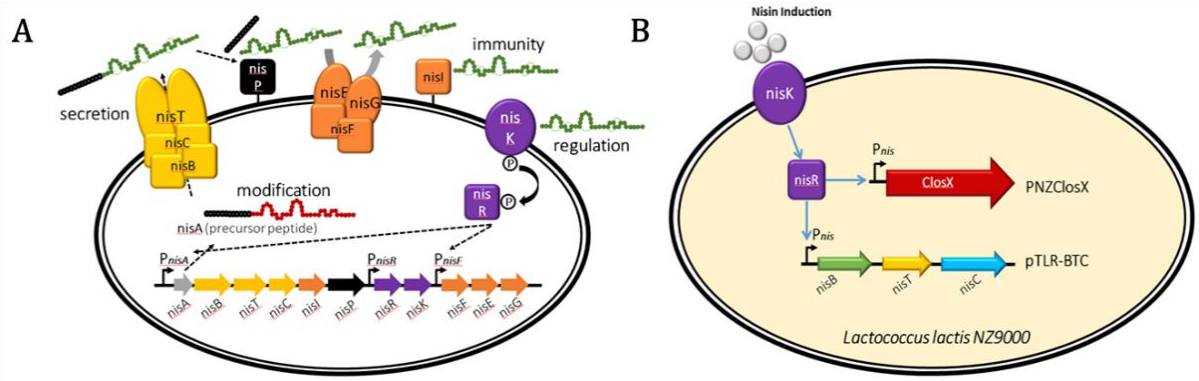

Fig. 4. A.) Wild-type Nisin expression system in *L.lactis*.
B.) Controlled nisin gene expression system

Next step after modification by NisB and NisC, the nisin precursor will be secreted outside of cell by NisT enzyme. NisT enzyme is a broad spectrum (poly) peptide transporter which able to export unmodified peptide independently without NisBC biosynthetic enzymes, and also partially or entirely modified nisin precursor after posttranslational modification (Kuipers et al., 2004). After bringing out of cell by NisT, the inactive peptide will be cleaved from leader by serine protease enzyme called NisP. This enzyme could recognize the conserved motif such as GAxPR (which x is variable amino acid) that conserved in a leader sequence and will cleave leader peptide at one site after the motif sequence (Majchrzykiewicz et al., 2010). Interestingly, this cleavage process could be done *in vitro* using a cell-membrane free extract of NisP overproducer strain and supernatant (SN) that contain inactive lantibiotic peptide (van der Meer et al., 1993; Seizen et al., 1995; Perez et al., 2014). As final result, the active peptide will be formed and show antimicrobial activity. Moreover, nisin-controlled expression system in *Lactococcus lactis* is very flexible and able to be modified with different lantibiotic enzymes such as GdmD (Gallidermin) produced by *Staphylococcus gallinarum* and MrsD (Mersacidin) produced by *Bacillus amyloliquefaciens,* to create a hybrid peptide (Zhao et al., 2016). This fact proved that nisin controlled expression system in *L.lactis* can be very useful and powerful as a tool for novel lantibiotics production.

Chapter 2. Materials and Methods

2.1 Bacterial Strains, Plasmids, And Growth Conditions

The strains used in this work listed in Table 1.

Strain	Characteristic	Purpose	Reference
Escherichia coli **TOP-10**	*mcrA, Δ(mrr-hsdRMS-mcrBC), Phi80lacZ(del)M15, ΔlacX74, deoR, recA1, araD139, Δ(ara-leu)7697, galU, galK, rpsL(SmR), endA1, nupG*	Cloning intermediate	Invitrogen
Lactococcus lactis **NZ900**	*pepN::nisRK*	sensitive strain	Kuipers et al., 1998
L. lactis **NZ9000 pIL3-253 pNZ-*nis*P8H**	EryR, CmR, NisP producer strain	Sensitive strain	Montalbán-López et al., unpublished
L. lactis **NZ9000 pTLR-BTC**	EryR, *pepN::nisRK* , *nis*BTC	cloning and expression host	Lab collection
L. lactis **NZ9000 pTLR-BTC pNZ8048**	EryR, CmR, *pepN::nisRK nis*BTC	Negative control strain with empty expression vector	Lab collection
L. lactis **NZ9000 pIL3-BTC pNZ8048-*nis*A**	EryR, CmR, NisA producer strain	Positive control Antimicrobial assay	Lab collection
Micrococcus flavus	-	sensitive strain	Lab collection
C. sporogenes **C22/10**	-	sensitive strain	Lab collection

Table 1. Strains used in this work

L. lactis NZ9000 were cultured in M17 (Difco) medium supplemented with 0.5% (wt/vol) glucose (GM17) at 30^0C. Minimal expression medium (MEM) was used for protein expression and purification (Rink et al., 2005). Chloramphenicol and/or erythromycin were added when necessary and used at 5 µg/ml and 10 µg/ml respectively. On the other hand, *E. coli* TOP-10 for amplification of pUC57-ClosMix plasmid (Table 2.) was cultured in LB medium at 37^0C with shaking for overnight. Ampicillin was used as a selective marker when necessary at 100 µg/ml. Chemocompetent cells preparation and transformation in *E. coli* was done according to (Sambrook & Russell, 2001).

GM17 medium was used for liquid cultures of indicator strains including *L. lactis* NZ9000 and *Micrococcus flavus,* incubated at 30^0C overnight for antimicrobial assay. Also,

to perform antimicrobial assay against *Clostridium* species, Reinforced Clostridium Medium (RCM) (Kemperman et al., 2003) was used to grow *Clostridium sporogenes* C22/10 anaerobically at 37^{0}C overnight and placed inside the anaerobic jar, Anaerocult A (Merck).

Vector	Characteristic	Purpose	Source
pUC57-*clos*Mix	AmpR,	Synthetic gen with the different putative lantibiotics inside separated by *XhoI* sites.	Genescript This work
pNZ-*nisA*	CmR, *nisA* gen cloned under *nisP* promoter	Putative lantibiotics cloning vector	van Heel et al., 2013
pNZ-*clos2*	CmR, putative peptide Clos2 cloned fused to nisin leader.	New lantibiotic expression	This work
pNZ-*clos4*	CmR, putative peptide Clos4 cloned fused to nisin leader.	New lantibiotic expression	This work
pNZ-*clos5*	CmR, putative peptide Clos5 cloned fused to nisin leader.	New lantibiotic expression	This work
pNZ-*clos12*	CmR, putative peptide Clos12 cloned fused to nisin leader.	New lantibiotic expression	This work
pNZ-*clos14*	CmR, putative peptide Clos14 cloned fused to nisin leader.	New lantibiotic expression	This work
pNZ-*clos15*	CmR, putative peptide Clos15 cloned fused to nisin leader.	New lantibiotic expression	This work
pNZ-*clos16*	CmR, putative peptide Clos16 cloned fused to nisin leader.	New lantibiotic expression	This work
pNZ-*clos17*	CmR, putative peptide Clos17 cloned fused to nisin leader.	New lantibiotic expression	This work
pNZ-*clos22*	CmR, putative peptide Clos22 cloned fused to nisin leader.	New lantibiotic expression	This work
pNZ-*clos24*	CmR, putative peptide Clos24 cloned fused to nisin leader.	New lantibiotic expression	This work
pNZ-*clos25*	CmR, putative peptide Clos25 cloned fused to nisin leader.	New lantibiotic expression	This work
pNZ-*clos*DP	CmR, putative double peptide lantibiotic cloned fused to nisin leader (each one).	New lantibiotic expression	This work

Table 2. Plasmids used in this work

CmR: chloramphenicol resistance. EryR: erythromycin resistance. AmpR: ampicillin resistance

Name	Sequence (5'→3')	PCR conditions	Description/ Purpose
Pep-fwPep	ATCTTGTTTCAG**U**TTCAAAAAAAGATTCAG GTGCTAGCCCACGT	1x 95°C 180'', 30x (95°C 30'',58°C 30'',68°C 60'') 1x 68°C 90''	Amplification of DNA fragments containing putative genes.
Pep-rvXhoI-USER-Rv	ACCGCATGCT**U**CTCGAGGGTTTTCTAATTT TGGTTCAAAG		
pN-USER-fw	AAGCATGCGG**U**CTTTGAACCAAAATTAGAA AACCAAGGCTTG	1x 95°C 180'', 30x (95°C 30'',58°C 30'',68°C 240'') 1x 68°C 300''	Vector backbone amplification
Leader-USER-rv	ACTGAAACAAGA**U**CAAGATTAAAATCTTTT GTTGAC		
pNZ-Cm-fw	CATGCAGGATTGTTTATGAACTCTATTCAG GAATTGTCAG	1x 95°C 180'', 30x (95°C 30'',58°C 30'',68°C 60'') 1x 68°C 90''	Colony PCR
pNZ-SphI-rv	TCGCCGCATGCTATCAATCAAAGCAACACG TGC		

Table 3: Primers and PCR conditions used in this work

2.2 Genome Mining

Identification of novel putative lantibiotic genes was done with BAGEL3 and Anti-SMASH genome mining software. Genomes from almost 600 *Clostridium* sps. from NCBI database had been screened. BAGEL3 software could be accessed on http://bagel2.molgenrug.nl/index.php/bagel3 and for Anti-SMASH via https://antismash.secondarymetabolites.org/#!/start. Selected putative lantibiotic genes (PLG) cloned fused to nisin leader and under nisin promoter control in pNZ8048 plasmid for their heterologous expression in *Lactococcus lactis* NZ9000.

2.3. Molecular Cloning

For molecular expression of putative lantibiotics, two plasmid expression systems were used, pTLR-BTC (with *nis*BTC) and a pNZ8048 plasmid containing the candidate genes fused to nisin leader. Putative core peptides were ordered from GenScript. The core peptides were codon optimized for *L. lactis* assisted by Jcat program (Grote et al., 2005). In case of double peptide antimicrobial (ClosDP), only the cores of each peptide were optimized, and the putative leader sequence of each one replaced by nisin leader. The PLG were fused with the nisin leader, replacing the core of nisin in pNZ8048-*nis*A using USER method (Bitinaite et.al, 2007). For this, the backbone (pNZ8048-*nis*A without the core nisin) and each new peptide were amplified with a couple of primers: Leader-user-

rv/pNZ-user-f and Pep-rv/ Pep-fw respectively (Table.3). pUC57-*clos*Mix vector was isolated from *E. coli* and digested with FD *XhoI* (Thermo Scientific) for 4h according to the suppliers. Afterwards, each fragment was amplified by PCR using specific primers for USER ligation and *Pfu7x* enzyme was added (Nørholm, 2010). PCR conditions and primers used in this work (for cloning and checking) listed in (Table.3). The backbone PCR products were digested with FD *DpnI* digestion enzyme to remove template DNA and avoid false positive. The PCR products were purified before the ligation using Nucleospin Gel & PCR-Clean Up kit (Macherey-Nagel©).

For USER reaction, 1:1 molar ratio of backbone and inserts were mixed with 1µl of USER enzyme and 1µl of 10X T4 DNA ligase buffer completing to 10 with MilliQ water. The mixture was left at 37˚C for 1h and then another hour at 24˚C (Bitinaite & Nichols, 2009). Finally, the ligation product was dialyzed for 20 minutes over MilliQ water and transformed in *L. lactis* NZ9000 pTLR-BTC competent cells. Preparation of competent cells and transformation in *L. lactis* was performed according to (Holo & Nes, 1995). Electroporation was performed using BioRAD Gene Pulser with the parameter 2.5kV, 200 Ω, 25 µF. After transformation, the cells spread on plates with the appropriate antibiotics, and incubated at 30˚C for 24-48h. 40 colonies were selected for screening by colony PCR step using *pNZ-Cm-fw* and *pNZ-SphI-rv* primers (Table 3.).

Plasmid isolation was done using a commercial plasmid isolation kit (Macherey-Nagel©). An additional step with lysozyme (40mg/ml) and incubation at 37˚C (20min) was added in case of *L .lactis* plasmid isolation. The constructs were checked by sequencing (Macrogen).

2.4. Peptide Expression and Purification

For peptide expression, *L. lactis* NZ9000 pTLR-BTC pNZ-Clos$_x$ were grown in 5ml of Minimal Expression Medium (MEM) supplemented with 0.5% glucose, 1% of vitamin also selected antibiotic markers (chloramphenicol and erythromycin). Afterwards, incubated at 30^{0}C for overnight (Rink et al., 2005). The overnight culture were transferred into the larger volume of the same medium (2% inoculum in 200ml total volume) and induced with 4 ng/ml nisin (Sigma) after initial incubation at 30^{0}C (OD600: 0.3-0.4). Subsequently, the cultures incubated 18h at 30^{0}C to continue the peptides production. Finally, the supernatants (SN) were separated from cells by centrifugation at 4 °C for 20 min, with 5000 rpm (Avanti J-25 Beckman-Coulter).

In a first approximation, the new peptides were concentrated from the SN using reversed phase chromatography by C18. Briefly, the SNs were applied to a column with two grams of C18 previously reconstituted in 100% of solvent B (Acetonitrile 0.1%TFA) and equilibrated with solvent A (MilliQ water 0.1% TFA) at 2 ml/min flow. Afterwards, the matrix were washed with 25 ml of water, and the peptides joined were eluted with 25ml of a gradual amount (10 to 60%) of solvent B in A. Each fraction was finally lyophilized using FreeZone 4.5 Liter Benchtop Freeze Dry System (LabConco) for 48h to remove all the solvents before the antimicrobial test assay.

Subsequently, a cationic interchange purification with SP Sepharose HiTrap was applied to the previous active fractions to obtain high purity peptides. Columns were washed with 25 ml of wash buffer (50mM lactate pH 6), then with 25ml of elution buffer (50mM lactate pH 4, 1 M NaCl) and another one with 25 ml of wash buffer. The active fractions from C-18 were mixed 1:1 with dilution buffer (100mM lactate pH2.5) and directly applied to the column at 1ml/min flow. Finally, the column was washed with 25 ml of wash buffer and peptides eluted with 12 ml of elution buffer.

High purity peptides were obtained by high-pressure liquid chromatography (HPLC, Agilent) on an Aeris wide pore phenomenex 250 C_{18} column (4.6mm 3.6um, XBC18). A linear gradient 20% to 60% of solvent B was applied for 25 min to separate the peptides. The fractions obtained from HPLC were lyophilized, and final purified peptides were dissolved in 500 µl of MQ water, then used for antimicrobial assay.

2.5. Purification of NisP

Nisin peptidase (NisP) was purified from the supernatant of the strain *L. lactis* NZ9000 pIL-253 pNZ8048-*nis*P8H. Briefly, 1L of MEM medium was inoculated at 10% with an overnight culture of *L. lactis* NZ9000 pIL-253 pNZ8048-*nis*P8H. When the OD600 reached 0.3-0.4, the culture induced with 5ng/ml of nisin and left at 30°C for 16 hours. The cells were removed by centrifugation and the supernatant was applied on Histrap Excel column previously equilibrated in 6 vol of NBB buffer. The column was washed with 4 vol of NWB buffer and then the peptidase were eluted with 12ml of NEB buffer and storage at -80°C (Table 4).

Solution(s)	Compositions
Solution A (10x)	200mM NaH2PO4, 5M NaCl, water to 1L
Solution B (10X)	200mM Na2HPO4, 5M NaCl, water to 1L
NPB (Native Purification Buffer) (5X)	250mM NaH2PO4, 2.5M NaCl, water to 200ml pH:8
Imidazole (100ml)	3M imidazole + 8.77ml of solution A + 1.23 ml of solution B. pH 6 water until 100ml
NBB (Native Binding Bufer)	30ml of NPB (1X)+ 100µl imidazole, pH 6.
NWB (Native Wash Buffer)	50ml NPB (1X)+ 335µl imidazole, pH 6
NEB (Native Elution Buffer):	13.75ml of NPB (1X)+1.25ml imidazole, pH 6

Table 4: Aqueous solutions for NisP purification

2.6. Antimicrobial Assay

5 µl of concentrate peptides were used to check the antimicrobial activity by the spot-on-lawn assays and 0.7% of GM17 and RCM (Reinforced Clostridium Medium) semisolid agar were used for the assays. To release the nisin leader peptide, 2 µl of purified NisP protease were added on the overlayer previously inoculated with the indicator strain (*L. lactis* and *M. flavus*) and then, 5 µl of HPLC purified peptides were dropped on the same spot, left until dried, and finally incubated at 30^0C overnight. Also, for *C. sporogenes* was incubated in RCM medium, anaerobically at 37^0C for overnight.

2.7. MALDI-TOF and LC-MS/MS Analysis

For checking the presence of peptides, matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) spectra analysis was performed. To do this, a mixture 1:1 vol : vol of the peptides fractions from HPLC with the matrix, α-cyano-4-hydroxycinnamic acid (CHC) were performed and spotted to dry in the MALDI plate. The matrix for MALDI was prepared to mix 3-4 mg/ml of CHC in 50% acetonitrile 0.1% TFA. Mass spectra were documented with a Voyager-DE Pro (Applied Biosystems) MALDI-TOF. To increase the sensitivity, the calibration was applied with six different peptides provided by the program (protein MALDI-MS calibration). For obtaining more accurate peptides identification especially their dehydration level and other modifications, Liquid Chromatography with Tandem Mass Spectrometry (LC-MS/MS) was applied to the purified peptides (contained nisin leader peptide) of each $Clos_x$ peptide. Preparation for LC-MS/MS samples were done by lyophilized a mixture of HPLC purified sample (1ml), and subsequently, the freeze-dried samples were sent to the LC-MS/MS operator in ERIBA building, Universitair Medisch Centrum Groningen (UMCG).

Chapter 3. Result and Discussion

3.1. Lantibiotic Gene Mining In *Clostridium* Spp.

This study focused on the identification of new lantibiotics codified in the genomes of several *Clostridium* spp. and subsequently, produce them under nisin controlled system in *L. lactis*. For this purpose, two bioinformatics program had been used. Firstly, a high screening genomic data has been accomplished using anti-SMASH and then small putative lantibiotic ORF has been identified using Bagel3. The selection of this genus has been decided based on the fact that antimicrobial peptides from *Clostridium* are difficult to produce due to the lack of information for biosynthesis and difficult growth conditions in the lab (Kemperman et al., 2003).

3.1.1) Selection Of Genomes For Gene Mining

The different genomes used in this work were searched in NCBI Genome for *Clostridium* (https://www.ncbi.nlm.nih.gov/genome/?term=clostridium). According to this page, 563 genomes from 110 *Clostridium* species that are completely sequenced and stored in Genebank NCBI (Fig.5) have been screened. The species of *Clostridium* used for this work were: *C. botulinum, C. perfringens, C. butyricum, C. novyi, C. beijerinckii, C. tyrobutyricum, C. pasteurianum, C. tetani, C. acetobutylicum, C. haemolyticum, C. intestinale, C. colicanis, C. sporogenes, C. celatum, C. carboxidivorans, C. baratii, C. cellulovorans, C. ljungdahlii, C. grantii, C. cavendishii, C. collagenovorans, C. estertheticum, C. acetireducens, C. ragsdalei, C. magnum, C. tepidiprofundi, C. homopropionicum, C. cylindrosporum, C. argentinense, C. akagii, C. hydrogeniformans, C. lundense, C. ihumii, C. senegalense, C. saccharobutylicum, C. autoethanogenum, C. paraputrificum, C. diolis, C. tunisiense, C. tetanomorphum, C. sartagoforme, C. arbusti ,C. cadaveris, C. saccharoperbutylacetonicum, C. bornimense, C. aceticum, C. fallax, C. amylolyticum, C. saudii, C. jeddahense, C. frigidicarnis, C. uliginosum, C. gasigenes, C. cochlearium, C. formicaceticum, C. taeniosporum , C. septicum, C. coskatii, C. neonatale, C. polynesiense, C. disporicum, C. sulfidigenes, C. algidicarnis, C. drakei, C. scatologenes, C. kluyveri, C. ventriculi, C. citroniae, C. clostridioforme, C. innocuum, C. hiranonis, C. hylemonae, C. ultunense, C. saccharolyticum, C. methylpentosum, C. papyrosolvens, C. bolteae, C. symbiosum, C. cellulosi, C. clariflavum, C. asparagiforme, C. leptum, C. spiroforme, C. lactatifermentans, C. populeti, C. polysaccharolyticum, C. fimetarium, C. lavalense, C. cocleatum, C. paradoxum, C. neopropionicum, C. glycyrrhizinilyticum, C. dakarense, C. purinilyticum, C. aminophilum, C. aerotolerans, C. saccharogumia, C. cellobioparum, C.*

viride, C. straminisolvens, C. sporosphaeroides, C. josui, C. indolis, C. sphenoides, C. celerecrescens, C. stercorarium, C. termitidis, C. propionicum and C. scindens. 43 strain of *Paeniclostridium sordelii* recently separated to the genera *Clostridium* also were analyzed.

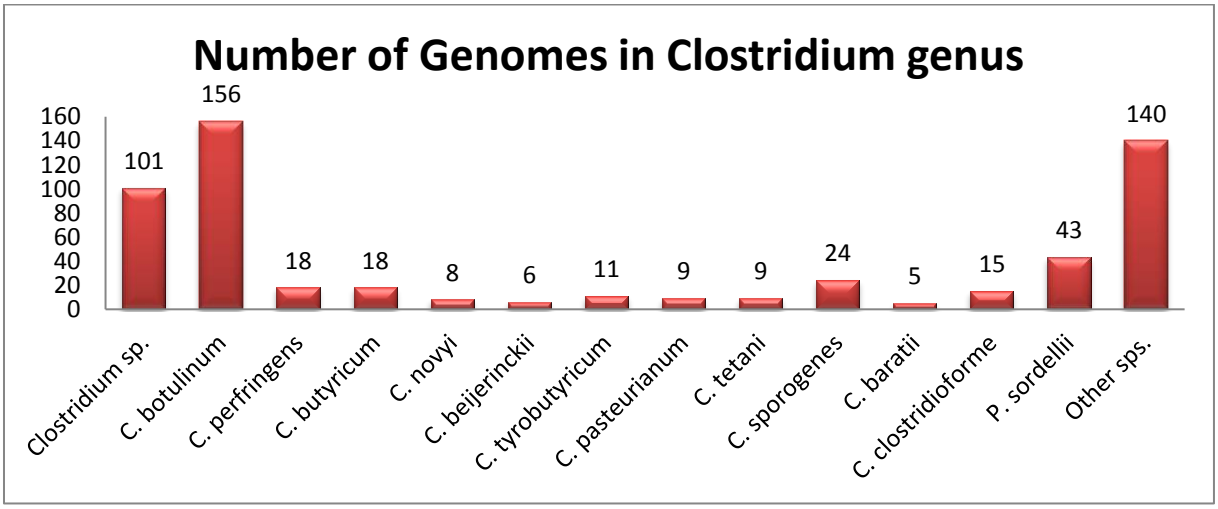

Fig.5: Number of genomes from different *Clostridium* species observed in this study.

All genomes were uploaded ten by ten to AntiSMASH website for first selection of possible lantibiotic sequences identification. From the 563 initial genomes, only 17 genomes were identified harboring lantibiotics of interest. They were downloaded and analyzed with Bagel3.

3.1.2) Putative Lantibiotic Areas Of Interest And Selected New Lantibiotics

54 PLG clusters were detected by AntiSMASH and Bagel3 (Table.5). Among all putative lantibiotic genes, only 12 genes selected for heterologous expression in *L. lactis* NZ9000 pTLR-BTC. For heterologous expression, the presence of all modification enzyme in the same gene cluster (Fig.6), the presence of some lantibiotics related domain (FDLD in the leader, GG cut site), and the presence of cysteines and serines/threonines in the correct position considered as selection criteria.

Strain name	Putative lantibiotic	Homology (BLASTp)
C. beijerinckii HUN142	MIKLDDFDLKIKKDDNKTGKVTPQVNSRYACTPGSCWKWVCFTTTAK	Streptin
	VGKLDDFDLDVKVKINSKKGIKPSYLSLTPKCTSLCPTNVFVCISKRCK	Gallidermin
	MAKLGDFDLDLKVKIKPKGGVTPATVSRFNCTLFGCIKVKDNI	Streptin
	MGKLDDFDLDVKVKATPKGGVKPSITSRILCTSSCYTQFIQCHDRV	Gallidermin
	MGKLDNFDLDVKIKKDEKRGVKPSVTSYSACTPGCATSLFRTCLTRSCKGC	Gallidermin
C. botulinum H04402 065	MKNNEVCKNAGFISEDELVELVDNSDISGGTAASAAAVSATVASATAVSALFTVTSACTTKCK	HP
	MIKNPIKRQSEDVKLPCGDTKVEITENQGLDVTGGTFSEGTISITLSVYMGNDGKVCTWTVECQNNCSHKK	Two peptide lantibiotic
	MYFNFYRVVFGFLCYLHCIDIEKDYIYIIKSQK	HP
C. botulinum strain CDC41370	MKNNEICKNAGFISEDELVELVDNNDISGGTASAISATVASATAVSALFTVTSACTKKCK	HP
	MIKNPIKRQSEDVKLPCGDTKVEITENQGLDVTGGTFSEGTISITLSVYMGNDGKVCTWTVECQNNCSHKK	Two peptide lantibiotic
	MRYRYIQGEDKFIYMLSIIDALDRSIIDYHMKFRCESEDVIELTDKGLIGRGWCNKTYYKN	HP
	LDILCLRLFIDISLALQAVQTPTEALILHSDLEYQHTSSSFKEYINRVLN	Transposase s
	MSNFNEFELDLQNEKIQNEAASERVKFTTWDCVASSIFNCPTLKCPTKGVLVCPQPPKPVNTKSQCSSTASCRTTFKK	Transposase s
	MEDKAQFGGHRGAASPKLQRKVPQSTAKYRKVGHSEWYNEYSDTPWHQAERVKTVQLVLGMPKKQIFCKI	HP
	MGKMDDFDLDLRKIAENGNSANALSASDMITSEIISKVTETITRTFKGQCVSVETPTTGMTSACCKKGGTDVEPQCVP	HP
C. cellulovorans 743B 1	MVSFCTAAFPWVAFSIGLAVFLTYTNSKTKLKKMENKNF	HP
	MLCSVWKFFLDAFYHKLSSMSISGLYFFYVIIYIE	HP
	MTNYKIGQTFEQKNYEEMASCQVAGDGFIFTTNQITVSYCPTLTIPHIPTITTPKLTIQ	HP
	MANYKIGAIFEQKNYEEMASSQMTGGDGFVTVTSPQYTLSCCITWTIPSIKLTV	HP
	MQNYESKAGFISEMELDELVSNKTVGGATTVPCAIAIIGITLSAGICPTSACSKDCPWNN	Two peptide lantibiotic
	MKNYEELFNEVNENASLQAELNGGSIATTIVCTIAQSLLGCVGSYVLGNKGYGCTVTNECMSNCR	Lantibiotic alpha
	MGSLKKITLEDLDLDFQIGDNNEEFLYLGGDSKGKLDLVGSPSVINSSLNFIQFIKTNRPVTKYTYSERGCC	HP
	MGDLKKLNLSDLDLDMQVGEINEEFINVSGEGKGDYSGSAASIAYSMMTLGQYWKGDTSTAKYTYSERGCC	HP
C. hylemonae DSM 15053	MQREEKNVEITGDLSLEFKEMQKLVDEEVGVPYSTWSKACTTFFTIICC	HP
	IIQTILSVCGGISIIGGTGAIVWKCCGRRSKLPSE	HP
C. ihumii AP5	MPNYKEFDLDIRNEKNNLKSMNSKKRSDGGTCYYSCGCKTNEGNSCGKVCFTDTIVCGTDFDGR	HP
	MPNYKDFDLDLQNNKLSKKYESVNRGNKTYNRDCGYKTHEPDSCGNSCFTSAKCDWA	HP
	MPNYKDFDLDIRNVRFQGRIDDRPGRTSIEVKEQSICIACKPKTGGSGSGGGSKTHEDSAW	HP
	MPNYKDFDLDIQNIKMNKINDKRRYPISDKRDDMSMCVCKKTDVCKTHETDSCNNGLCFESGKCTWV	HP
	MPNYKDFDLDIQNSKLGVDSSRKVLPPTFSYEYDKLSECRCRPKTQTCATHCSCATYCNGSCNQHTDCAL	HP
	MPNYKEFDLDIRNSKNGINMYGPSAVIVPATDGGGKKTVCGRTCNGSACNPNSCQTRCIKPAD	HP
	MPNYKDFDLDIQNNKSSVNSIKTTTMPPTFSYEYDQYSECVCKPKTRNSCVTYCNGSCNQHTDCTL	HP
	MPNYKDFDLDLRNNTVKGNNSKNENIQGKKKTDGCYTYGNRSCPNTMCAY	HP
	MPNYKDFDLDIQNNKGSSKTSLGKELSNTGNYYDPLSECRCKPKTYTKRLKTCNVKECMY	HP
	MPKYNDFNLDIQTDNKNCHTTKLTIEVKHKENKGGNMATWSTHCY	HP
	MPNYKDFDLDLTNKKVITNEIKDKTIQANIARTYGPVKSCQYICRLSENIEDCQ	HP
C. josui JCM 17888	MCGPWELYLKYTFLSSIFPHSFLAVCLLSDESGGVDKIGVSISIVE	HP
C. perfringens B ATCC 3626	MSEIDSKKIVGDTFEDMSIWEMTMVQGSGDMEPNSLTVASAVLMSAAATGSFSIAVTKTVKGKC	Cytolysin
	MSEINMKKIVGDTFEDMSIAEMTLVQGSGDVNGEVTTSPACVYVSVAASRASSQKCGQAAGAIASFVSTAVLSAVKC	Cytolysin
C. perfringens CPE str. F4969	MMKQLDKKSKTGIYVQVASDKELELLVGGAGAGFIKTLTKDCPEVVSQVCGSFFGWVSACKNC	Lantibiotic type II
C. perfringens D JGS1721	MMKQLDKKSKTGIYVQVASDKELELLVGGAGAGFIKTLTKDCPEVVSQVCGSFFGWVSACKNC	Columbicina A
	LFYKVLLLLGWRDKMKLIRIISGVVSIFFIGCAAYGYYSSKTLYLADVILGLIAISVFAFSFLKNSKNN	HP
C. senegalense JC122	MSNYNDFDLDLKMVSENGAQSKGVSDYTVDIITSALTCWVKISKALNCTNGRECAMPTKDRPAASCHRAMAGAVQA RC	HP
C. sordellii W10	MSNFNEFELDLQNEKIQNEAASERVKFTTWDCVASSIFNCPTLKCPTKGVLVCPQPPKPVNTKSQCSSTASCRTTFKK	HP
Clostridium sp. ASF502	MRTACTRRSSTTGRICARNPCARDRAGIRKRHGVTAQAGSRCQE	HP
	MSTSLYQNLIQTANQFCNQYPSCPYDSCSIK	HP
Clostridium sp. BNL1100	MTSLLSPLKISIVLNPTFYTIILRKANGIKNIRKLCWTSAIHINPKTSSTNN	HP
	LVLWTVPKVCCGGKLIGYNLRGLFPDVNKACFHK	HP
Clostridium sp. KLE 1755	MGKMDDFDLDLRKIAENGNSANALSASDMITSEIISKVTETITRTFKGQCVSVETPTTGMTSACCKKGGTDVEPQCVP	HP
	LLAKTRKTENNLFSNHNIEGIKLCQREICYTAPKRYGGEVCESD	HP
Clostridium sp. BR31	MDDFDLDLRKIAENGNSANALSASDMITSEIISKVTETITRTFKGQCVSVETPTTGMTSACCKKGGTDVEPQCVP	HP
Clostridium sp. KNHs205	MEVKEMTTKVTRVKTGNQFNHPAGDIPAEISEIVSLRESKNPDAIYTITVGCSGFLTLICC	HP
C. saccharobutylicum DSM 13864	MRETLLVVLMLLFVILAAAMIFPMPPIIYGAIMSLIILCIIIIISIYILIIKKNH	HP

Table.5. List of putative lantibiotic genes discovered in the genus of *Clostridium*

Name	Source	Core peptide	MW	pI	GRAVY	S+T	C
Clos2	*C.beijerinckii* HUN142	YLSLTPKCTSLCPTNVFVCISKRCK	2805.42	9.21	0.34	6	4
Clos4		ITSRILCTSSCYTQFIQCHDRV	2574.97	7.97	0.15	6	3
Clos5		VTSYSACTPGCATSLFRTCLTRSCKGC	2817.28	8.80	0.27	9	5
Clos12	*Clostridium ihumii* AP5	TCYYSCGCKTNEGNSCGKVCFTDTIVCGTDFDGR	3649.04	4.68	-0.28	7	6
Clos14		ISDKRDDMSMCVCKKTDVCKTHETDSCNNGLCFESGKCTWV	4619.26	5.57	-0.58	8	6
Clos15		TFSYEYDKLSECRCRPKTQTCATHCSCATYCNGSCNQHTDCAL	4844.38	6.60	-0.67	10	8
Clos16		ATDGGGKKTVCGRTCNGSACNPNSCQTRCIKPAD	3414.82	8.78	-0.73	6	5
Clos17		TFSYEYDQYSECVCKPKTRNSCVTYCNGSCNQHTDCTL	4392.82	5.45	-0.81	9	6
Clos22	*C. perfringens* D JGS1721	AGAGFIKTLTKDCPEVVSQVCGSFFGWVSACKNC	3554.13	7.91	0.45	5	4
Clos24	*Clostridium sp.* BR31	VTETITRTFKGQCVSVETPTTGMTSACCKKGGTDVEPQCVP	4291.91	6.20	-0.23	11	4
Clos25	*Clostridium sp.* KNHs205	ESKNPDAIYTITVGCSGFLTLICC	2548.97	4.37	0.65	5	3
ClosDP 1	*C.botulinum* H04402 065	TAASAAAVSATVASATAVSALFTVTSACTTKCK	3062,5	8,88	0,96	12	2
ClosDP 2		TFSEGTISITLSVYMGNDGKVCTWTVECQNNCSHKK	3982,49	6,42	-0,33	9	3

Table.6. Characteristic of core peptides from selected lantibiotic gene candidates

Genes represented in this work were based on the number of observed genes, except for putative class II two-component lantibiotic which described as ClosDP. From 12 putative lantibiotic genes observed, eight genes were identified as putative Class I lantibiotics group, and four genes were identified as putative Class II lantibiotics. Characteristic of selected core peptides was analyzed by ProtParam Tool (ExPASy) (Gasteiger et al., 2005) and putative leader cleavage manually identified according to other putative cleavage sites (Table.5). Furthermore, for expression system, all putative genes were fused into the common nisin leader sequence and cloned into *L. lactis* NZ9000 by USER cloning approach (Bitinaite & Nichols, 2009).

Theoretical molecular weight of each peptide could be predicted and used as references when doing mass spectra analysis. Theoretical molecular weight is important to be known in case of novel peptides analysis since renowned molecular weight references about them are still unknown. Based on molecular weight prediction, all of the putative lantibiotics observed in this study could be considered as small peptides which have a molecular weight less than 5kDa (McAuliffe et al., 2001) with Clos25, had the lowest molecular weight while Clos15 had the highest amount. Next, the theorical isoelectric point of each peptide also could be estimated by this tool. In general, most of the lantibiotics are cationic antimicrobial peptides which dominantly assembled by positively charges amino acid residues, and thus they usually have high pI value, but there are lantibiotics which considered as negatively charged such as the type-B lantibiotics such as mersacidin (Islam et al., 2012). In case of lantibiotics, the information about pI is not just related to the purification process, but also can give the insight about mode of action from lantibiotics, for example, the positive charges in lantibiotics play a vital role for binding on targeted membranes with high affinity (Hasper et al., 2004). From the data shown in Table.6, most of putative lantibiotic candidates have high pI value while a few of them have low pI value due to the excess amount of negatively charged amino acids in their core sequences. Another valuable property of putative peptides is their interaction with water molecules. The term that been used to know this property is GRAVY (Grand Average of Hydropathicity), the more positive the value, the more hydrophobic are the peptides. Hydrophobicity could affect how peptides will interact with targeted cell membranes. In case of nisin, hydrophilic residues of peptide will bind to phospholipid groups of cell membranes and followed with penetration of hydrophobic side chains of peptide into hydrophobic core of membranes leading to pore-formations (Weidemann et al., 2001). Nevertheless, hydrophobicity of each putative lantibiotic in this study shown in Table.6. In addition, the number of serines/threonines/cysteines residues of each peptide are also important to be known, because the number of these residues representing the theoretical dehydration level or modification that may emerge for each peptide (Table.6).

3.1.3) The Organization Of Gene Operon From Selected Putative Lantibiotics

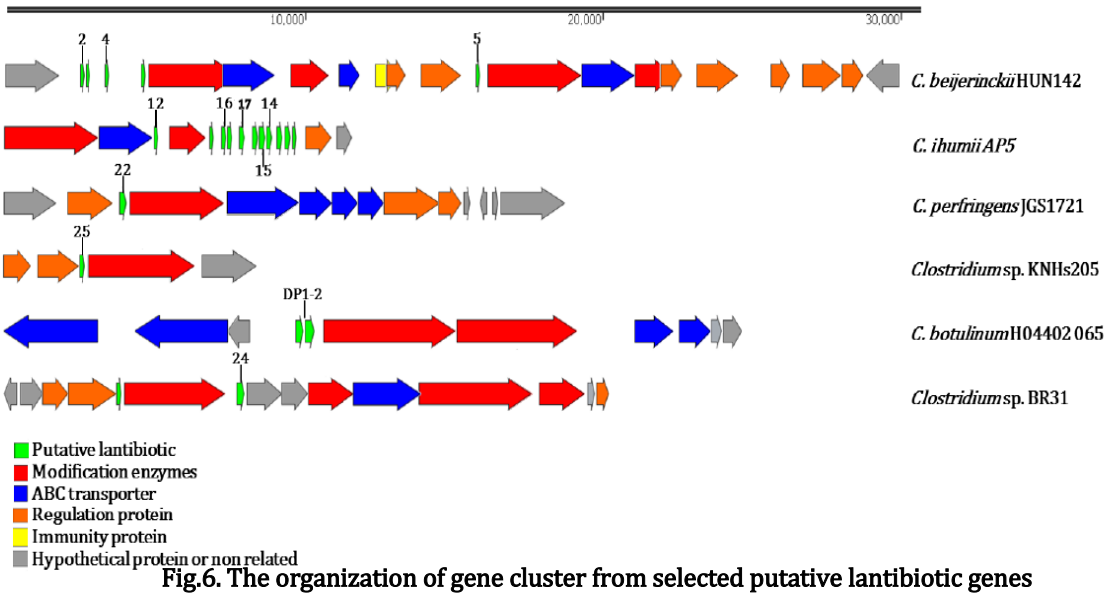

Fig.6. The organization of gene cluster from selected putative lantibiotic genes

In silico analysis of each selected putative lantibiotics also uncovered their gene clusters organization. In case of putative lantibiotics from *C. beijerinckii* HUN142, observed genes designated as *clos*2, *clos*4, and *clos*5 in this study. There are complete set of biosynthetic genes including putative modification enzymes, ABC Transporters, and regulator proteins (Fig.6). Also, putative immunity protein observed in this gene cluster showing similarity to the typical class I lantibiotic biosynthetic genes cluster, in this case, gallidermin/nisin like family based on BlastP homology (NCBI) analysis. Putative class I lantibiotic genes also discovered in *C. ihumii* AP5. *clos*12, *clos*14, *clos*15, *clos*16, and *clos*17 are positioned adjacent to each other and organized together with putative modification enzymes, ABC Transporter and putative regulation protein. Additionally, *clos*14 showed peculiar characteristic as lantibiotic since it has two methionine residues in the core sequence and also it is an anionic peptide. One single component lantibiotic from Class II lanthipeptides also found in *C. perfringens* D JGS1721 coded as *clos*22 and identified as type A(II) lantibiotic which known has two domains, a linear N-terminal region, and globular C-terminal region. This type of lantibiotics show slightly different mode of action to the type A(I) lantibiotic which is only could bind lipid II and inhibit cell wall synthesis. Notable example of this group is Nukacin ISK-1 (Asaduzzaman et al., 2009) and most of lantibiotics in this group are unable to execute pores formation (Islam et al., 2012) or just create unstable pores such as streptococcin A-FF22 (Jack et al., 1994). Other putative lantibiotic genes also found in *Clostridium sp.* BR31 and *Clostridium sp.* KNHs205 these genes coded as *clos*24 and *clos*25 respectively. Taken from the newest study about *Clostridium sp.* BR31, this species previously was known as novel species in the genus of *Clostridium* as it still listed in *Clostridium* genome database when this study started, but recently it had a new order in taxonomy and declared as a new genus in

Clostridium cluster XIVa, in the family *Lachnospiraceae*. This species now is designated as *Merdimonas faecis gen. nov., sp. nov* (Seo et al., 2017). Interestingly, the genome mining in this study also could observe the putative class II lantibiotic, a unique two-component lantibiotic found in one organism, *Clostridium botulinum* H04402 065. Both of these genes coded as *clos*DP in this work.

3.2. Peptides Purification Result

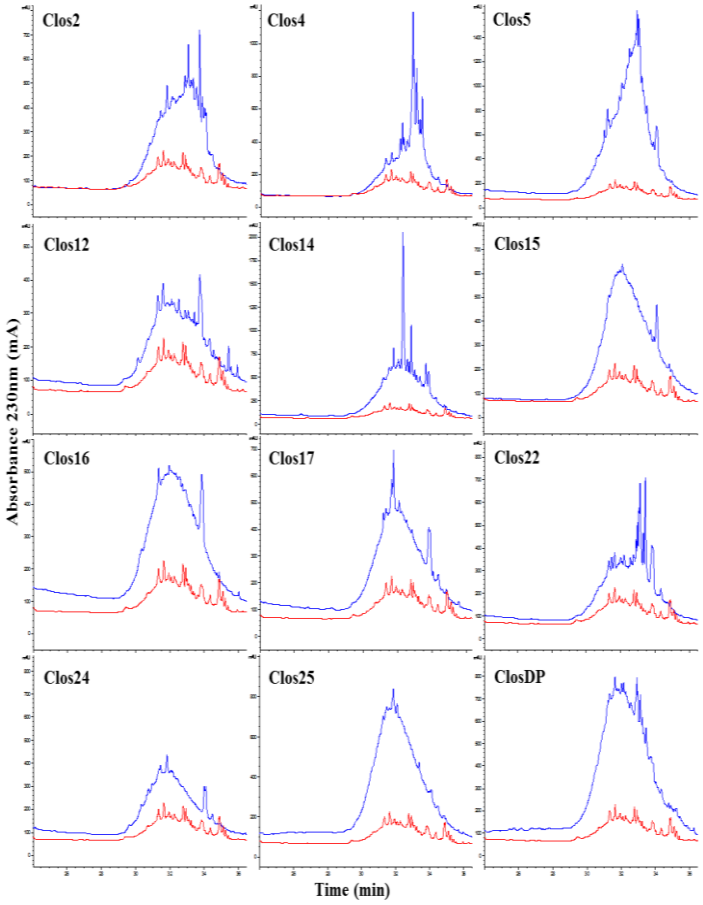

Fig.7. The HPLC profile of all twelve novel clostridia lantibiotics
Red lines are negative control

After the initial purification step with C18 and sephadex, finally the peptides were purified to homogeneity by HPLC. This step resulting high purity of each peptide, and the negative control generated from the same strain but without any genes of interest or empty expression vector (*L. lactis* NZ9000 pTLR-BTC-pNZ8048). The peptides production level varies for each peptide which most of them were produced in low amount with 200ml cultures except for Clos4,Clos5 and Clos14, the HPLC graphs also showed different typical motifs for each peptides meaning that twelve putative clostridia lantibiotics were successfully produced (Fig.7).

3.3. Antimicrobial Assay

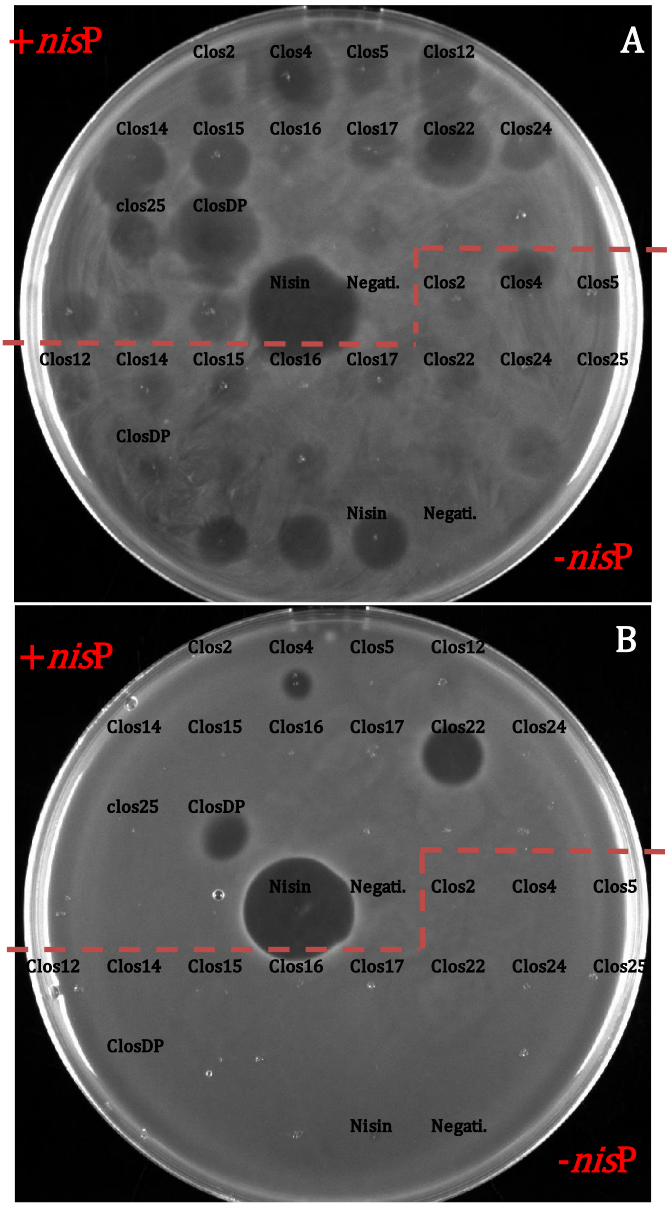

Fig.8. Antimicrobial assay against *L.lactis* NZ9000 (A) and *M.flavus* (B) strain using HPLC purified peptides from 200ml cultures
positive control : *L. lactis* NZ9000 pIL3-BTC pNZ8048-*nis*A peptide
negative control : *L.lactis* NZ9000 with empty pNZ8048 expression vector peptide

Based on antimicrobial assay against *L.lactis* NZ9000, eleven novel lantibiotics produced in this study displayed antimicrobial activity after cleavage of leader peptide (except Clos16). Both positive control and negative control worked properly in this experiment. In case of ClosDP antimicrobial activity, the release of nisin leader peptide was necessary since the negative result without NisP. Despite some activity showed on the side without NisP, the activity of purified peptides were better after cleavage of leader peptide.

Furthermore, for antimicrobial activity against *M.flavus* (Fig.8B) Clos4, Clos22, ClosDP (and to a lesser extent Clos12) also displayed potent antimicrobial activity. In case of assay against *C. sporogenes*, there were no significant inhibition from the samples that produced in 200ml cultures. Additionally, the potent activity against the two indicator strains might indicate that the lanthionine rings formed in eleven peptides, except for Clos16. The lanthionine rings are essential in mode of action especially for lipid-II binding and also the stability of peptides against protease (Hsu et al., 2004; Kluskens et al., 2005).

3.4. Characteristics Of Novel Lantibiotics Based on MALDI-TOF and LC-MS/MS analysis

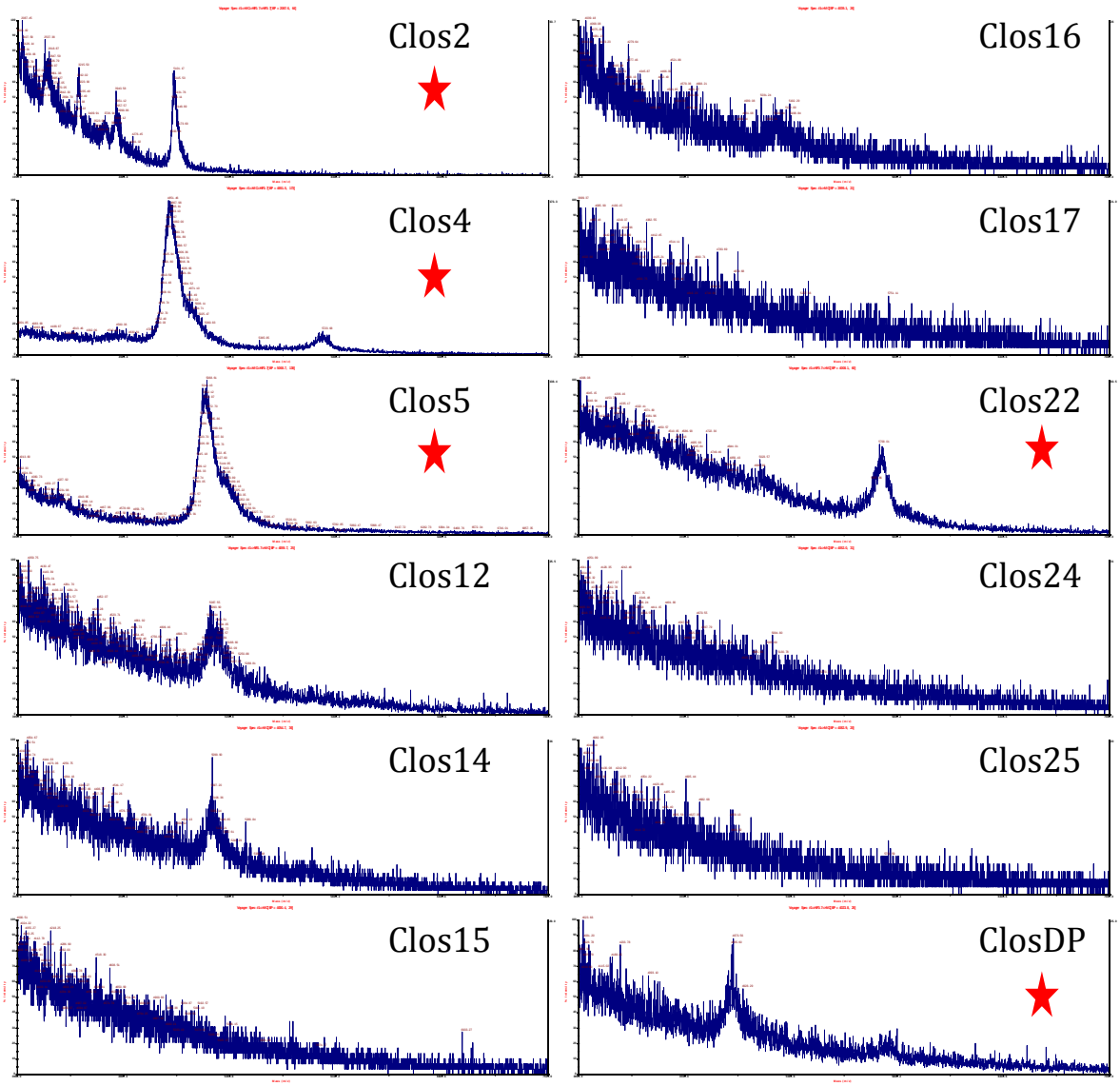

Fig.9. The MALDI-TOF spectra analysis of all the purified peptides

To confirm novel lantibiotics production, MALDI-TOF mass spectra analysis were applied. Despite almost all of purified peptides showed antimicrobial activity but only five novel clostridia lantibiotic were detected (Clos2, Clos4, Clos5, Clos22 and ClosDP marked with a red star in Fig.9). Furthermore, to achieve more accurate peptide mass analysis, LC-MS/MS also used in this study. LC-MS/MS technique could increase sensitivity therefore resulting high throughput and high confidence in data quality. Recently, LC-MS/MS is gaining more attraction from the scientist primarily in the discovery of new drugs (Espada et al., 2008). In this study, this technique has been chosen to reveal dehydration level of these novel lantibiotics. The principle of this method is similar with MALDI-TOF by analyzing the mass of each peptide. Technically, one dehydration of serine-threonine resulted -18 Dalton of the mass peptide. Otherwise, the presence of methionine residues could increase the peptide mass by 16 Dalton (van Heel et.al, 2016). Methionine residues also may affect to peptides, because this amino acid could be oxidized, thus changed peptide forms. Nevertheless, oxidation also could weaken the peptide antimicrobial activity. Based on the LC-MS/MS analysis these five novel clostridia lantibiotics showed different characteristic each other against the negative control. The negative control was purified peptide generated from L. lactis NZ9000 pTLR-BTC-pNZ8048 empty expression vector (Fig.10 & Fig.11)

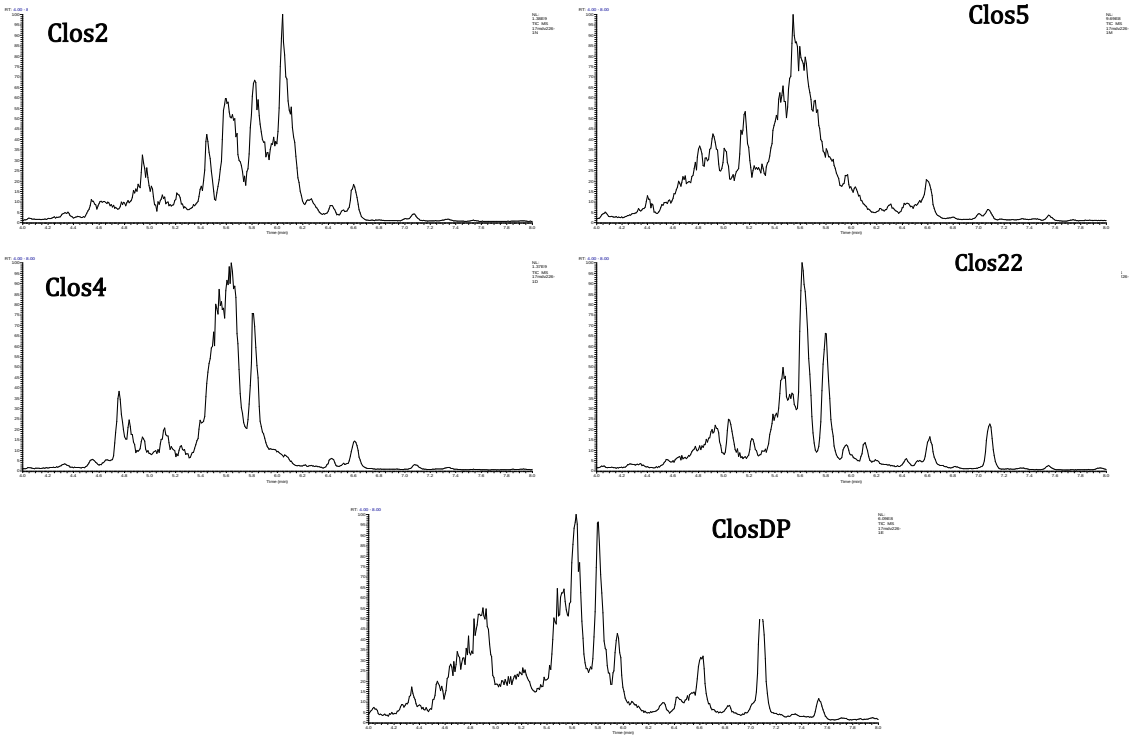

Fig.10. Chromatogram of five novel clostridia lantibiotics showed in LC-MS/MS.

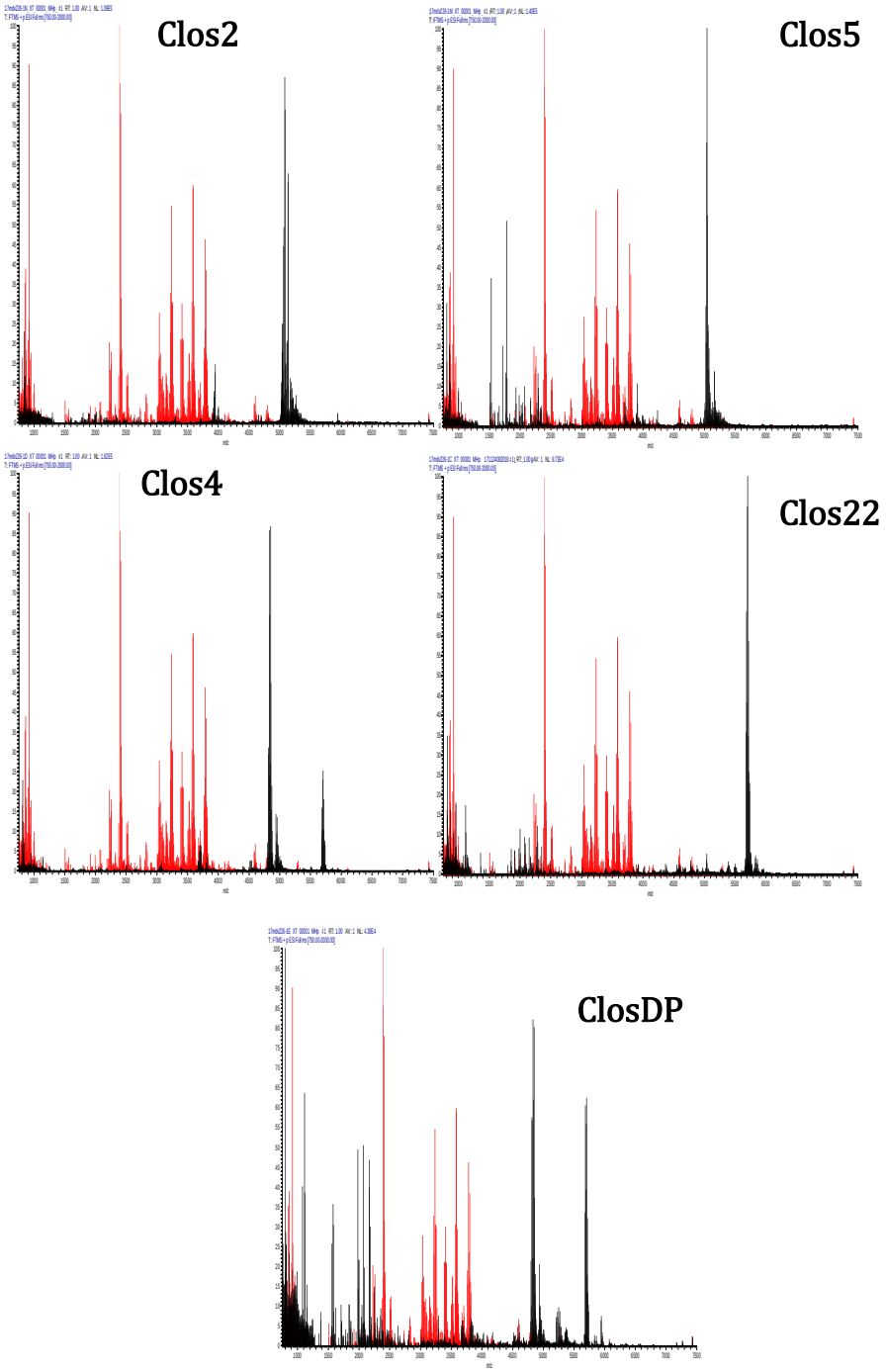

Fig.11. Masss spectra of five novel clostridia lantibiotics in LC-MS/MS graph. Red graph indicated the negative control.

Moreover, dehydration levels of 5 known novel lantibiotics in this study described in Table 7. The presence of dehydration levels varied in Clos2, Clos4, Clos5, Clos22, and ClosDP. The percentage showed in Table 7. represented the proportion of peak area from each dehydration level respect to the total of all peak areas. As shown in the Table 7., all of the novel lantibiotics produced in this study modified by NisB enzyme sufficiently.

Two of five possible dehydrations observed as the highest entity in Clos22. Other peptides reached the complete dehydration levels, but just in low amount. In Clos2, 3 dehydration levels are the highest entity, while in Clos4, 3 or 4 dehydrations, Clos5 6 dehydrations, ClosDP-1 9 dehydrations, and ClosDP-2 8 dehydrations respectively. Regarding to the number of cysteines, dehydration levels that observed in this study are still enough for the peptide to execute lanthionine ring formation. This study revealed that NisB enzyme could modify the core peptides at least 50-80% degree of modifications. The presence of dehydroamino acids following by lanthionine rings formation was essential for structural feature of lantibiotics. The structure of lantibiotic is strongly correlated to antimicrobial activity, especially in the fully modified lantibiotic (Kastin, 2013). In general, Ser-Thr dehydrations were influenced by the flanking amino acids (Moll et al., 2010). The residue positioned at the N-side of Ser/Thr residues seemed to be more governing on the extent of dehydration rather than the C-side. Therefore the dehydration started from N-terminal region after leader peptide (Rink et al., 2007; Khusainov et al., 2011). The more hydrophobic amino acid flanking to Ser-Thr is positively contribute to dehydration, while in contrast, a Ser-Thr residue which flanked by two hydrophilic residues could not be dehydrated. In fact, Ser residue is more difficult to be dehydrated than Thr residue (Rink et al., 2005). Based on core sequences of five novel lantibiotics, most of Ser-Thr residues were flanked to the hydrophobic amino acids that helped in achieving a mid-to-high degree of modifications for each peptide.

The unmodified peptides which had no dehydration of Ser and Thr residues also observed in Clos2, Clos5, Clos22, and ClosDP-1 (first part of the two components lantibiotic). Nevertheless, the unmodified peptides still can be obtained after purification due to the capability of NisT enzyme which could export both unmodified or partially and fully posttranslational modified forms of lantibiotic or non-lantibiotic peptides (Kuipers et al., 2004).

Peptide Sequence	S+T	Dehy Levels	Expected Mass	Observed Mass	%
Clos2 YLSLTPKCTSLCPTNVFVCISKRCK	6(3)	0 1 2 3 4 5 6	5136,63 5118,62 5100,61 5082,60 5064,59 5046,58 5028,57	5136,61 5118,60 5100,59 5082,59 5064,58 5046,57 5028,56	12,72 3,69 4,49 44,22 19,90 11,95 3,02
Clos4 ITSRILCTSSCYTQFIQCHDRV	6(3-4)	1 2 3 4 5 6	4887,40 4869,39 4851,38 4833,37 4815,36 4797,35	4887,37 4869,39 4851,38 4833,37 4815,36 4797,34	0,38 10,24 37,50 37,10 14,60 0,18
Clos5 VTSYSACTPGCATSLFRTCLTRSCKGC	9(6)	0 1 2 3 4 5 6 7 8 9	5148,47 5130,46 5112,45 5094,44 5076,42 5058,41 5040,40 5022,39 5004,38 4986,37	5148,42 5130,42 5112,41 5094,41 5076,40 5058,40 5040,39 5022,38 5004,38 4986,37	1,48 1,80 1,05 1,29 3,74 11,67 63,75 12,89 1,18 1,14
Clos22 AGAGFIKTLTKDCPEVVSQVCGSFFGWVSACKNC	5(2)	0 1 2	5883,86 5865,85 5847,84	5883,79 5865,80 5847,80	1,70 20,39 77,91
ClosDP-1 TAASAAAVSATVASATAVSALFTVTSACTTKCK	12(9)	0 1 2 3 4 5 6 7 8 9 10 11 12	3060,54 3042,53 3024,52 3006,51 2988,50 2970,49 2952,48 2934,47 2916,46 2898,45 2880,44 2862,43 2844,42	3056,84 3039,23 3026,46 3008,25 2989,12 2970,46 2954,08 2937,42 2919,31 2901,65 2883,20 2864,51 2845,44	1,82 0,94 0,53 0,61 0,27 0,56 2,71 18,47 31,70 38,38 3,17 0,45 0,39
ClosDP-2 TFSEGTISITLSVYMGNDGKVCTWTVECQNNCSHKK	9(8)	1 2 3 4 5 6 7 8 9	3961,80 3943,79 3925,78 3907,77 3889,76 3871,75 3853,74 3835,73 3817,72	3965,18 3944,59 - 3904,48 3886,31 3869,48 3854,43 3837,92 3817,90	10,11 8,14 - 10,88 8,51 10,58 8,04 34,83 8,91

Table.7. Modifications in novel clostridia lantibiotics analyzed by LC-MS/MS.
A number in bracket indicating the most abundant form of dehydration levels for each lantibiotic.

3.5. Remarks

The production system with 500ml batch cultures was performed to Clos2, Clos14 and ClosDP lantibiotics in the previous experiment of this study. The maturation process for each peptide was done by mixing NisP supernatant from *L.lactis* NZ9000 pIL3-253 pNZ-*nis*P8H strain and the supernatants of each peptide production directly after centrifugation in proportion 1:10 (NisP: Peptide SNs), and subsequently incubated at 30^0C for 18h. The purification steps were the same as described previously in Chapter 2. Antimicrobial assay was done by overlay assay using HPLC purified peptides resuspended with 500 µl of MQ water, against *M.luteus* and *C.sporogenes* C22/10 as sensitive strains. MALDI-TOF mass spectra were applied to check the presence of modifications by NisB enzyme and to confirm the production of the novel lantibiotics. In fact, the MALDI-TOF analysis could reveal the characteristic of Clos14 (Fig.14). Based on the mass analysis, Clos14 had two levels of dehydration which showed the highest peak of relative intensity and the presence of oxidized form due to methionine residues in this peptide were confirmed (Table 8. and Fig.14).

Peptide Sequence	S+T	Dehy Level(s)	Expected Mass Without Oxidation	Observed Mass Without Oxidation	Expected Mass With 1 Oxidation	Observed Mass With 1 Oxidation	Expected Mass With 2 Oxidation	Observed Mass With 2 Oxidation
Clos14	8(2)	0	4615,97	4613,10	4631,97	4632,99	4647,97	4647,60
ISDKRDDMSMCVCKKTDVCKT		1	4597,96	4596,33	4613,96	4613,09	4629,96	-
HETDSCNNGLCFESGKCTWV		2	4579,95	4581,91	4595,95	4596,33	4611,95	4613,09
		3	4561,94	4561,61	4577,94	4576,19	4593,94	-
		4	4543,93	4545,15	4559,93	4561,61	4575,93	4576,19
		5	4525,92	4526,46	4541,92	4540,25	4557,92	4556,83
		6	4507,91	-	4523,91	-	4539,91	-
		7	4489,90	-	4505,9	-	4521,9	-
		8	4471,89	-	4487,89	-	4503,89	-

Table 8. Modifications in Clos14 lantibiotic analyzed by MALDI-TOF which confirmed the presence of 2 dehydration levels as the most abundant form observed.

Interestingly, Clos14 also displayed potent antimicrobial activity against *M.luteus* and *Clostridium sporogenes* C22/10 (Fig.14). On the other hand, Clos2 and ClosDP lantibiotics which produced in 500ml culture also showed potent antimicrobial activity against *Clostridium sporogenes* C22/10 and *M.luteus* (Fig.12 & Fig.13).

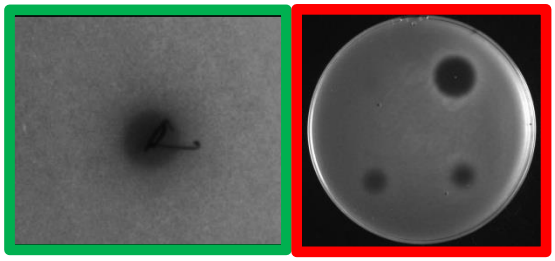

Fig.12 Antimicrobial activity of Clos2 from 500ml batch against *M.luteus* (green) and *Clostridium sporogenes* C22/10 (red)

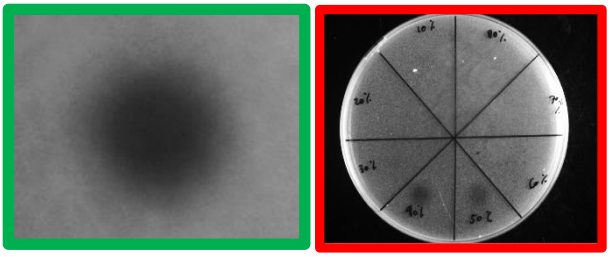

Fig.13 Antimicrobial activity of ClosDP from 500ml batch against *M.luteus* (green) and *Clostridium sporogenes* C22/10 (red)

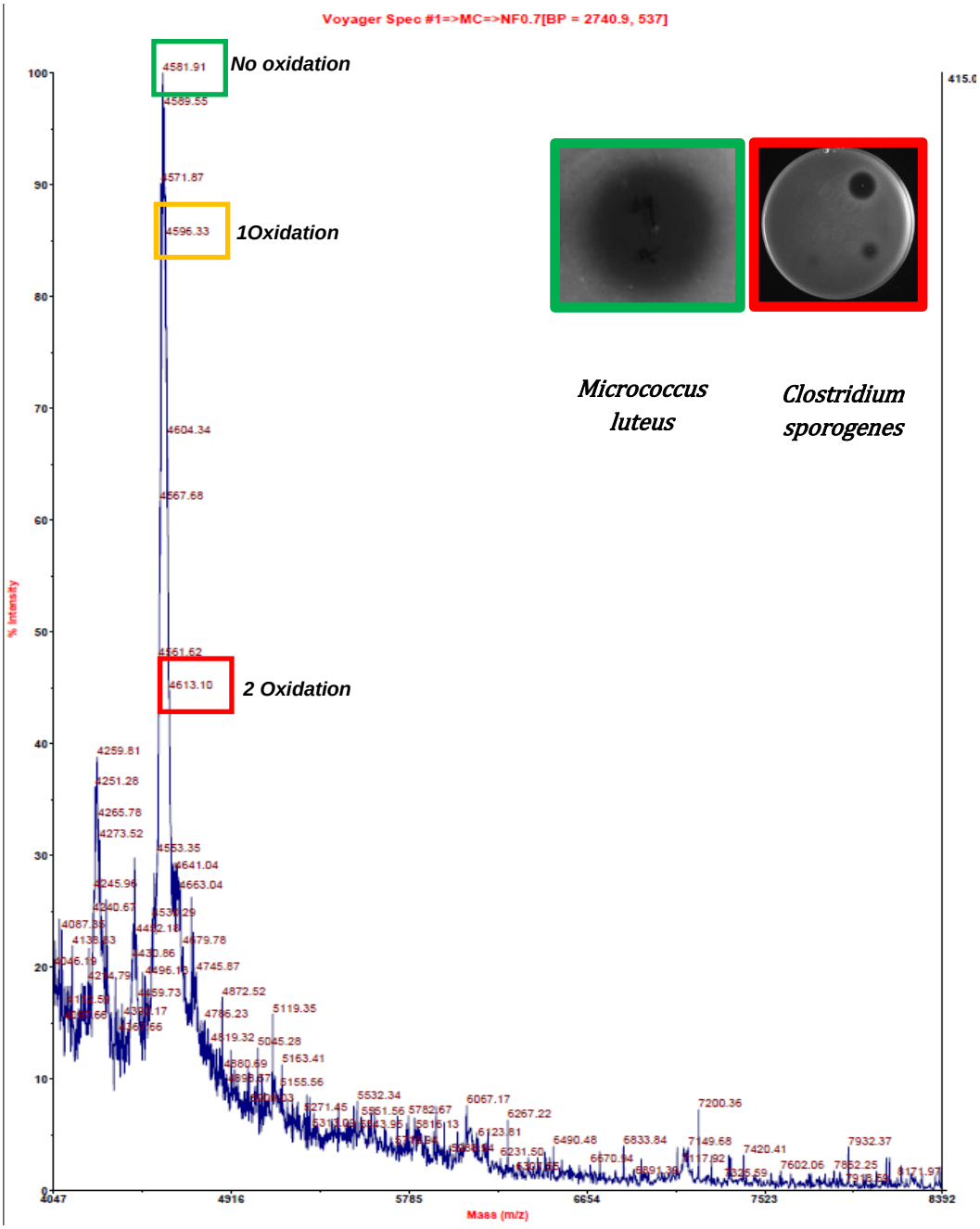

Fig14. The MALDI-TOF mass spectra of Clos14, all oxidation forms of this peptide were observed. Antimicrobial activity of Clos14 against *M.luteus* (green box) and *C.sporogenes* (red box)

Chapter 4. Conclusions and Future Perspective

The current situation of antibiotic resistance in pathogenic bacteria which threat the global health urgently needs to be solved using new approaches. The discovery of novel antimicrobial compounds become scientific challenges for academia, since the exodus of pharmaceutical industries due to economic factors. Nonetheless, synthetic biology era opens an excellent opportunity for academia to create new-to-nature antimicrobials by modifying the biological system in nature. Lantibiotics are known having low resistance level due to their multiple mode of actions: lipid II (a precursor for cell wall synthesis) sequestration and pore formations on cell membrane. Nisin controlled gene expression (NICE) in *L. lactis* is a powerful tool to produce new lantibiotics by fusing non-related to nisin lantibiotic gene sequence into normal nisin leader sequence. The promiscuous modification enzymes NisB and NisC will process the biosynthesis and resulted novel compounds. This approach also could be used to re-activate the silent lantibiotic genes in some organism such as in *Clostridium* species.

As the result of this study, genome mining using Anti-SMASH and BAGEL3 discovered 54 putative lantibiotics genes from the total of 563 genomes and 109 plasmids from Clostridium available in NCBI GenBank. 12 putative clostridial lantibiotic genes used as selected candidates and the construction of host production cell for these putative clostridia lantibiotic genes were successfully created using USER cloning approach in *L. lactis* NZ9000, confirmed with DNA sequencing. Based on antimicrobial assay against indicator strain *L. lactis* NZ9000, eleven peptides: Clos2, Clos4, Clos5, Clos12, Clos14, Clos15, Clos17, Clos22, Clos24, Clos25 and ClosDP showed antimicrobial activity after cleavage of leader peptide, except Clos16. Furthermore, *Micrococcus flavus* is also susceptible to Clos4, Clos22, ClosDP and in minus extent Clos12. Regarding to the remarks about previous experiment, Clos2, Clos14, ClosDP which produced from 500ml batch cultures showed activity against *M. luteus* and *C. sporogenes*.

Despite the fact that almost all peptides were active only six novel lantibiotics were able to be characterized using MALDI-TOF and LC-MS/ MS: Clos2 had 3 dehydration levels, Clos4 had 3-4 dehydration levels, Clos5 had 6 dehydration levels, Clos22 had 2 dehydration levels, ClosDP had 9 dehydration levels for the first component and 8 dehydration levels for the second component. However, Clos14 had 2 dehydration levels based on MALDI-TOF mass spectra analysis from the previous experiment. These results

confirmed successful production of novel lantibiotics from *Clostridium* species by nisin synthetic machinery.

Additionally, the future development of the lantibiotic bioengineering could explore in how to design special probes that could be used for fishing the active peptide or inactive peptide which commonly mixed up thus make it difficult to sort out. For example, by designing expression vectors with specific multiple tags as investigated by (Pastrana et al., 2017). Two modified expression vectors were used in *L. lactis* with AVI-tag and His_6 -tag combination constructed for purification and fluorescent labelling, while another vector allows removal of N-terminal Strep-or His_6 -tags from expressed proteins. This system could distinguish the peptides by enzyme-linked immunosorbent assay.

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