CHARACTERISATION AND MECHANISM OF ACTION OF LANTIBIOTICS PRODUCED BY *STREPTOCOCCUS SALIVARIUS*

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FACULTY OF SCIENCE
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ABSTRACT

Salivaricins are lantibiotics produced by *Streptococcus salivarius*, some strains of which have significant antimicrobial effects. *S. salivarius* strains in this study were isolated from Malaysian subjects and showed variable antimicrobial activity, metabolic profile, antibiotic susceptibility and lantibiotic production. New *S. salivarius* strains isolated from Malaysian subjects with potential as probiotics were investigated and reported. Safety assessment of these strains included their antibiotic susceptibility and metabolic profiles. Genome sequencing using Illumina's MiSeq system was performed for two strains namely NU10 and YU10 and showed the absence of any known streptococcal virulence determinants indicating that these strains are safe for subsequent use as probiotics. Strain NU10 was found to harbour loci for biosynthesis of the lantibiotics specifically salivaricins A and 9 while strain YU10 harboured genes encoding salivaricins A3, G32, streptin and slnA1 lantibiotic-like protein. The full locus for biosynthesis of salivaricin G32 was sequenced and analysed for the first time in this study. The enhancement of lantibiotic production was achieved by using a newly developed medium buffered with 2-(N-morpholino)ethanesulfonic acid (MES). This showed better biomass accumulation compared with other commercial media. Furthermore, salivaricin 9 from strain NU10 and salivaricin G32 from strain YU10 were extracted and purified using *S. salivarius* cells grown aerobically in this medium. Additionally, salivaricins A2 and B were also recovered from strain K12 and purified to homogeneity in this study to understand the mechanism of inhibition on selected microorganisms. The binding mechanism of antimicrobial peptides was better understood using a new peptide-membrane model developed in this study using cecropin B as a model of small cationic antimicrobial peptide. This was investigated by following the fluorescence variation of tryptophan in energy and time domains. The fluorescence showed enhancement of the peak intensity of cecropin B upon mixing with the bacterial membrane accompanied by a blue shift indicating perpendicular penetration of cecropins B from its Lys side where the Trp residue of cecropin B is immersed in the bacterial membrane vesicles. Unlike cecropin B, the lantibiotic salivaricin B was not able to penetrate bacterial membrane vesicles. This was established by measuring the fluorescence of the tryptophan residue at position 17 when salivaricin B interacted with bacterial membrane vesicles. The absence of a fluorescence blue shift indicates a failure of salivaricin B to penetrate the membranes. The potential membrane permeabilization by salivaricin B was also probed in *Streptococcus pyogenes* and *Micrococcus luteus* using the molecular probe SYTOX Green. The results showed that unlike nisin A, salivaricin B did not induce pore formation in susceptible cells. Flow cytometric analysis using DiOC2(3) revealed that salivaricin B did not dissipate membrane potential in sensitive cells. On the other hand, salivaricin B interfered with cell wall biosynthesis as shown by the accumulation of the final soluble cell wall precursor UDP-MurNAc-pentapeptide which is the backbone of the bacterial peptidoglycan. Transmission electron microscopy of salivaricin B-treated cells showed a reduction in cell wall thickness together with signs of aberrant septum formation in the absence of visible changes to cytoplasmic membrane integrity.

ABSTRAK

Salivaricins adalah lantibiotik yang dihasilkan oleh *Streptococcus salivarius*, merupakan beberapa strain yang memiliki efek probiotik yang jelas. Strain *S. salivarius* dalam penelitian ini diisolasi dari subjek Malaysia dan menunjukkan variasi dalam aktiviti antimikrobia, profil metabolik, kerentanan antibiotik dan produksi lantibiotik. Strain *S. salivarius* baru yang diisolasi dari subjek Malaysia mempunyai potensi sebagai probiotik dikaji dan dilaporkan. Sensitiviti terhadap antibiotik dan profil metabolik adalah antara penilaian keselamatan yang dinilai. Sistem Illumina MiSeq merupakan sistem yang digunakan untuk sekuensi genom bagi dua strain iaitu NU10 dan YU10 telah menunjukkan ketidakhadiran sebarang virulensi streptokokus dan kedua dua strain ini selamat untuk digunakan sebagai probiotik. Strain NU10 mempunyai lokus untuk biosintesis bagi lantibiotik salivaricins A dan 9 manakala strain YU10 mengekod gen bagi salivaricins A3, G32, streptin dan slnA1 lantibiotik-serupa protein. Dalam kajian ini, biosintesis untuk keseluruhan lokus bagi salivaricin G32 disekuansi dan dianalisis untuk pertama kalinya. Penambahan dalam produksi lantibiotik dicapai dengan penemuan media baru menggunakan 2- (N-morfolino) asid ethanesulfonic (MES). Ini menunjukkan bahawa akumulasi biomassa yang lebih baik berbanding dengan yang media yang ada di komersil. Selain itu, salivaricin 9 daripada strain NU10 dan salivaricin G32 daripada strain YU10 diekstrak dan ditulenkan dengan menghidupkan sel *S.salivarius* dalam media ini secara aerobik. Tambahan pula, penghasilan lantibiotik bagi salivaricins A2 dan B daripada strain K12 dan ditulinkan sehingga homogenik dalam kajian ini untuk mengkaji mekanisme bagi menyekat pertumbuhan pada mikroorganisma yang dipilih. Pemahaman yang baik terhadap mekanisma interaksi dapat dicapai menggunakan inovasi model baru peptida-membran menggunakan cecropin B yang berfungsi sebagai peptida antimikrobia kationik kecil. Penyelidikan dibuat dengan berdasarkan terhadap variasi fluoresen tryptophan mengikut tenaga dan masa domain. Hal ini mengakibatkan peningkatan intensitas puncak cecropin B apabila dicampur dengan membran bakteria disertai dengan peralihan biru menunjukkan penetrasi tegak lurus dari cecropins B dari sisi Lys yang mana residu Trp dari cecropin B tenggelam dalam vesikel membran bakteria. Tidak seperti cecropin B, lantibiotik salivaricin B tidak mampu menembusi vesikel membran bakteria. Ini dicapai dengan mengukur fluoresensi dari residu triptofan pada posisi 17 ketika salivaricin B berinteraksi dengan vesikel membran bakteria. Tidak adanya peralihan fluoresensi biru menunjukkan kegagalan salivaricin B menembusi membran. Potensi membran permabilisasi oleh salivaricin B juga dikaji dalam *Streptococcus pyogenes* dan *Micrococcus luteus* menggunakan probe molekuler SYTOX Green. Hasil penelitian menunjukkan bahwa tidak seperti nisin A, salivaricin B tidak menginduksi pembentukan pori di sel rentan. Analisis Aliran Cytometric menggunakan DiOC2 (3) menunjukkan bahawa salivaricin B tidak menghilang potensi membran dalam sel sensitif. Di sisi lain, salivaricin B mengganggu biosintesis dinding sel, seperti yang ditunjukkan oleh akumulasi daripada dinding sel larut akhir prekursor UDP-MurNAc-pentapeptide yang merupakan tulang belakang kepada peptidoglikan bakteria. Mikroskop elektron transmisi dari salivaricin sel B yang dirawat menunjukkan pengurangan ketebalan dinding sel bersama-sama dengan tanda-tanda pembentukan septum menyimpang dengan ketiadaan perubahan yang terlihat pada integritas membran sitoplasma.

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LIST OF SYMBOLS AND ABBREVIATIONS

°C	:	Degree Celsius
µg	:	microgram
µlµL	:	Microliter
µM	:	Micromolar
ATCC	:	American type culture collection
bp	:	Base pair
BSA	:	Bovine serum albumin
Da	:	Dalton
EDTA	:	Ethylenediaminetetraacetic acid
mg	:	Milligram
mlmL	:	Milliliter
mM	:	Millimolar
MurNAc	:	Monosaccharide derivative of N-acetylglucosamine
nM	:	Nanomolar
nm	:	Nanometer
OD	:	Optical density
PBS	:	Phosphate-buffered saline
PCR	:	Polymerase chain reaction
PG	:	Peptidoglycan
RAST	:	Rapid Annotation using Subsystem Technology
rpm	:	Revolutions per minute
Trp	:	The amino acid tryptophan
w/v	:	weight / volume
χ^2	:	Chi-square

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

Lantibiotics are ribosomally-synthesized antimicrobial peptides containing intramolecular ring structures introduced through the thioether-containing lanthionine (Lan) and/or methyllanthionine (MeLan) residues formed by post-translation modification (Bierbaum and Sahl, 2009). Although most of the currently-described lantibiotics are produced by Gram-positive bacteria (Jack *et al.,* 1995) certain isolates of *Streptomyces* have also been shown to produce lantibiotics or lantibiotic-like peptides (Kodani *et al.*, 2004; Völler *et al.*, 2012). Lantibiotics are widely considered to assist the survival of the host bacteria in their favoured ecosystem by suppressing the growth of competitor bacteria in that particular ecological niche.

The most well-known lantibiotic is nisin produced by *Lactococcus lactis*, which was first described in 1928 (Rogers and Whittier, 1928) and then subsequently widely used in the dairy industry as an efficient and safe preservative (Delves-Broughton *et al.*, 1996). Lantibiotics from gram-positive bacteria are classified into two major groups based on their modes of action and structural variations (Brötz and Sahl, 2000). Nisin, epidermin and Pep5 are members of the type A lantibiotic group and they act mainly by forming pores in the cytoplasmic membrane of the targeted bacterial cells (Moll *et al.*, 1996). On the other hand, type B lantibiotics such as mersacidin form complexes with their membrane-bound substrates and inhibit peptidoglycan synthesis (Brotz *et al.*, 1995; Brotz *et al.*, 1998; Altena *et al.*, 2000; Hsu *et al.*, 2003; Bottiger *et al.*, 2009). While type AI lantibiotics (the nisin group) are elongated and flexible, type AII (the lacticin 481 group) display an unbridged N-terminal extremity and a globular C-terminal part. Type AIII lantibiotics consist of lactosin S and the two-component system lantibiotics (Dufour *et al.*, 2007; Sahl *et al.*, 1995; Uguen *et al.*, 2000).

Salivaricins are type AII lantibiotics produced by *S. salivarius* and having a ring topology similar to that of the *L. lactis* lantibiotic, lacticin 481 (Bravo *et al.*, 2009; Dufour *et al.*, 2007; Hyink *et al.*, 2007).

S. salivarius is a commonly-occurring member of the human oral microbiota, typically colonizing the mouth and upper respiratory tract within a few hours of birth. Some *S. salivarius* are equipped to compete with predominant bacterial pathogens involved in upper respiratory tract infections due to their production of various lantibiotics, which include salivaricin A, salivaricin B, salivaricin G32 and salivaricin 9 (Barbour and Philip, 2014; Barbour *et al.*, 2013; Heng *et al.*, 2011; Hyink *et al.*, 2007; Wescombe *et al.*, 2012; Wescombe *et al.*, 2012; Wescombe *et al.*, 2006). Salivaricin B is particularly potent, with a broad inhibitory spectrum that includes all 9 standard indicator strains used in the production (P-) typing method that was developed specifically for the categorization of bacteriocin-producing streptococci (Hyink *et al.*, 2007; Tagg and Bannister, 1979).

One important characteristic of the members of the lacticin 481 group is that they contain a mersacidin-like lipid II binding motif and in this regard salivaricins are no exception (Dufour *et al.*, 2007; Hyink *et al.*, 2007; Knerr *et al.*, 2012). Although salivaricins and lacticin 481 are classified as class AII lantibiotics they also contain an important membrane binding motif found in class B lantibiotics, which makes it interesting to study the mechanism of action of these lantibiotics and to determine whether they follow the typical pore formation activity of class A lantibiotics or interfere with cell wall biosynthesis like class B lantibiotics (Brötz and Sahl, 2000).

Developing novel lantibiotic-producing oral probiotics is crucial for the management of oral and upper respiratory tract health disorders. Currently, there is no information available to address the persistence of lantibiotic determinants in Malaysian or South

East Asian populations. Understanding the biosynthesis of lantibiotics can lead to the development of alternative antibiotics in view of the emerging antibiotic resistance.

Furthermore, the mode of action of the lantibiotic salivaricins (especially salivaricin B) is largely unexplored. It is not known whether these salivaricins attack their likely targets by pore formation similar to other lantibiotics investigated in the past or perhaps it has a different mode of bacterial inhibition.

In this study, the various characteristics of lantibiotic-producing *S. salivarius* strains isolated from healthy Malaysian subjects were described. These strains showed antagonism against selected Gram-positive bacteria associated with dental implications and halitosis. The safety assessment study of these strains did not detect any streptococcal virulence genes and demonstrated the susceptibility of the *S. salivarius* strains to a few classes of antibiotics. The stability of the metabolic profiles was also investigated in this study and showed some variations among the strains. To estimate the distribution of lantibiotic genes precursors among Malaysian subjects, one hundred strains of *S. salivarius* were isolated from 107 healthy Malaysian children. These strains were evaluated for their lantibiotic production and the genes encoding them.

S. salivarius strains isolated in this study showed to produce the levan-sucrase enzyme. The *de novo* amino acid sequence of the enzyme showed similarity to that produced by other lantibiotic producing *S. salivarius* probiotic strains. However, genome sequencing of strain YU10 helped to fully characterise the gene encoding levan-sucrase production. Due to the high level of levan-sucrase production and the secretion of lantibiotics, strains presented in this study can play a great role in pharmaceutical applications as a source of lantibiotics that can be used as probiotics and/or prebiotics to improve human oral health. This study also led to the development of a new medium to obtain higher biomass levels of *S. salivarius* and lantibiotic

production during aerobic fermentation when compared with other commercial media. This new medium can be used to enhance lantibiotic production by *S. salivarius* which may help to develop new oral probiotics.

To better understand how small antimicrobial peptides attack the membranes of the targeted bacterial pathogens, a new peptide-membrane binding model was developed in the current study. This was achieved using modified cecropin B as the control antimicrobial peptide and targeting membrane vesicles of *Pseudomonas aeruginosa*. The mode of action was investigated based on the intrinsic fluorescence of tryptophan. The same model was applied to the lantibiotic salivaricin B to investigate whether this lantibiotic can penetrate the bacterial membrane vesicles and induce pore formation.

In the present study, molecular probes were used to examine whether salivaricin B disrupts bacterial cell membrane integrity or dissipates the membrane potential of targeted cells. The spectrofluorometric analysis was also carried out to determine whether the tryptophan residue of salivaricin B plays any role in the peptide-membrane interaction. It is concluded that salivaricin B interferes with cell wall biosynthesis by deregulating the cell envelope and interfering with septum formation. This study reports the first lantibiotic within the salivaricins family exhibiting such a mode of antibacterial action.

CHAPTER 2: LITERATURE REVIEW

Lantibiotics are ribosomally synthesized post-translationally modified antimicrobial peptides containing lanthionine and methyllanthionine structures produced by many genera of bacteria most of which are Gram-positive. Major classes of lantibiotics are Type-AI, Type-AII, Type-B and the two peptide lantibiotics group. Additionally, there are class III morphogenetic peptides and some lantibiotics which have not yet been classified.

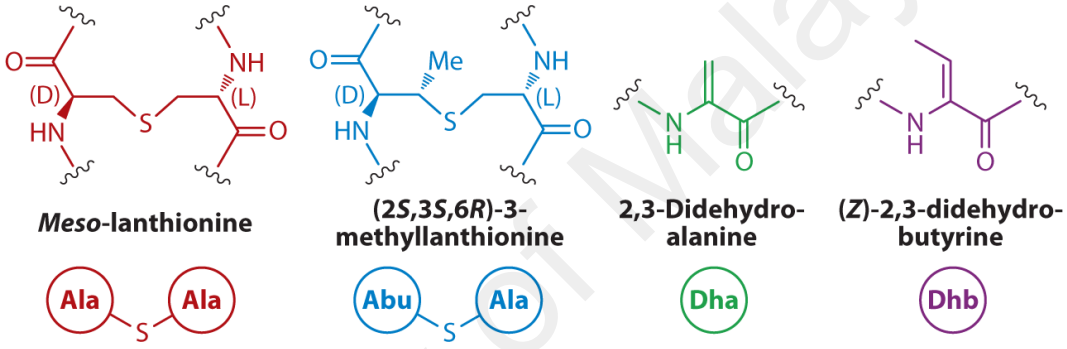

Figure 2.1: Characteristic lantibiotic posttranslational modifications. Abu, 2-aminobutyric acid; Dha, 2,3-didehydroalanine; Dhb, (Z)-2,3-didehydrobutyrine. This figure was taken from Knerr and van der Donk (2012).

After the lantibiotic modification enzyme dehydrate selected serine and threonine residues, lanthionine and methyllanthionine bridges are formed by the intramolecular addition of cystine thiols to the resultant unsaturated amino acids.

Lanthionine is comprised of two alanine residues which are cross-linked by a thioether linkage. This molecule is a monosulfide analog of cystine first isolated in 1941 by treating wool with sodium carbonate. However, lanthionine was found in the bacterial cells as part of the ribosomally synthesized lantibiotics. Methyllanthionine is composed of one alanine and one 2-aminobutyric acid residue.

2.1 Type AI lantibiotics

This class of lantibiotics includes nisin, epidermin and pep5 groups.

2.1.1 Nisin group

In 1928, Rogers and Whittier found that during the fermentation process of *Streptococcus lactis* (now known as *Lactococcus lactis*), the bacterium was inhibited by a compound (not an organic acid) with defined inhibitory effect. This compound was described as a "diffusible substance limiting *St. lactis* growth" (Rogers and Whittier, 1928). Some five years later, it was reported that the starter culture added during cheese making process was inhibited by overnight stored bulk milk and later this inhibitory factor was said to be nisin derived from the initial classification of the inhibitory substance as Lancefield Group *N* inhibitory substance (Mattick and Hirsch, 1944). Later in 1947, Mattick and Hirsch proved that nisin is an effective antimicrobial agent during *in-vivo* trials and they described a method for large-scale preparation of nisin and its activity against pathogenic microorganisms (Mattick *et al.*, 1947). By the year 1969, experts from the World Health Organisation (WHO) and the Food and Agriculture Organisation (FAO) stated that nisin is a safe and natural food additive.

The first structural study of nisin was carried out in 1971 by Gross and Morell who addressed for the first time the sulphide bridges contributed by lanthionine and β-methyllanthionine (Gross and Morell, 1971). In 1988, nisin was given the status of generally recognised as safe (GRAS) by the Food and Drug Agency (FDA) in the USA and by 1996 more than 50 countries worldwide started to use nisin as a food additive (Delves-Broughton *et al.*, 1996). Buchman and co-workers cloned the nisin precursor gene for the first time and gave the first insight into its leader peptide with confirmation of nisin identity at a genetic basis (Buchman *et al.*, 1988).

Nisin A and nisin Z are the most common forms of nisin, both produced by *Lactococcus lactis* strains and differing from each other by only one amino acid at position 27 (histidine in nisin A and asparagine in nisin Z) (de Vos *et al.*, 1993; Mulders *et al.*, 1991). Nisin Q is another variant and it differs from nisin A by two amino acids in the leader peptide and four positions in the mature peptide (Zendo *et al.*, 2003). Nisin F is a homolog to nisin Q with two amino acids differing at position 15 (alanine in nisin F instead of valine in nisin Q) and position 21 (methionine in nisin F instead of leucine in nisin Q) (de Kwaadsteniet *et al.*, 2008). Figure 2.2 shows a comparison between the secondary structures of nisinA, nisin Q and nisin Z.

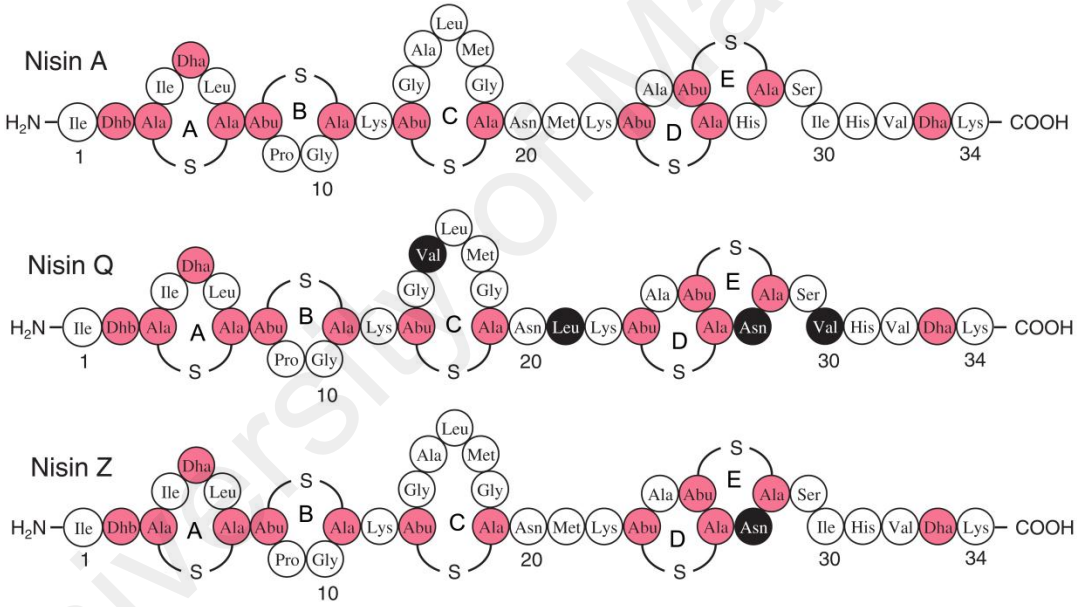

Figure 2.2: The lantibiotic nisin A and its variants nisin Q and nisin Z. Modified residues are indicated in red. Residues differ from nisin A are indicated in black. Rearranged and coloured from Fukao *et al*. (2008).

The most recent nisin variant to date is nisin H produced by a porcine gut-derived isolate of *Streptococcus hyointestinalis*. Nisin H differs from prototype nisin A by 5 amino positions and represents an intermediate variant between lactococcal and streptococcal nisins (O'Connor *et al.*, 2015).

The first reported streptococcal nisin variant is nisin U produced by *Streptococcus uberis* with 89% similarity to nisin A (Wirawan *et al.*, 2006). Nisin operons found in both *Streptococcus gallolyticus* subsp. *pasteurianus* and *Streptococcus suis* showed to encode another nisin variant called nisin P that differs from nisin U by three amino acids at positions 1, 20 and 21 (Wu *et al.*, 2014; Zhang *et al.*, 2012). Interestingly, a nisin-like lantibiotic designated as salivaricin D produced by *Streptococcus salivarius* showed to have 62% homology to nisin Q, 59% to nisin A and 55% to nisin U (Birri *et al.*, 2012).

2.1.1.1 Nisin biosynthesis and genetic organization

Nisin is a ribosomally synthesized antimicrobial peptide generated from the linear precursor peptide (NisA) encoded by the structural gene *nisA* (Kaletta and Entian, 1989). After NisA is produced, a dehydratase enzyme called NisB (encoded by *nisB* gene) is involved in the peptide maturation process by dehydrating eight serines and threonines in NisA core region to yield dehydroalanine (Dha) and dehydrobutyrine (Dhb) residues (Karakas Sen *et al.*, 1999; Ortega *et al.*, 2015). Subsequently, five lanthionine and methyllanthionine crosslinks are formed by NisC cyclase (encoded by *nisC* gene) which catalyses cyclization by coupling of cysteines to the formed Dha and Dhb residues (Li *et al.*, 2006; Rink *et al.*, 2007). Finally, nisin protease NisP (encoded by *nisP* gene) cleavages the leader peptide sequence yielding the mature nisin lantibiotic (van der Meer *et al.*, 1993).

The nisin gene cluster of *Lactococcus lactis* contains in addition to the biosynthesis genes mentioned above (*nisA, nisB, nisC* and *nisP*) three more genes named as *nisT*, *nisI* and *nisR*. *nisT* encodes NisT protein which plays a great role in the translocation of nisin lantibiotic outside the producing cell with evidence of production of nisin inside the cytoplasm when NisT is blocked (Qiao and Saris, 1996).

NisI encoded by the *nisI* gene is a self-protection and non-related lipoprotein acting as nisin sequestering protein. However, there are other genes involved in nisin immunity called *nisFEG* genes encoding NisFEG proteins acting like ABC transporter proteins by exporting nisin molecules from the cytoplasmic membrane into the outside environment (Nawrot *et al.*, 2003).

NisK encoded by *nisK* gene and NisR encoded by the *nisR* gene is a two-component regulatory system of nisin. NisK is a histidine kinase sensor that can recognize the extracellular nisin signals which lead to autophosphorylation induction of the histidine residue within NisK. The energetic potential of the phosphoryl group is then transferred to NisR. Subsequently, the transcription of nisin gene and the other modification and immunity genes as well are up-regulated by binding of NisR to some distinct promoters (Alkhatib *et al.*, 2012; Kleerebezem *et al.*, 1997). Figure 2.3 shows organization and genomic comparison of nisin loci and other types of lantibiotics including subtilin, pep5 and epidermin locus.

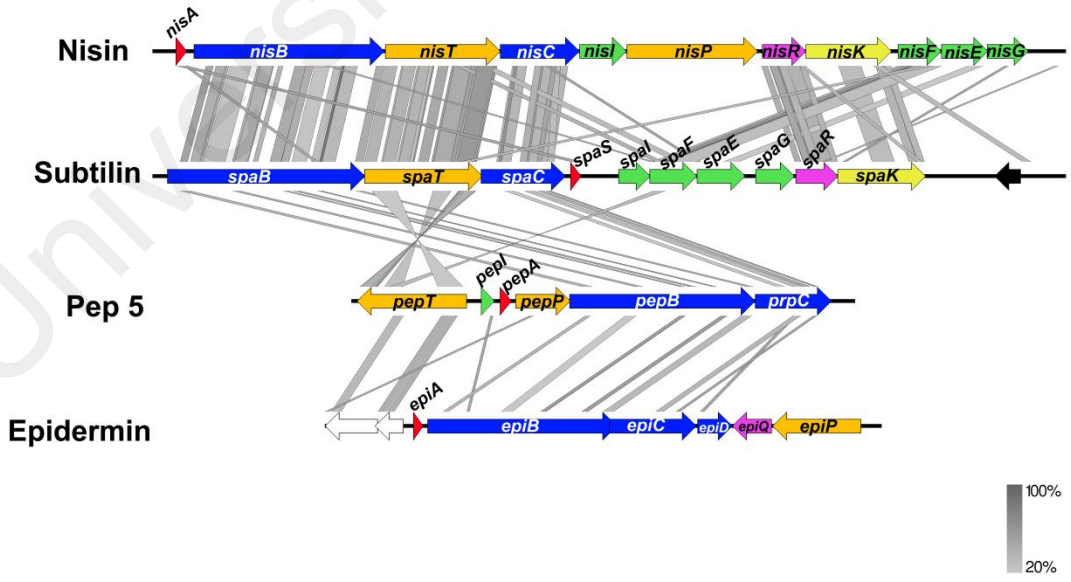

Figure 2.3: Genomic comparison of different lantibiotic loci. Lantibiotic modification and maturation genes are indicated in blue, transportation genes are indicated in orange, structural genes are red, lantibiotic immunity genes are indicated in green, respond regulator genes are in pink and histidine kinase encoding genes are in yellow. This figure was produced using Easyfig software.

2.1.1.2 Nisin mode of action

The first study on the mode of action of nisin was carried out by Reisinger and co-workers who found that nisin inhibits the peptidoglycan (murine) synthesis by forming complex with undecaprenyl pyrophosphate-activated intermediates of murein synthesis (lipid I and lipid II) (Reisinger *et al.*, 1980). This study showed that at low concentrations of nisin (20 µg/mL), the peptidoglycan biosynthesis did not decrease but rather was activated. However, the formation of peptidoglycan was dramatically inhibited when a higher concentration of nisin was used. Apparently, inhibiting the peptidoglycan biosynthesis is not the only killing mechanism of nisin. This polypeptide antibiotic was found to cause an immediate collapse of the cytoplasmic membrane potential of targeted gram-positive bacterial cells. After one minute of nisin addition to the susceptible cells, rapid and complete efflux of amino acids and radiolabelled Rb+ (a tracer used in place of K^+) from the cytoplasm was achieved. The influence of nisin on cytoplasmic membrane vesicles strongly suggested that the main target of nisin is the cytoplasmic membrane (Ruhr and Sahl, 1985) (Figure 2.4).

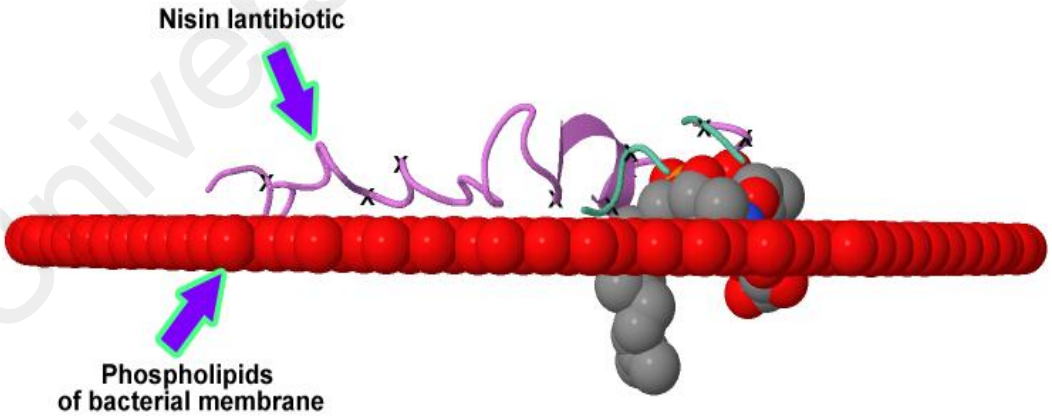

Figure 2.4: Nisin-phospholipids binding model. This figure was taken from orientations of proteins in membranes database (OPM) based on a study by Bonev *et al.* (2000).

Using black lipid membrane studies, nisin was found to form defined pores with diameters of 2 to 2.5 nm. This pore formation took place after the addition of both *trans*-negative and *trans*-positive membrane potential and remained stable for hours in the presence of lipid II (Wiedemann *et al.*, 2004). However, the nisin pores had millisecond life time range and 1 nm diameter when lipid II was absent with threshold potential of −100 mV (Benz *et al.*, 1991; Kordel and Sahl, 1986). Studies on different variants of nisin e.g. nisin Z suggested that this lantibiotic uses the same docking site as vancomycin (the membrane-anchored cell wall precursor Lipid II). As a result, the transglycosylation step in the cell wall biosynthesis is inhibited by nisin which sequesters lipid II from the cell division site (Breukink *et al.*, 1999; Hasper *et al.*, 2006).

Recently, NMR studies have suggested that nisin interacts from its N-terminal side which contains the first two lanthionine rings A & B with the pyrophosphate moiety of lipid II via five intermolecular hydrogen bonds.

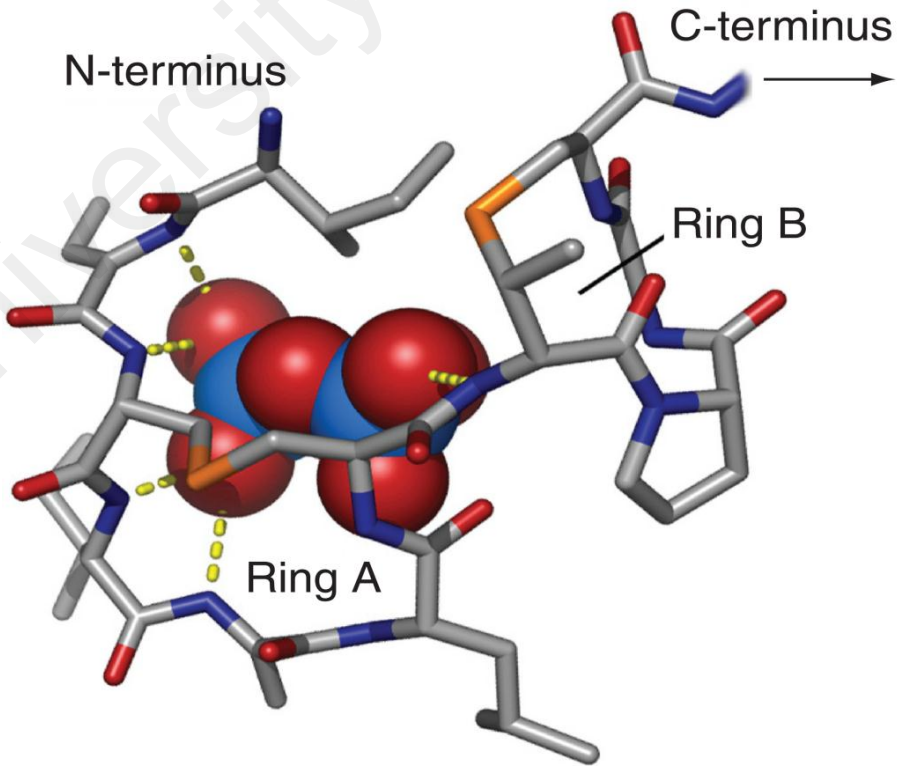

Figure 2.5: The interaction between nisin N-terminus nisin and the pyrophosphate moiety of lipid II via a hydrogen-bounding network. Hydrogen bonds are indicated in yellow. The figure was from Martin and Breukink (2007).

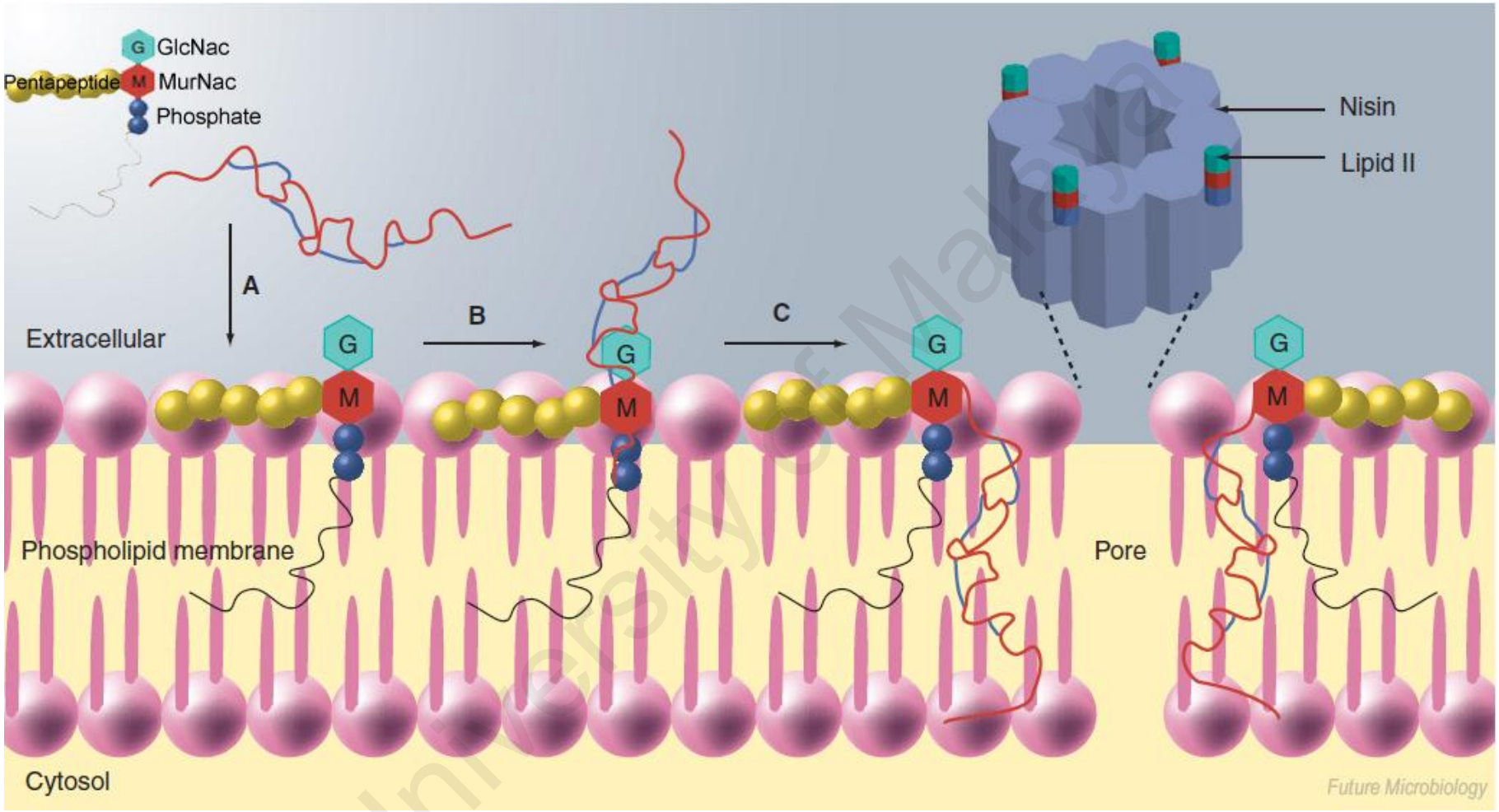

Figure 2.6: Mode of action of the lantibiotic nisin. After nisin approach the cytoplasmic membrane environment of the targeted bacteria (A) it binds to lipid II (B) and induces pore formation (C). During or after the formation of nisin-lipid II complex, four additional nisin molecules are added to form the pore complex. This figure is taken from Breukink and de Kruijff (2006).

2.1.1.3 Subtilin biosynthesis, genetic organization and mode of action

Subtilin is a 3317 Da ribosomally synthesized antimicrobial peptide produced by *Bacillus subtilis* strain ATCC66 (Jansen and Hirschmann, 1944). In 1951, Alderton and Fevold had isolated lanthionine from subtilin hydrolysates (Alderton and Fevold, 1951) and hence, this antimicrobial peptide belongs to the lantibiotics family (Guder *et al.*, 2000; Sahl and Bierbaum, 1998). Structurally, subtilin is related to nisin with more than 60% similarity (Hurst, 1981). The subtilin structural gene *spaS* encoding a 56-residue peptide precursor was reported in 1988 and it showed to contain a leader peptide region with an unusual hydropathic character for an exported protein (Banerjee and Hansen, 1988). Later on, three more open reading frames *(spaB*, *spaC*, and *spaT*) were identified in subtilin-producing *Bacillus subtilis* strain ATCC66 with high homology to genes identified near epidermin lantibiotic structural gene. However, defects in *spaB* and *spaC* due to mutations resulted in subtilin-negative cells while *spaT* mutant cells retained subtilin production (Klein *et al.*, 1992). Like nisin, the *spaB* gene encodes the subtilin dehydratase, *spaC* encodes subtilin cyclase and *spaT* encodes subtilin transporter. Subtilin has the two-component regulatory system just like nisin designated as *spaR* (sensor-regulator) and *spaK* (histidine kinase) and both are essential for subtilin induction. Deletion mutations introduced in either of the two genes resulted in a failure of *spaB* expression (Gutowski-Eckel *et al.*, 1994). Sequencing analysis downstream of the structural gene *spaC* revealed the presence of three additional genes (*spaI*, *spaF*, and *spa*G) involved in the self-protection similar to *nisI*, *nisF* and *nisG* in nisin-immunity system (Klein and Entian, 1994).

The first evidence of nisin and subtilin interfering with the cell wall biosynthesis was published (Linnett and Strominger, 1973) before the nisin-lipid complex was described clearly as mentioned above (Reisinger *et al.*, 1980). Like nisin, subtilin also showed to form pores in the targeted bacterial membranes with a lifetime of several hundred

milliseconds and a pore diameter of 2 nm irrespective of the orientation of the potential. However, the essential threshold potential of -90 to -100 mV was deduced for subtilin action on intact bacterial cells with more potency on energized cells rather than starved cells (Schuller *et al.*, 1989).

2.1.1.4 Subtilin-like lantibiotics

Stein and co-workers identified a lantibiotic gene cluster in *Bacillus subtilis* A1/3 with strong homology to subtilin gene cluster which was identified earlier in *Bacillus subtilis* ATCC6633. This new gene cluster showed to have a replication of two subtilin-like genes encoding two lantibiotic-like peptides named ericin S (3,442 Da) and ericin A (2,986 Da). Ericin S differs from subtilin by only four amino acids with similar lanthionine-bridging pattern and antibiotic activity. However, ericin A showed low antibacterial activity with two different C-terminal rings compared to that of lanthionine pattern of subtilin (Stein *et al.*, 2002).

2.1.1.5 Microbisporicin biosynthesis, genetic organization and mode of action

Microbisporincin (commercially known as lantibiotic NAI-107) produced by *Microbispora coralline* is another potent lantibiotic belonging to the nisin group. This lantibiotic contains chlorinated tryptophan and dihydroxyproline residues and showed to have the typical lantibiotics fashion of biogenesis. Molecular analysis of the gene cluster showed that the structural gene *mibA* encoding 57 amino acids prepropeptide of microbisporicin followed by *mibB* gene encoding the lantibiotic dehydratase with some homology to those found in *Bacillus clausii* and other actinomycetes. MibC gene product encoded by *mibC* is a lantibiotic cyclase similar to SpaC of subtilin and EpiC of epidermin. MibD which is involved in S-[(Z)-2-aminovinyl]-D-cysteine moiety formation at the C-terminus of microbisporicin is encoded by *mibD* gene resembling MrsD in mersacidin. *mibT* and *mibU* genes encode MibTU which plays a role as a two-

component ABC transporter. MibEF encoded by *mibE* and *mibF* apparently confer immunity to the microbisporicin producer by transporting the lantibiotic away from its target (membrane lipid II) in the producing cell. *mibH* and *mibS* encode MibH (flavin-dependent tryptophan halogenases) and MibS (flavin reductases), respectively. The gene cluster of microbisporicin also contains many other genes which might not be part of the *mib* cluster. However, *mibRWX* genes are believed to encode regulatory proteins. MibX is an RNA polymerase σ factor that responds to extracellular signals and directs RNA polymerase to particular promoter sequences while MibW is an anti-σ factor suppressing MibX activity (Foulston and Bibb, 2010). Microbisporicin showed to form complexes with bactoprenol-pyrophosphate coupled precursors of the bacterial cell wall and interfered with peptidoglycan biosynthesis with a slow depolarization of the cell membrane (Münch *et al.*, 2014).

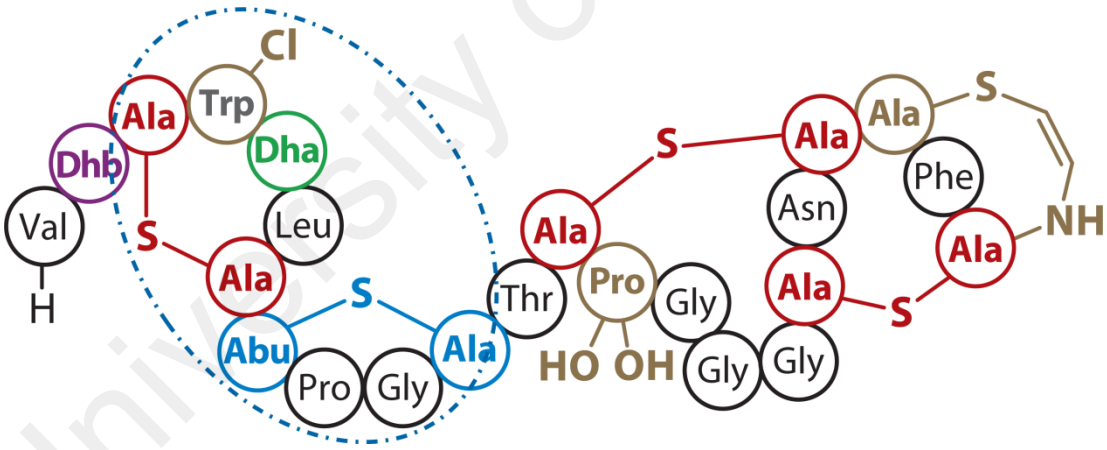

Figure 2.7: Secondary structure of the lantibiotic microbisporicin. The nisin-lipid II-binding motif is indicated in blue-dashed circle. Adapted from Knerr and van der Donk (2012).

2.1.2 Epidermin Group

Epidermin is a 22 amino acids staphylococcal lantibiotic containing one residue each of Dhb and MeLan and two residues of Lan synthesized ribosomally as a polycyclic peptide distinguished by a unique C-terminus (mono-carboxy, di-amino acid, AviCys (S-[(Z)-2-aminovinyl]-d-cysteine)) (Allgaier *et al.*, 1985). Epidermin-like peptides are shorter than nisin group peptides with potent antimicrobial activity. Epidermin group consists of epidermin, gallidermin, staphylococcin T, mutacin B-Ny266, mutacin 1140, mutacin I, mutacin III and streptin (Bierbaum and Sahl, 2009).

2.1.2.1 Epidermin biosynthesis, genetic organization and mode of action

Epidermin and gallidermin produced by *Staphylococcus epidermidis* and *Staphylococcus gallinarum* respectively have the same genetic organization. In this section, the gene cluster of epidermin will be discussed and the involved genes will start with "*epi"*. However, the same organization can be applied on gallidermin using "*gdm"* referring to the gallidermin gene cluster. *epiA* is the structural gene encoding epidermin production followed by the modification genes *epiBCD* in the same orientation. At the opposite orientation regulatory gene *epiQ* and protease gene *epiP* are located. EpiBC encoded by *epiBC* have the same functions of NisBC in nisin. EpiD encoded by the *epiD* gene in epidermin is a unique flavoenzyme involved in the formation of S-[(Z)-2-aminovinyl]-D-cysteine structure by catalyzing the removal of two reducing equivalents from the cysteine residue of the C-terminal *meso*-lanthionine (Kupke *et al.*, 1992). EpiD is a low substrate-specific enzyme which works on any peptide having (V/I/L/(M)/F/Y/W)-(A/S/V/T/C/(I/L))-C sequence at the carboxy terminus (Kupke *et al.*, 1995). The *epiT* encoding epidermin transporter EpiT is defected by two frameshift deletions while the homolog *gdmT* in gallidermin system has an entire sequence. When *gdmT*-complemented clone was introduced in the heterologous host *Staphylococcus carnosus*, an increased epidermin production was observed (Peschel *et al.*, 1997).

However, the *epiH* gene was also shown to play a major role in enhancing epidermin production (Peschel *et al.,* 1996). The self-protection to epidermin and/or gallidermin is mediated by *epiF*, *epiE* and *epiG* genes located upstream of *epiH*. EpiFEG is ABC transporter system works like hydrophobic vacuum cleaner expelling epidermin/ gallidermin from the cell membrane (Bolhuis *et al.,* 1997; Otto *et al.,* 1998; Peschel *et al.*, 1996). The leader peptidase EpiP is a serine protease encoded by *epiP* and processes epidermin leader peptide to produce the functional lantibiotic. When different mutations were introduced in *epiP*, the pre-epidermin was not processed and the mutant cells were unable to produce the active lantibiotic (Geissler *et al.,* 1996; Krismer *et al.,* 2012). Epidermin regulator, EpiQ, was shown to activate most of the epidermin gene cluster with no apparent activation of *epiQ* by a sub-lethal concentration of epidermin (Peschel *et al.,* 1993; Peschel *et al.,* 1996). EpiQ does not contain the conserved aspartic acid residue unlike other response regulators in conventional lantibiotics but is essential for epidermin production. Previously it was shown that additional copies of *epiQ* increased epidermin production in *Staphylococcus epidermidis* Tü3298 (Peschel *et al.,* 1993).

Epidermin interacts with lipid I, II, III (undecaprenol-pyrophosphate-N-acetyl-glucosamine), and IV (undecaprenol-pyrophosphate-N-acetyl-glucosamine-N-acetyl-mannosamine). Hence, epidermin can inhibit peptidoglycan and wall teichoic acid biosynthesis (Brotz *et al.,* 1998; Muller *et al.,* 2012). Epidermin is shorter than nisin, therefore, is unable to span the cytoplasmic membrane completely. It was demonstrated that pore formation mediated by epidermin/ gallidermin is membrane thickness-dependent and pore formation is not likely the main mode of action of epidermin (Bonelli *et al.,* 2006).

2.1.2.2 Epidermin-like lantibiotics and other members of epidermin group

Staphylococcin T (StT) is a 2166 Da natural gallidermin variant lantibiotic produced by *Staphylococcus cohnii*. Interestingly, stT interfere with the cytoplasmic membrane by causing efflux of ions and block in macromolecular synthesis (Furmanek *et al.*, 1999). Besides staphylococci, some streptococcal strains can also produce lantibiotics similar to epidermin.

Mutacin B-Ny266 produced by *Streptococcus mutans* is a 22.7kDa lantibiotic that differs from epidermin by 5 amino acids at positions 1, 2, 4, 5 and 6. Mutacin B-Ny266 was the first mutacin lantibiotic to be fully sequenced and it was shown to contain two Lan residues and one of each MeLan, Dha, Dhb and one S-[(Z)-2-aminovinyl]-d-cysteine (MotaMeira *et al.*, 1997).

Mutacin 1140 produced by *Streptococcus mutans* JH1140 belongs to epidermin group of lantibiotics. Based on NMR studies and restrained molecular dynamics simulations, it was deduced that this lantibiotic adopts a compact and small structure of multiple forms conferring an activity of membrane disintegration (Smith *et al.*, 2003). It was found that mutacin 1140 forms a stable complex with lipid II without forming pores into membranes of susceptible bacterial cells. However, mutacin 1140 showed disruptive activity towards artificial bacterial membrane system (Smith *et al.*, 2008).

Mutacin II and mutacin III produced by *Streptococcus mutans* are also members of the epidermin group of lantibiotics with some differences in the hinge region of the mature peptide. Hence, these two lantibiotics confer different hydrophobic properties and different inhibitory activity (Nicolas *et al.*, 2007; Qi *et al.*, 1999, 2000).

Streptin lantibiotic belonging to the epidermin group is a 23 amino acid peptide produced by *Streptococcus pyogenes* with a mass of 2,424 Da. This lantibiotic has a

2,821-M_r form with three additional amino acids at the N-terminus. Streptin precursor is encoded by the structural gene *srtA* which was found in many strains of *Streptococcus pyogenes* and some strains of *Streptococcus salivarius* as well (Barbour and Philip, 2014; Wescombe and Tagg, 2003).

2.1.3 Pep5 Group

Pep5 is a basic bactericidal lantibiotic produced by *Staphylococcus epidermidis* strain 5 (Sahl and Brandis, 1981). This peptide was shown to be a complex mixture of closely related peptides rather than a homogenous peptide containing dehydrobutyrine, lanthionine and 3-methyllanthionine (Sahl *et al.,* 1985). The pre-Pep5 was the first lantibiotic pre-peptide to be isolated and it was shown that this pre-peptide is mostly associated with the cytosol. This group contains three more lantibiotics namely epilancin K7, epicidin 280, and epilancin 15X that are produced by different strains of *Staphylococcus epidermidis* (Ekkelenkamp *et al.,* 2005; Heidrich *et al.,* 1998; Vandekamp *et al.*, 1995).

2.1.3.1 Pep5 biosynthesis, genetic organization and mode of action

Pep5 is matured from the precursor peptide pre-Pep5 encoded by *pepA* structural gene which was shown previously to be plasmid encoded (Kaletta *et al.*, 1989). The genetic arrangement of Pep5 biosynthesis is *pepTIAPBC*. PepT is the translocator of the ABC transporter family, PepI is the immunity protein, PepA is the peptide precursor, PepP is the serine protease and PepBC are the modification enzymes. In Pep5 biosynthesis, PepT can be replaced by other host-encoded translocators as its deletion showed 10% less Pep5 production. However, inactivation of PepP resulted in a significant reduction of Pep5 activity while PepBC deletion led to the accumulation of the incorrectly modified pre-peptide inside the cells (Meyer *et al.*, 1995).

Pep5 was shown to depolarise bacterial membranes and planer lipid bilayers as well in a voltage-dependent manner. It was demonstrated that Pep5 requires energized membranes to exert its biological activity (Kordel and Sahl, 1986; Sahl, 1985). However, it was proven that Pep5 dissipate the membrane potential of *Staphylococcus cohnii* 22 cells optimally at pH 7.5 at - 130 mV. Reduction in the activity was observed at pH 5.5 or when cells energized to approximately -100 mV were used. Below -100 mV, Pep 5 showed to have little effect on planer lipid membranes, whereas the activity increased dramatically above -100 mV. Pep5 showed to form short-lived pores with the diameter of 0.1-1 nm (Kordel *et al.*, 1988).

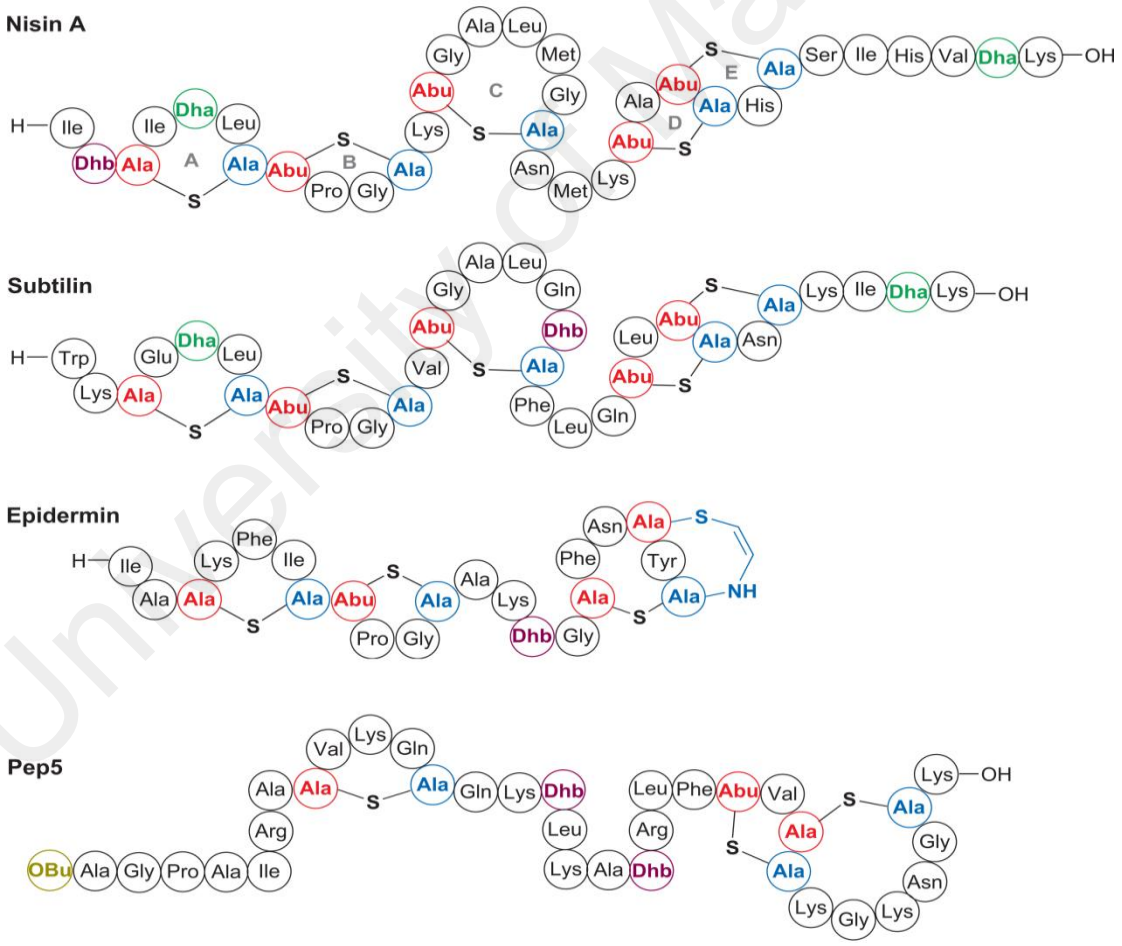

Figure 2.8: Structures of nisinA, subtilin, epidermin and pep5. Adapted from Willey and van der Donk (2007).

2.2 Type AII lantibiotics

This class of lantibiotics includes lacticin 481 group.

2.2.1 Lacticin 481 group

This is the largest group of lantibiotics consisting of more than 16 members such as lacticin 481, ruminococcin A, macedocin, streptococcin A-FF22, mutacin II, nukacin ISK-1 and salivaricins (A, B, G32, 9).

2.2.1.1 Lacticin 481 biosynthesis, genetic organization and mode of action

Lacticin 481 is a lantibiotic produced by *Lactococcus lactis* subsp. *lactis* CNRZ 481 (Piard *et al.*, 1992). Lacticin 481 is encoded by the *lct* gene which includes the transcription of 51 amino acids pre-peptide (24 residues leader peptide and 27 residues propeptide) (Piard *et al.*, 1993). Two-dimensional NMR studies suggested that lacticin 481 contains both lanthionine and β-methyl-lanthionine structures with a molecular weight of 2901 Da (Piard *et al.*, 1993).

Lacticin 481 is maturated by a modification enzyme encoded by the *lctM* gene and exported by transporter encoded by the *lctT* gene. These genes were found to be organized as an operon encoded by a 70-kb plasmid (Rince *et al.*, 1994). Additionally, this operon was shown to contain three more genes namely *lctF*, *lctE*, and *lctG* which are associated with lacticin 481 immunity. Strains lacking any of these genes failed to confer immunity to lacticin 481 (Rince *et al.*, 1997).

Lacticin 481 was shown to be a potent inhibitor of peptidoglycan biosynthesis eliminating the PBP2 (penicillin-binding protein 2) activity completely *in vitro*. Lacticin 481 can form a stable complex with lipid-II indicating that this lantibiotic uses lipid-II as a docking site to facilitate the interaction with bacterial membranes (Böttiger *et al.*, 2009). Flow cytometric analysis showed that lacticin 481 is not able to induce pore

formation into targeted bacterial membranes, unlike nisin which is a potent pore formation inducer (Knerr *et al.*, 2012).

2.2.1.2 Members of the lacticins 481 group

Ruminococcin A is a 2,675 Da lantibiotic which belongs to lacticin 481 family and produced by a strictly anaerobic strain of *Ruminococcus gnavus* isolated from human feces (Dabard *et al.*, 2001). Butyrivibriocin OR79A is another lantibiotic which belongs to the same family and produced by *Butyrivibrio fibrisolvens* OR79. The structural gene *bvi79A* encodes 47 amino acids of prepeptide and 25 amino acids of a mature peptide. A gene involved in the lanthionine formation was found downstream of the *bvi79A* (Kalmokoff *et al.*, 1999).

Bactericidal lantibiotic Mutacin II produced by group II *Streptococcus mutans* was first described in 1995. This peptide has a unique mechanism of action which differs from the typical pore formation of type A lantibiotics. Mutacin II acts mainly by inhibiting essential enzyme functions at the level of metabolic energy generation (Chikindas *et al.*, 1995). Genetic analysis revealed that Mutacin II biosynthetic genes cluster consists of seven open reading frames: *mutR* (the regulator), *mutA* (structural gene encoding pre-pro-peptide), *mutM* (encoding modification enzyme involves in the maturation of mutacin II), *mutT* (encoding ABC transporter) and *mutFEG* (self-protection and immunity determinants) (Chen *et al.*, 1999). Structure-Activity Study of mutacin II using mutagenesis revealed that the hinge region of this lantibiotic is essential for its biological activity (Chen *et al.*, 1998).

Nukacin ISK-1 is a member of this lantibiotic group and was well studied and characterised previously. This lantibiotic is produced by *Staphylococcus warneri* ISK-1 as a 27 amino acids peptide (Sashihara *et al.*, 2000). Two modes of action were proposed for nukacin action. The first study demonstrated that nukacin ISK-1 exhibited

a bacteriostatic mode of action towards *Bacillus subtilis* cells without pore formation or cytoplasmic membrane dissipation (Asaduzzaman *et al.*, 2009). However, an alternative bactericidal mechanism of action of nukacin ISK-1 was also reported against *Micrococcus luteus* cells in a separate study with potent pore formations (Roy *et al.*, 2014). Nukacin ISK-1 has a lipid II binding motif located at its ring A which suggests that nukacin ISK-1 uses lipid II as a docking site to bind to the cytoplasmic membrane of targeted bacterial cells. This lantibiotic showed to inhibit peptidoglycan biosynthesis in susceptible cells by accumulating the cell wall precursor UDP-MurNAc-penta peptide (Islam *et al.*, 2012). Replacing the ring forming residues of nukacin ISK-1 caused complete loss of bioactivity. However, Gly5, His12, Asp13, Met16, Asn17 and Gln20 residues of nukacin ISK-1 was shown to be essential for antimicrobial activity (Islam *et al.*, 2009). The N-terminus of nukacin ISK-1 contains three lysine residues giving this lantibiotic 3^+ overall charges. Replacing these three lysine residues with alanine residues resulted in significant reduction in nukacin activity. Deletion of only one of the three lysine residues did not abolish the bioactivity of nukacin ISK-1 suggesting that the magnitude of charge is not necessary for the activity as long as nukacin ISK-1 maintains an overall positive charge (Asaduzzaman *et al.*, 2006; Ross and Vederas, 2011). Furthermore, nukacin ISK-1 showed significantly higher affinity to anionic membranes than to zwitterionic membranes (Asaduzzaman *et al.*, 2006). The nukacin ISK-1 is encoded on pPI-1 plasmid and the biosynthesis gene cluster consists of eight genes that includes response regulator (*nukR*), structural gene (*nukA*), modification enzyme (*nukM*), transporter (*nukT*), immunity determinants (*nukFEG*) and a second immunity system (*nukH)* (Aso *et al.*, 2004). The immunity gene nukH was shown to synergistically confer self-protection and host immunity with the *nukFEG* system. When both systems *nukFEG* and *nukH* were co-expressed, they conferred a

significantly higher level of nukacin ISK-1 immunity than to expressing each of the two systems individually (Aso *et al.*, 2005).

The first member of lacticin 481 group to be produced by *Streptococcus pyogenes* is streptococcin A-FF22 (Tagg *et al.*, 1973). *S. pyogenes* is an oral pathogen associated with a sore throat and can cause severe illness and acute pharyngitis. This β-hemolytic bacterium which belongs to group A streptococci (GAS) was shown previously to produce inhibitory substances by some of M-type 49 strains (Tagg and Skjold, 1984). *S. pyogenes* produces streptococcin A-FF22 like lantibiotics to achieve better dominance in the oral cavity during infections. The genetic organization of streptococcin A-FF22 biosynthesis comprises of sensor kinase, regulator, structural gene, modification enzyme, transporter and immunity genes. These genes are designated as a *scnKRAMTFEG* cluster (Hynes *et al.*, 1993; McLaughlin *et al.*, 1999). The mode of action of streptococcin A-FF22 was intensively studied by Ralph Jack and his colleagues who reported that this lantibiotic can bind to non-energized artificial phospholipids vesicles and induce efflux of radiolabelled amino acids from energized membrane vesicles. This study suggested that unlike lacticin 481, streptococcin A-FF22 can generate unstable pores with diameters of 0.5-0.6 nm and induce membrane potential disruption in susceptible cells (Jack *et al.*, 1994).

Mutacin II is a bactericidal lantibiotic of lacticin 481 group produced by *Streptococcus mutans*, unlike streptococcin A-FF22, mutacin II does not form pores in susceptible bacterial membranes but rather inhibits essential enzyme functions at the level of metabolic energy generation (Chikindas *et al.*, 1995). Mutacin II biosynthesis gene cluster *mutRAMTFEG* is similar to that of lacticin 481 members in both organization and functions (Chen *et al.*, 1999).

2.2.1.3 Consensus structure of lacticin 481 group

Salivaricin A is the smallest lantibiotic in this group with a molecular mass of 2315 Da (Ross *et al.*, 1993) while the biggest is mutacin II 3245 Da (Chikindas *et al.*, 1995). The primary structure of active lacticin 481 has been elucidated (van den Hooven *et al.*, 1996) showing that this lantibiotic contains four unusual residues: one each of Dhb and MeLan and two Lan. The same structure was also found in mature mutacin II lantibiotic (Krull *et al.*, 2000). Both lacticin 481 and mutacin II share the same thioether bridging pattern located at the C-terminal side and occupying two-thirds of the molecule. Out of the seven modified residues, four are conserved in most of the propeptides in this group. However, in some cases, serine residues at positions 11 and 18 are replaced by threonine which is found dehydrated. For example, the Thr18 of streptococcin A-FF22 was shown to form MeLan structure (Jack *et al.*, 1994). Although streptococcin A-FF22 contains tow MeLan and one Lan residues instead of two Lan and one MeLan but it shares the same bridging pattern and ring topology of lacticin 481 and mutacin II (Jack *et al.*, 1994; Sahl and Bierbaum, 1998). The C-terminal part of salivaricin A is four residues shorter than the other members of lacticin 481 group and it contains three MeLan residues while it lacks in Threonine at position 24 which gives rise to a Dhb when present (Dufour *et al.*, 2007). There is no evidence if MacA1 can be found as a functional mature lantibiotic. However, the translation of its structural gene revealed that the threonine at position 24 is replaced by serine and hence it could generate a Dhb residue (Papadelli M *et al.*, 2005). Unlike most of the lantibiotics of lacticin 481 group, Dabard and co-authors suggested that ruminococcin A has a different pattern (Dabard *et al.*, 2001) when compared with the typical modification of the seven residues found in variacin, nukacin ISK-1, warnericin RB4, butyrivibriocin OR79A and macedocin (Georgalaki *et al.*, 2002; Kalmokoff *et al.*, 1999; Kimura *et al.*, 1998; Minamikawa *et al.*, 2005; Pridmore *et al.*, 1996; Sashihara *et al.*, 2000).

Salivaricins A, B, 9 and G32 are all produced by strains of *Streptococcus salivarius* and were reported previously to contain lanthionine and methyl-lanthionine structures. The biosynthesis gene clusters of SalA, SalB and Sal9 were elucidated and fully sequenced previously and it were shown to match what was reported for the members of lacticin 481 group. However, the genetic basis of salivaricin G32 was partially sequenced and analysed (Heng *et al.*, 2011; Hyink *et al.*, 2007; Ross *et al.*, 1993; Wescombe *et al.*, 2012). In most cases salivaricin lantibiotics were shown to be encoded on megaplasmids rather than the bacterial chromosome (Wescombe *et al.*, 2006). These molecules are of great importance to control the oral cavity hygiene and to maintain upper respiratory tract health.

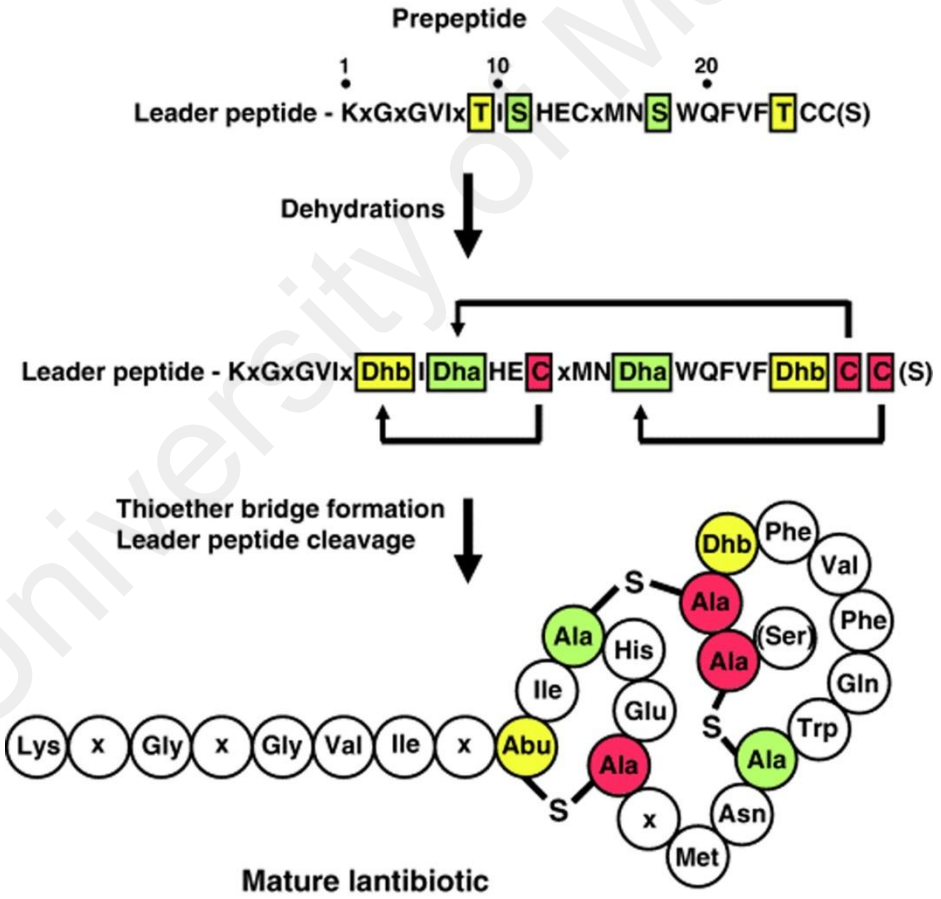

Figure 2.9: Primary structure of lantibiotics of lacticin 481 group and their maturation. Adapted from Dufour *et al.*(2007).

Interestingly, some members of the type-AII lantibiotics such as bovicin HJ50 and thermophilin 1277 produced by *Streptococcus bovis* and *Streptococcus thermophiles*

respectively possess disulfide bond replacing a lanthionine. However, they maintain ring topology similar to lacticin-481 (Kabuki *et al.*, 2009; Xiao *et al.*, 2004).

Bovicin HJ50 was shown to bind to lipid II precursor and induced pore formation in the targeted bacterial membrane (Xiao *et al.*, 2004; Zhang *et al.*, 2014).

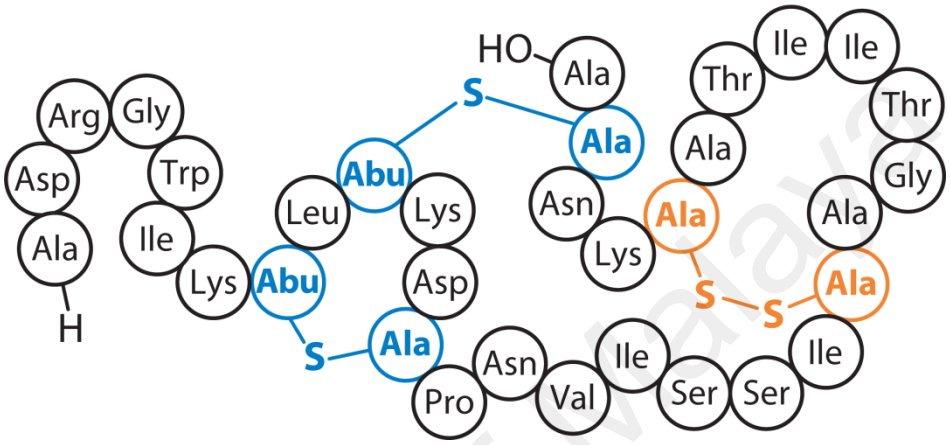

Figure 2.10: The secondary structure of bovicin HJ50. Disulfide bond (Ala-S-S-Ala) is indicated in orange. Adapted from Knerr and van der Donk (2012).

2.3 Type B lantibiotics

2.3.1 Mersacidin group

This group of lantibiotics comprises of four lantibiotics; mersacidin, actagardine, Ala(O)-actagardine and michiganin A.

During a screening scheme for novel antibiotics discovery, Chatterjee and co-workers isolated a new lantibiotic called mersacidin produced by *Bacillus* species HIL Y-85,54728. The 1824 Da peptide was shown to contain MeLan structure (Chatterjee *et al.*, 1992). This lantibiotic was shown to be active particularly against *Staphylococcus aureus* strains including MRSA and other Gram-positive bacteria but not the Gram-negative bacteria. The mode of action of mersacidin was elucidated and shown to involve in the inhibition of the peptidoglycan biosynthesis. A study carried out by Brötz and co-workers revealed that the biosynthesis of DNA, RNA and protein were not affected in mersacidin treated-*Staphylococcus simulans* model. However, incorporations of glucose and D-alanine were inhibited with a reduction in the cell wall thickness of the treated cells (Brotz *et al.*, 1995).

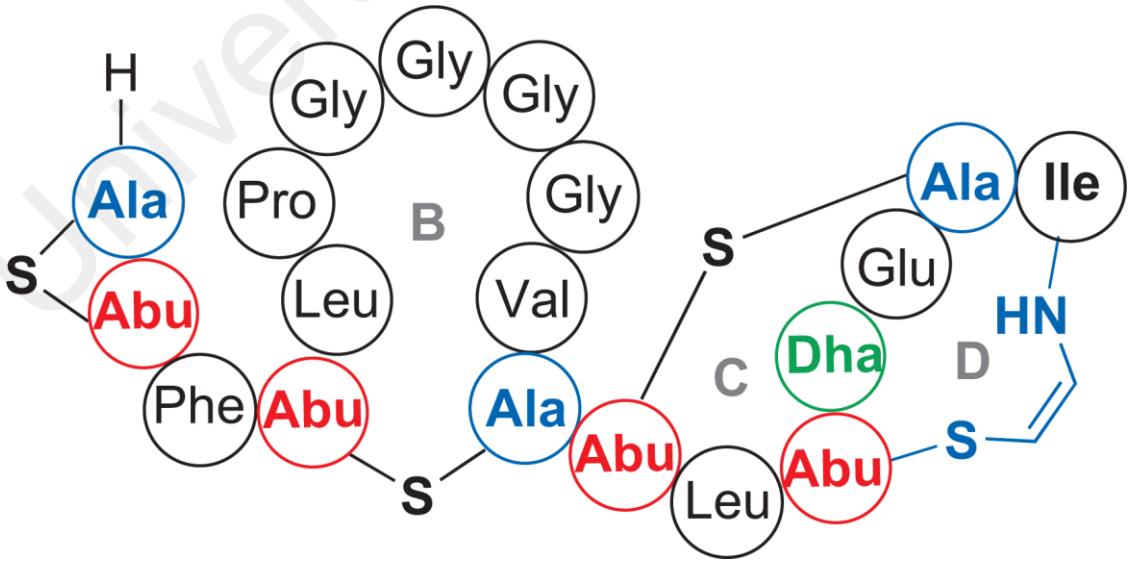

Figure 2.11: Secondary structure of the lantibiotic mersacidin. Adapted from Knerr and van der Donk (2012).

The 12.3 kb biosynthesis gene cluster of mersacidin lantibiotic consists of 10 open reading frames located on the chromosome of the producer strain *Bacillus* species HIL Y-85,54728. In addition to the structural gene *mrsA* encoding the mersacidin precursor, the gene cluster contains two genes *mrsM* and *mrsD* encoding modification enzymes playing a role in the maturation of the lantibiotic, one transporter encoding gene *mrsT* and three genes *mrsFGE* encoding ABC-transporting system involves in mersacidin immunity (Altena *et al.*, 2000). The *mrsR2* and *mrsK2* genes are two-component regulatory system involve in the self-protection of the producer cells while mrsR1 is an important regulator of mersacidin production (Guder *et al.*, 2002; Schmitz *et al.*, 2006) Figure 2.12.

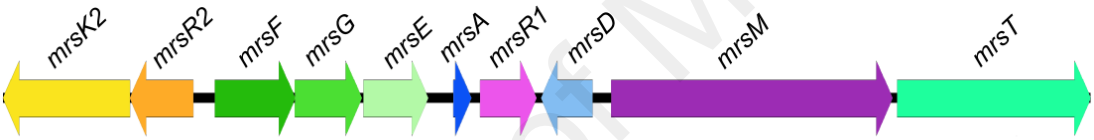

Figure 2.12: The genetic organization of mersacidin biosynthesis. The figure was produced using easyfig software (Sullivan *et al.*, 2011).

Actagardine (formally known as gardimycin) is another example of type-B lantibiotics produced by *Actinoplanes liguriae*. This molecule was first described in 1995 by Zimmermann and co-workers. Actagardine structure was elucidated by NMR and it was found to contain one Lan and three overlapping β-MeLan bridges (Zimmermann *et al.*, 1995). What makes actagardine a good candidate for clinical and pharmaceutical development is the high effectiveness and low toxicity of this antimicrobial agent (Arioli *et al.*, 1976; Coronelli *et al.*, 1976; Parenti *et al.*, 1976).

The mode of action of actagardine was reported previously and shown to have a lysing effect towards susceptible bacterial cells. Furthermore, actagardine was shown to interfere with the cell wall biosynthesis by inhibiting peptidoglycan formation with

bactericidal effect on actively dividing cells rather than resting cells (Somma *et al.*, 1977).

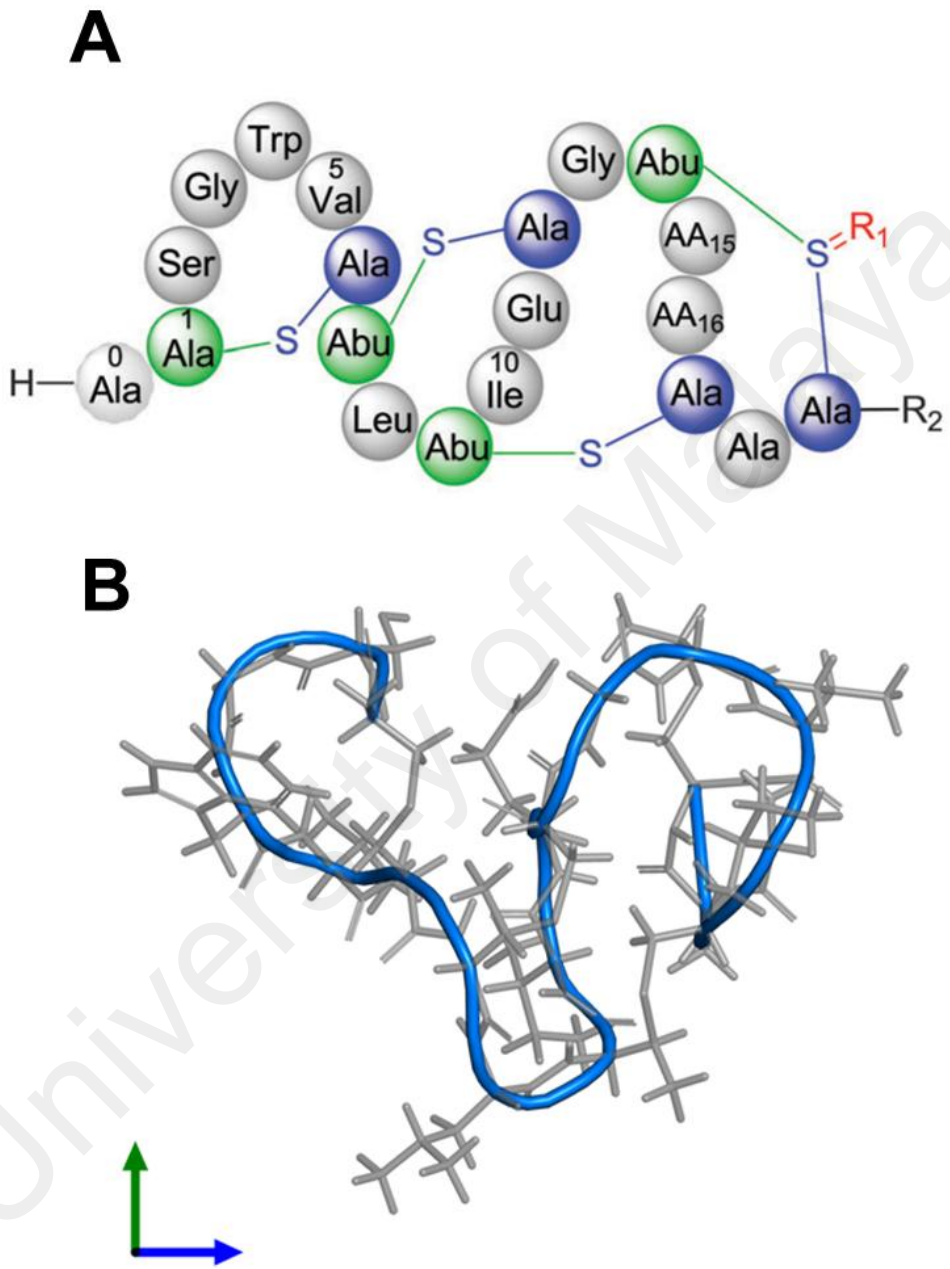

Figure 2.13: Secondary structure of the lantibiotic actagardin (A). The structure of the lantibiotic actagardin taken from Protein Data Bank in Europe (B). The peptide is highlighted and viewed from the front. The structure was determined at high resolution by homonuclear and heteronuclear two-dimensional and three-dimensional NMR spectroscopy (Zimmermann and Jung, 1997).

2.3.2 Cinnamycin group

Members of this group including cinnamycin, duramycin and ancovenin are type-B lantibiotics ranging in sizes of 1951-2008 Da. Cinnamycin and duramycin produced by *Streptomyces cinnamoneus* bind to phosphatidylethanolamine (PE) in a 1:1 stoichiometry (Machaidze and Seelig, 2003). Cinnamycin is a 19 amino acid lantibiotic containing one Lan and two MeLan ring structures. Additionally, cinnamycin contains lysinoalanine (Lal) bridge and an erythro-3-hydroxy-L-aspartic acid. All these modified structures are crucial to enable cinnamycin- phosphatidylethanolamine binding (Ökesli *et al.*, 2011). It was shown that these peptides bind exclusively to small membrane vesicles of high curvature and when large multilamellar liposomes were exposed to duramycin they were tubulated (Iwamoto *et al.*, 2007).

Ancovenin is also a member of this group and it was shown to contain Lan and MeLan structures in addition to dehydroalanine (Kido *et al.*, 1983; Wakamiya *et al.*, 1985).

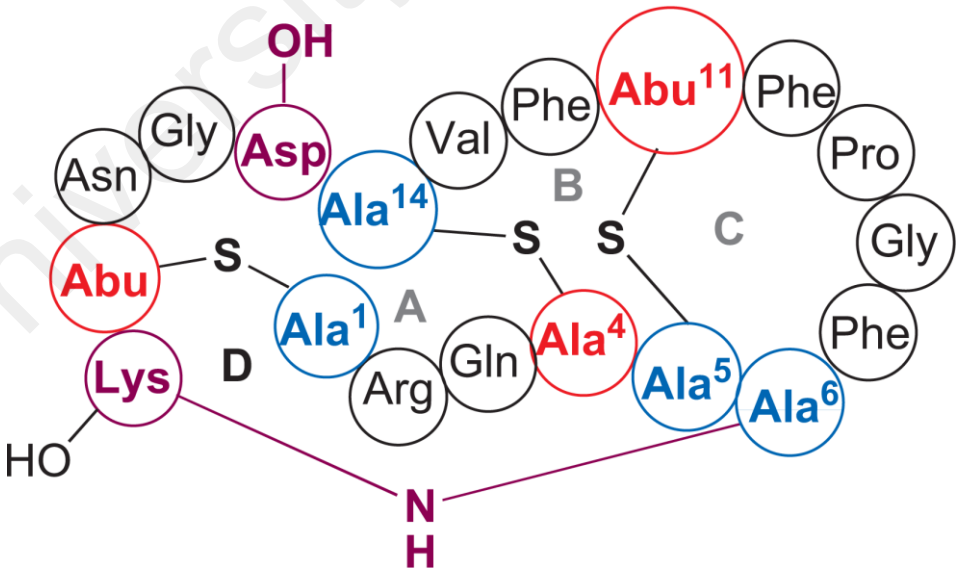

Figure 2.14: Secondary structure of the lantibiotic cinnamycin. lysinoalanine (Lal) bridge can be seen in ring D in addition to erythro-3-hydroxy-L-aspartic acid indicated in purple (Asp-OH). Adapted from Knerr and van der Donk (2012).

2.4 Type III lantibiotics

This class includes lantibiotics which are produced by actinomycetes such as SapT, SapB, LabA1 and LabA2. This kind of lantibiotics is morphogenetic peptides which facilitate aerial mycelium formation like SapB lantibiotic produced by *Streptomyces coelicolor* (Kodani *et al.*, 2004).

Recently, the lantibiotics avermipeptins, erythreapeptins, and griseopeptins produced by *Saccharopolyspora erythraea*, *Streptomyces avermitilis* and *Streptomyces griseus* respectively were characterised and shown to contain lanthionine and labionin structures. Dual-mode of peptide cyclisation is involved in the maturation of these lantibiotics (Völler *et al.*, 2012).

Labyrinthopeptin is a family of lantibiotics containing labionin structure produced by *Actinomadura namibiensis* (Muller *et al.*, 2010).

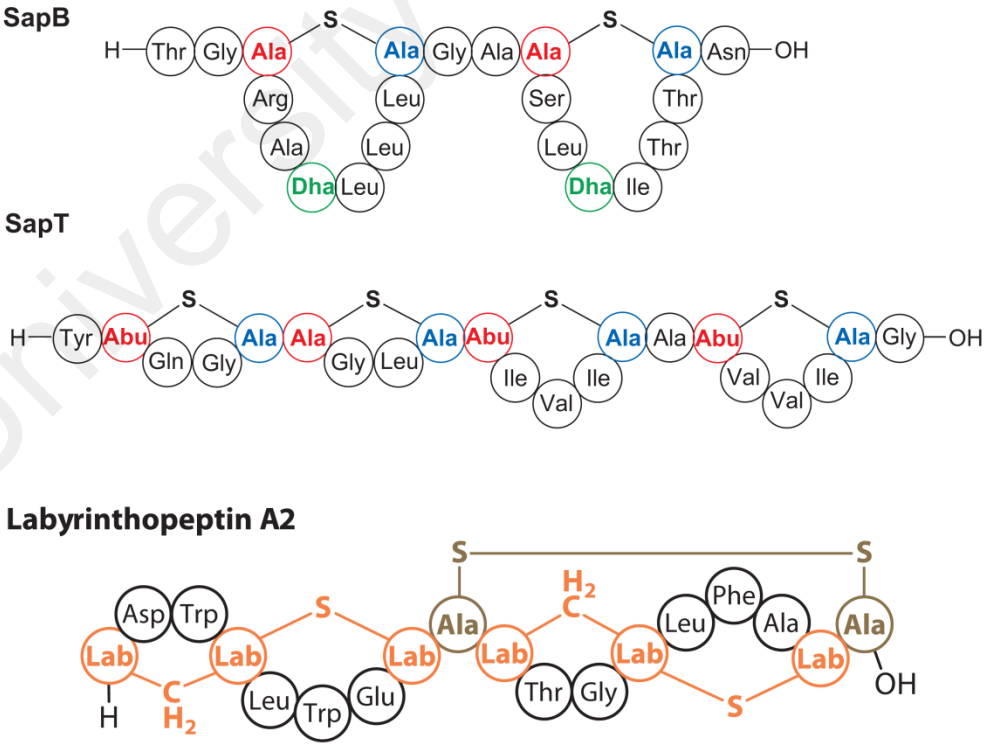

Figure 2.15: Examples of class III lantibiotic. Lab residues represent labionin structure (Willey and van der Donk, 2007).

2.5 Two-peptide lantibiotics

This group consists of lantibiotics which function by consequence of the synergistic activity of two peptides most of which showed to be potent at nano molar levels against multi-drug resistant pathogens (Lawton *et al.,* 2007). Examples of this type of lantibiotics are lacticin 3147, staphylococcin C55, plantaricin W, Smb, BHT-A, haloduracin and lichenicidin (Dischinger *et al.*, 2009; Holo *et al.*, 2001; Hyink *et al.*, 2005; Lawton *et al.*, 2007; Navaratna *et al.*, 1998; Ryan *et al.*, 1996; Yonezawa and Kuramitsu, 2005).

Lacticin 3147 (comprise of two peptides A1 and A2) produced by *Lactococcus lactis* DPC3147 was described in 1995 and it was used in cheddar cheese manufacture (Ryan *et al.*, 1996). This lantibiotic is bactericidal in action and exerts biological activity by dissipating the membrane potential of the targeted bacterial cells by pore formation. The pores were shown to be selective for K^+ ions and other inorganic phosphate and the activity was enhanced with energized cells (McAuliffe *et al.*, 1998).

Lacticin 3147 was shown to be effective *in vivo* and it showed to prevent systemic spread of *Staphylococcus aureus* in a murine infection model (Piper *et al.*, 2012). The potency of lacticin 3147 includes the inhibition of important clinical pathogens such as *Clostridium difficile,* methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant enterococci (VRE) (Piper *et al.*, 2009; Rea *et al.*, 2007).

Multidimensional NMR spectroscopy showed structural differences between the two components of the lantibiotic. Lacticin 3147 A1 was shown to resemble mersacidin-like type-B lantibiotics in the bridging pattern whereas Lacticin 3147 A2 was shown to belong to the elongated type-A lantibiotics (Martin *et al.*, 2004).

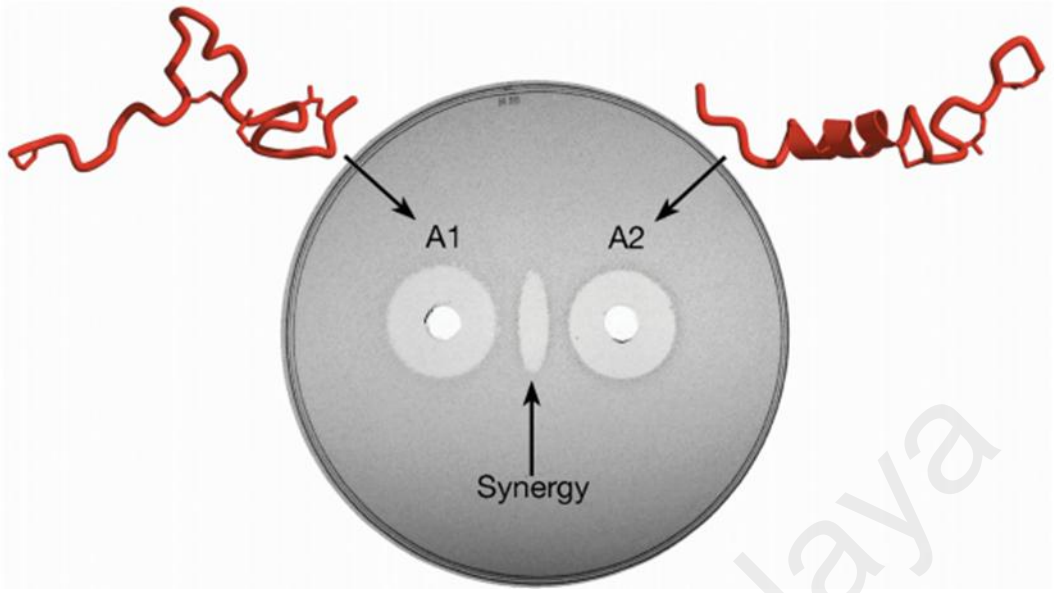

Figure 2.16: The structure and the synergistic activity of lacticin 3147-A1 and A2 against *Lactococcus lactis* subsp. *crimoris* HP in well diffusion assay. This figure was taken from Martin and Breukink (2007).

Lacticin 3147 showed a potent inhibitory activity when the two peptides A1 and A2 were combined whereas modest activity was observed when each peptide was administrated individually. Like nisin, lacticin 3147 was shown to have lipid II-mediated mode of action and the pore formation was observed when the two peptides were combined. The study revealed that A1 peptide binds first to the lipid II precursor to form A1-lipid II complex followed by a subsequent binding of the A2 peptide to this complex to initiate pore formation (Wiedemann *et al.*, 2006).

Staphylococcin C55 is another two-peptide lantibiotic produced by *S. aureus* C55 is related to the lantibiotic lacticin 3147 (O'Connor *et al.*, 2007). Like lacticin 3147, the two-peptides of staphylococcin C55 (C55α and C55β) work synergistically. A cured derivative of strain C55 exhibited a significant reduction in the lantibiotic inhibitory activity (Navaratna *et al.*, 1998).

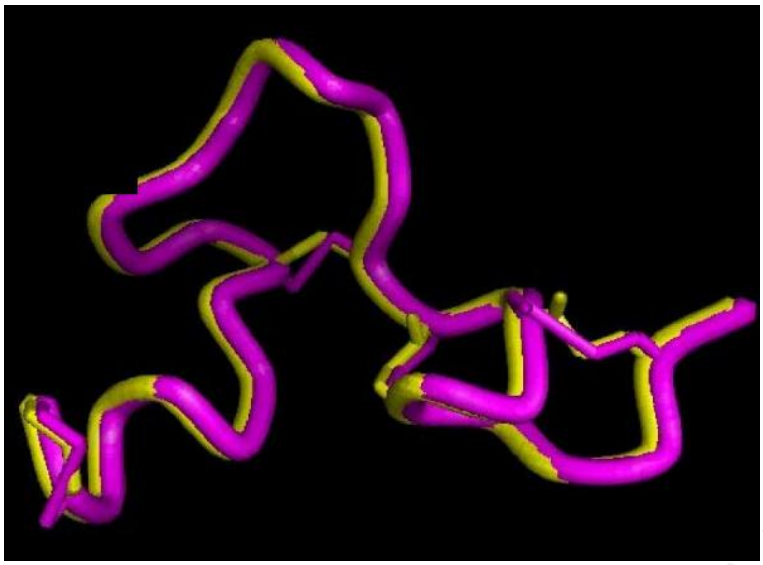

Figure 2.17: Homology modeling of LtnA1 (yellow) and C55α (purple). Adapted from O'Connor *et al.* (2007).

Haloduracin is another example of this group of lantibiotics and it was intensively studied previously. This two-component lantibiotic was identified in the genome draft of *Bacillus halodurans* C-125. The two peptides Halα and Halβ were heterologously expressed in *E. coli* overexpression system, purified and processed by modification enzymes HalM1 and HalM2. The biological activity of haloduracin is largely dependent on the presence of both mature peptides (McClerren *et al.*, 2006). It was found that Halα peptide contains highly conserved lipid-II binding motif (CTLTXEC) in its ring B. Replacing the glutamate residue in this motif for alanine or glutamine completely abolished the biological activity (Cooper *et al.*, 2008). Halα was also shown to Binds the Peptidoglycan Precursor Lipid II with 2:1 Stoichiometry (Oman *et al.*, 2011).

The two-peptide lantibiotic haloduracin showed a potent activity in a 1:1 stoichiometry similar to the activity of lantibiotic nisin. Structurally, haloduracin differs to lacticin 3147. However, both two-peptide lantibiotics share the same mode of action. Halα first binds lipid-II precursor of the Gram-positive bacteria followed by Halβ which induces pore formation and potassium efflux (Oman and van der Donk, 2009).

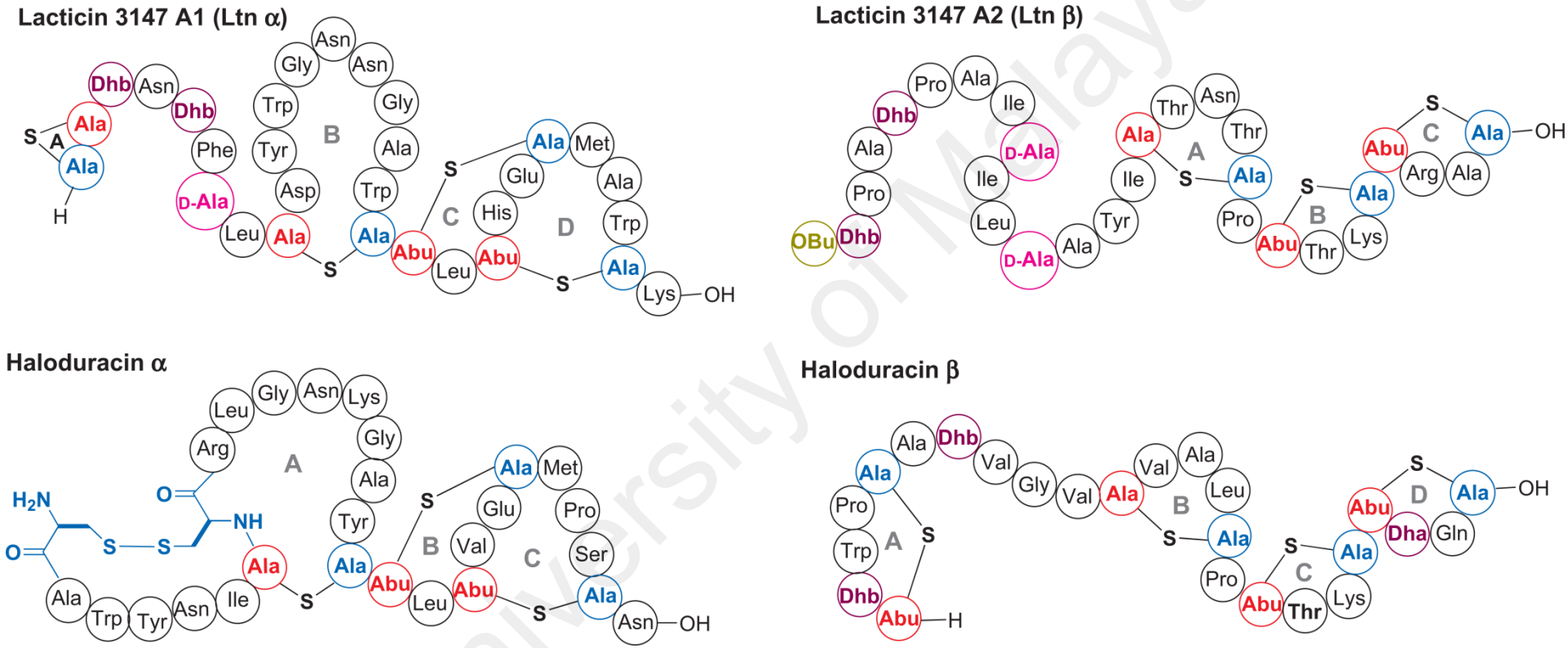

Figure 2.18: Secondary structures of haloduracin (Halα & Halβ) and lacticin 3147 (Ltnα & Ltnβ). Adapted from Willey and van der Donk (2007).

2.6 Other unclassified lantibiotics

There are lantibiotics which have not yet been assigned to a group such as lactocin S, bovicin HC5 and others.

Lactocin S is a 37 residues lantibiotic and was first shown to be produced by *Lactobacillus sakei* (Mørtvedt *et al.*, 1991). This lantibiotic includes unique post-translational modification like D-alanine, α-ketoamide and α-hydroxyamide N-terminal caps. The unique structure of lactocin S makes it distinct to any other lantibiotic classes.

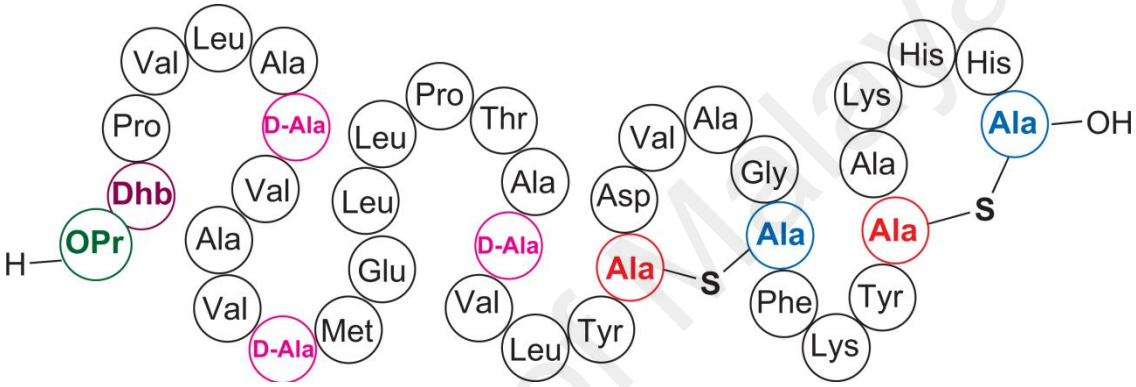

Figure 2.19: Secondary structure of the lantibiotic lactocin S (Willey & van der Donk, 2007).

Bovicin HC5 produced by *Streptococcus bovis* was shown to have a broad spectrum of activity against Gram-positive bacteria. However, this lantibiotic has only 55% homology with a lantibiotic precursor of *S. pyogenes* SF370. Bovicin HC5 has a lipid II-binding motif at its N-terminus and hence it was shown to bind to bacterial membranes. Nevertheless, Bovicin HC5 induced pore formation only in thin membranes and induced the segregation of lipid II into domains in giant model membrane vesicles (Paiva *et al.*, 2011). It was shown that Bovicin HC5 can auto-regulate its own biosynthesis through activation of BovK/R two-component system (Teng *et al.*, 2014)

2.7 Bioengineered lantibiotics

The bioengineering of lantibiotics is less straightforward than that of non-modified bacteriocins and other antimicrobial peptides. In the case of lantibiotics, other biosynthesis determinants must be considered. Beside the lantibiotic structural gene, the expression system must include the lantibiotic modification and maturation enzymes, immunity, regulatory and transportation proteins. The expression of the structural genes for nisin A, nisin Z, gallidermin, epidermin and Pep5 was described previously (Kuipers *et al.*, 1996). Site-directed mutagenesis enabled the discovery of residue and motifs which are crucial for the lantibiotic bioactivity, biosynthesis and immunity. The lantibiotic mutagenesis includes thiol bridge formation, Dha and Dhb modification, charge alteration and hinge region manipulation (Cotter *et al.*, 2005).

Generally, eliminating a thiol ring will significantly reduce the biological activity in lantibiotics. For example, the biological activity of epidermin was eliminated when hydroxyamino acids involved in thiol bridge formation were replaced by non-hydroxyamino acids (Ser3Asn and Ser19Ala) (Ottenwalder *et al.*, 1995). Similarly, mutacin II activity vanished when the Thr10 residue was replaced by alanine. However, mutation of this threonine residue to serine produced correctly modified and processed lantibiotic with a strong activity resembling the wild-type mutacin (Chen *et al.*, 1998).

Dehydration of serine to dehydroalanine (Dha) and of threonine to dehydrobutyrine (Dhb) is the most common post-translation modifications in lantibiotics. Dha and Dhb residues interact with cysteine to form intramolecular lanthionine and β-methyllanthionine structures respectively. Attempts to alter serines or threonines involved in Lan/MeLan formation eliminated the lantibiotic activity (Field *et al.*, 2008).

Incorporation of negatively charged residues in lantibiotics generally leads to the elimination of the inhibitory activity. However, less severe outcome or even enhanced

activity was achieved when positively-charged residues were introduced. For example, the introduction of arginine into the hinge region of nisin (NZ9800pCI372nisAN20R, M21R and K22R) significantly reduced the bioactivity whereas introduction of histidine at N20H and K22H displayed strong inhibitory activity (Field *et al.*, 2008).

The hinge region of natural nisin contains various hydrophobic amino acids (Met21 in nisin A & Z, Leu21 in nisin Q, Pro20/Leu21 in nisin U/U2). Mutated producer strains with introduced leucine (K22L), isoleucine (N20I, M21I) or methionine (K22M) retained high levels of bioactivity. The bioactivity of M21L producer strain was decreased (58–76%) comparing with wild-type nisin which may explain the variation in the activity of nisin Q, nisin U and nisin U2 which naturally possess a leucine residue at this location. The M21V variant strain exhibited increased levels of bioactivity against *S. aureus* ST528 (135%) (Field *et al.*, 2008).

Another study used the same approach and proposed that mutant nisin A analogues with hinge region consisting of AAK, NAI and SLS displayed enhanced antimicrobial activity against a variety of targeted bacterial strains. Hence it was concluded that the incorporation of small chiral amino acids within the hinge region can ultimately lead to derivatives with enhanced antimicrobial properties (Healy *et al.*, 2013).

A new method was developed for the preparation of improved lacticin 481 analogues containing non-proteinogenic amino acids. This bioengineering methodology is based on fusing the leader peptide of lacticin 481 to the modification enzyme LactM which allows the resultant protein to process the mature (core) peptide without a leader peptide (Oman *et al.*, 2012). Using an *in vitro* biosynthetic platform, lacticin 481 analogues were generated with both improved and reduced activities. This study suggested that the conserved glutamate residue at position 13 (Glu13) in the ring A of lacticin 481 is essential for the antimicrobial activity of this lantibiotic. Mutating the Glu13 to alanine

abolished the inhibitory activity. Similar results were achieved with different lantibiotics containing mersacidin-like lipid II binding motif (Knerr *et al.*, 2012).

It was suggested that inclusion of potassium bromide salt in the production medium of either the *Actinoallomurus* or the *Microbispora* producer strains of the lantibiotic NAI-107 can generate brominated variants of this lantibiotic with enhanced antimicrobial potency against selected bacterial strains. This unique approach can alter the composition of the molecule by incorporating brominated tryptophan within the ring A without affecting the post-translation modifications of this lantibiotic (Cruz *et al.*, 2015).

2.8 Developing lantibiotic-producing probiotics

Probiotic is a term derived from the Greek meaning "for life". It was first referred to substances produced by one microorganism that stimulate the growth of another which is the opposite of the term "antibiotic". It was also used to describe tissue extracts stimulating microbial growth. In 1974 probiotics were defined as "Organisms and substances which contribute to the intestinal microbial balance". Later on, probiotics were established in food products, infant formulae and therapeutic applications (Fuller, 1992; Goldin and Gorbach, 1992; Lee and Salminen, 1995).

Nowadays probiotics refer to "live organisms, which when administered in adequate amounts, confer a health benefit on the host" (Organization/WHO., 2002). Usually, probiotics are bacteria of intestinal origin such as bifidobacteria and lactobacilli with a beneficial impact on the GI tract. However, fostering healthy oral microbiota has attracted probiotic researchers to overcome other problems associated with oral health such as periodontal disease, dental caries and candidosis or even cardiovascular disease and rheumatic fever in some cases (Gerber *et al.*, 2009; Maharaj and Vayej, 2012).

The problem with intestinal-derived probiotics (when used for oral health applications) is that they lack oral persistence. Therefore, it was necessary to develop probiotics for oral health benefits derived from the oral cavity of healthy humans. The key factor influencing the development of oral probiotics is to choose non-pathogenic commensals which can produce inhibitory activity against oral pathogens such as *S. pyogenes*. These characteristics were found in *Streptococcus salivarius* which is a pioneer colonizer of the human oral cavity and a predominant member of the native microbiota that persists there throughout the life of the human host (Favier *et al.*, 2002).

S. salivarius strain K12 was the first strain to be developed as oral probiotic. This strain produces two kinds of lantibiotics namely salivaricin A2 and salivaricin B that belong to the lacticin 481 family of lantibiotics Type AII (Hyink *et al.*, 2007).

S. salivarius strain M18 is the second available oral probiotic that can produce anti-*Streptococcus mutans* lantibiotic designated as salivaricin M. This makes it an excellent choice to control tooth decay (Burton *et al.*, 2013; Heng *et al.*, 2011). Additionally, this strain harbours other lantibiotic biosynthesis gene clusters for salivaricins A2 and 9 productions and the putative large bacteriocin salivaricin MPS. The persistence of both K12 and M18 probiotics was demonstrated previously and it was shown that lantibiotic loci and adhesion factors are encoded in transferable megaplasmids in these strains (Burton *et al.*, 2013).

Safety assessment of strain K12 proved the stability of the metabolic profile and absence of streptococcal virulent determinants. Developing antibiotic resistance was not observed for this probiotic strain (Burton *et al.*, 2006).

The importance of developing lantibiotic-producing *S. salivarius* stems from its ability to control the oral health by inhibiting halitosis associated bacteria and reducing the

numbers of other streptococcal commensals which might cause many health implications in the upper respiratory tract.

Limited information is available regarding the biosynthesis and characteristics of lantibiotics produced by *S. salivarius* and their modes of action.

CHAPTER 3: METHODOLOGY

3.1 Bacterial strains and culture media

S. salivarius strains NU10, YU10 and GT2 were isolated from the oral cavity of healthy Malaysian subjects and their 16S rRNA gene sequences were deposited in the NCBI gene bank under accession numbers KC796011, KC796012 and KC796010 respectively. *S. salivarius* strain K12 was provided by John Tagg (University of Otago, BLIS technologies, New Zealand). Indicator strains including *Bacillus cereus* ATCC14579, *Lactococcus lactis* ATCC11454, *Lactococcus lactis subsp. cremoris* HP, *Micrococcus luteus* ATCC10240, *Streptococcus constellatus* ATCC27823, *Streptococcus dysgalactiae* subsp. *equisimilis* ATCC12388, *Streptococcus pneumonia* ATCC6301, *Streptococcus pyogenes* ATCC12344, *Streptococcus pyogenes* ATCC12348, *Streptococcus sanguinis* ATCC10556 were purchased from American Type Culture Collection (ATCC). *Listeria monocytogenes* NCTC10890 was acquired from National Collection of Type Cultures. Other indicator strains such as *Actinomyces naeslundii* TG2, *Corynebacterium spp* GH17, *Enterococcus faecium* C1, *Haemophilus parainfluenza* TONEJ11, *Lactobacillus bulgaricus* M8, *Staphylococcus aureus* RF122, *Streptococcus gordonii* ST2, *Streptococcus mutans* GEJ11, *Weissella confusa* A3 were taken from the culture collection of Microbial Biotechnology Laboratory, Division of Microbiology, Institute of Biological Science, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia. Todd-Hewitt broth (THB) (Difco) was used to propagate some of the bacterial strains in this study namely *S. salivarius*, *S. pyogenes* and *S. mutans*. Mitis Salivarius agar (MSA) (Difco) was used to isolate pure colonies of *S. salivarius* strains. M17 (Merck), MRS (Merck), Brain Heart Infusion (BHI) (Difco) media were used to study the growth kinetics of *S. salivarius* strains. Columbia blood agar base (Difco) supplemented with 5% (v/v) defibrinated sheep blood and 0.1% $CaCO_3$ (Baca) was used to carry out the deferred antagonism test. For MIC tests, M17

medium supplemented with 1% glucose (w/v) (GM17) was used to propagate the streptococcal strains. All streptococci were grown in a microaerophilic atmosphere using GasPak EZ CO_2 Container System, BD at 37°C. *M. luteus* and *Corynebacterium* spp. were grown aerobically at 37°C. *Lactococcus lactis* subsp. *cremoris* HP was propagated in GM17 at 30°C.

3.2 *S. salivarius* isolation and identification

Sterile cotton swabs were used to take samples from the tongue surfaces of the subjects. The samples were then streaked on MSA plates before they were incubated at 37°C for 18 hours aerobically with 5% CO_2. Blue and dome-shaped colonies were selected for further sub-culture on MSA. After pure colonies had been obtained, the *S. salivarius* strains were subjected to 16S rRNA gene sequencing to confirm the identity using the universal primers 27F, 5'-AGA GTT TGA TCC TGG CTC AG-3' and 1492R, 5'-CGG TTA CCT TGT TAC GAC TT-3'. The inclusion criteria of the subjects were as follows: a) healthy Malaysian children, b) age 4-15 years, c) races (Malay, Indian and Chinese) and d) gender (male and female). Exclusion criteria included a) children with dental problems e.g. tooth decay, b) children with upper respiratory tract diseases, c) children above 16 years old, d) Children with anemia and e) children with a sore throat.

3.3 Ethical approval for *S. salivarius* sampling

The subjects were required to sign a consent form to permit isolation of *S. salivarius* from their tongue surfaces using sterile cotton swabs. The ethics committee IRB Reference Number is OF BS1401/0017 (L) for our Institution (University of Malaya). The protocol used complied with Good Laboratory Practices (GLP).

3.4 Deferred antagonism test

The method was first described by Tagg and Bannister (1979) and performed in this study with some modifications. Bacteriocin producer strains were streaked on different

production media agar plates as 1 cm wide strip using sterilized cotton swabs. The producers were then incubated aerobically with 5% CO_2 for 18 hours at 37°C. Sterile cotton swabs were used to remove the bacteriocin producer bacteria before the plates were sterilized by inverting the plates over filter paper soaked with chloroform for 30 minutes. The plates were aired for another 30 minutes to remove any chloroform residues. Indicator bacterial strains of OD_{600} = 0.1 were streaked at a right angle across the producer streak. The plates were re-incubated under the same conditions mentioned above for 18 hours. Zones of no bacterial growth were recorded as antagonism activity due to bacteriocin production. The antagonism assay for each strain was repeated twice using different production media.

3.5 DNA extraction and PCR screening for genes encoding salivaricins production

Single *S. salivarius* colony grown on MSA medium for 18 hours was transferred to 10 mL of sterilized THB and incubated aerobically with 5% CO_2 for 18 hours at 37°C before the pellets were centrifuged at 8000 × *g* for 5 min at 4°C and suspended in 400 µL of 0.85% NaCl in water (w/v). The suspension was heated at 70°C for 30 min and the bacterial pellets were collected at 8000 × *g* for 5 min at 4°C. The pellets were suspended in lysing buffer (2mM Tris-HCl pH 8, 0.2 mM EDTA, 1.5% Triton ×100 (v/v) and 1000 U of mutanolysin) and incubated at 37°C for 2 hours. 50 µg/mL of lysozyme was added and the samples were further incubated for one hour at 37ºC. After three freeze-thaw cycles, the samples were then processed using DNeasy Blood and Tissue kit (Qiagen) following manufacturer's instructions for DNA extraction from Gram-Positive bacteria. PCR conditions for salivaricin genes amplifications were applied as described previously (Wescombe *et al.*, 2006; Heng *et al.*, 2011; Wescombe *et al.*, 2012) with some modifications to the reaction composition which included using

Top Taq™ Master Mix (QIAGEN). This experiment was performed using Applied Biosystems® Veriti 96-Well Thermo Cycler.

Table 3.1: Primers used for lantibiotic genes amplification.

Primer	Lantibiotic precursor	Sequencing
salAUS	Salivaricin A	GTAGAAAATATTTACTACATACT
salADS		GTTAAAGTATTCGTAAAACTGATG
salBF	Salivaricin B	GTGAATTCTCTTCAAGAATTGACTCTT
salBR		AAAATATTCATACCGCTCTTCC
sivF	Salivaricin 9	AAAAAGGCGCTTCTATATCCATGA
sivR		ATCTTTACCTCAAACTTTTAAGTCCATT
G32F	Salivaricin G32	GTAGTTATCGAATCAATTAAAGAAG
G32R		GCAACATGTAGCAAGAAATGCC
srtUS	Streptin	CTTTTTTTCTGAACARATTCTGAACT
srtDS		TAAAGGAGCAAACTAAAAWCAGTCTACT

3.6 Biochemical characterization of *S. salivarius* isolates

All the biochemical tests were performed using 50 CH and 20 strep API® kits to study the metabolic profiles of *S. salivarius* strains as shown in Table 2. The API kits were used according to manufacturer's instructions (API®-bioMeriex).

3.7 Antibiotics susceptibility test

The antibiograms of the three strains NU10, YU10 and GT2 were tested by the antibiotic disc diffusion assay according to Clinical and Laboratory Standards Institute (CLSI) (Vol. 32, No.3, Jan, 2012). *S. salivarius* cultures were grown on Mueller-Hinton Agar (Difco, USA) supplemented with 5% sheep blood (Liofilchem srl, Italy) for 20 hours at 37 °C in 5% CO_2 atmosphere using BD Gas Pak™ EZ CO_2 container system. Bacterial suspensions were prepared from morphologically identical colonies grown on the agar plates and suspended in saline solution (0.85% NaCl (w/v) in water). The resultant bacterial suspensions were adjusted to the turbidity of 0.5 McFarland before

bacterial lawns were performed on the same blood agar plates mentioned above. Antimicrobial susceptibility test discs (OXOID, UK) were placed on top of the pre-seeded plates using sterile forceps and the plates were incubated as indicated above. Antibiotics used in this test are penicillin G, penicillin V, amoxicillin, ofloxacin, tetracycline, erythromycin, gentamicin, clindamycin, streptomycin, vancomycin, novobiocin and chloramphenicol. Both original cultures stored at -80 °C from two years and weekly subcultures used in the lab routinely were tested to check for any differences following storage. Strain K12 was also tested as a control since it was reported previously (Burton *et al.*, 2006) for susceptibility against the same and additional antibiotics used in this study. Measurement of the diameters of zones of complete inhibition (as judged by the unaided eye) including the diameter of the disc are listed in Table 3. This experiment was repeated in triplicates and showed almost identical results.

3.8 Preparation of the first genome drafts for strains NU10 and YU10

Genome sequencing was carried out using Illumina's compact MiSeq® system at the High Impact Research Center, University of Malaya, Malaysia. Genomic libraries were prepared using the Nextera kit Illumina (Illumina, Inc., San Diego, CA) which produced a mean insert size between 800 and 1,200 bp. Total of 379-fold and 204-fold coverages were generated for strains NU10 and YU10 respectively. Approximately 85% of these reads were assembled using CLC Bio Genomic Workbench Software Version 6.0.5. Genome annotation was performed using RAST Version 4.0 (Aziz *et al.*, 2008). The genome analysis included the virulence assessment for YU10 and NU10 strains to prove the absence of any streptococcal virulence determinants within both strains genomes (Table 4).

3.9 Developing new bacteriocin-production medium

Different media e.g. M17, MRS, THB and BHI were used to study the growth kinetics of *S. salivarius* grown aerobically at 37ºC. Each *S. salivarius* strain was grown on BACa plates (Columbia agar base supplemented with 5% (v/v) whole human blood and 0.1% (w/v) $CaCO_3$ for 18 hours at 37°C. Then the bacteria were washed from the agar plates using phosphate buffer saline at pH 7 and centrifuged to pellet the cells at 5000 × *g* for 10 minutes. Bacterial pellets were washed twice with the same buffer using the same conditions mentioned above before re-suspending in the same buffer. The bacterial suspension was then diluted using the same buffer to 0.5 McFarland before 20 µL of each bacterial suspension was used to inoculate 180 µL of each medium into a 96-well sterile plate. The plate used in this experiment was a flat base well plate and covered with a sterile plastic lid to prevent contamination. The growth was monitored by measuring the OD at a wavelength of 600 nm using a Multiskan™ GO Microplate Spectrophotometer (Thermo Scientific) over 24 hours. The spectrophotometer was set at medium speed shaking for 20 seconds before each photometric measurement. This experiment was carried out in triplicates and average of each triplicate measurement was used for growth kinetics (Figure 2). YNS medium (1% yeast extract, 1% neopeptone and 1% sucrose) and PTNYMES medium w/v (1% peptone, 1% tryptone, 1% neopeptone, 1% yeast extract, 1% sucrose, 1% MES (2-(N-morpholino)ethanesulfonic acid), 0.2 g/L NaCl, 0.5 g/L ascorbic acid, 0.25 g/L magnesium sulphate and 0.2 g/L sodium acetate) were also used in this study. Typical compositions of all media used in this study are listed in Table 5.

3.10 Lantibiotics production and purification

PTNYMES medium (adjusted before autoclaving at pH 6.5 using concentrated NaOH) was inoculated with 5% of *S. salivarius* cultures grown for 18 hours in the same medium. 4000 mL shaking flasks were used for this experiment at 37°C for 22 hours

with 150 rpm orbital shaking aerobically. The cultures were adjusted to pH 5.8 and incubated for 1 hour at 4ºC to adsorb levels of lantibiotics secreted into the liquid medium to the producer's cells. Then the cultures were centrifuged at 8500 × *g* for 30 minutes and the cells were re-suspended in 95% methanol (adjusted to pH 2 with concentrated HCl). The cell suspensions were stirred gently overnight at 4 °C for 18 hours before the cells were collected by centrifugation at 1000 × *g* for 30 minutes. The supernatant was evaporated using a rotary evaporator at 45°C and the crude lantibiotic was assayed for antimicrobial activity. The crude preparation was concentrated 10 fold and then diluted 1:5 (v/v) with 20 mM sodium phosphate buffer pH 5.8. This final preparation was subjected to FPLC ÄKTA purifier (GE Healthcare, Malaysian Genome Institute MGI) using HiTrap SP FF strong cation exchanger column pre-equilibrated with 20 mM sodium phosphate buffer pH 5.8 (buffer A). The column was washed with a 10× column volume of buffer A before a leaner gradient of buffer B (1 M NaCl in buffer A) was applied. Eluted fractions were collected using auto collector and the separation was monitored using three different UV wavelengths (207, 214 and 280 nm). Active fractions from 5 FPLC runs were pooled and concentrated before subjecting to Chromolith® SemiPrep RP-18e 100-10 mm column using Waters HPLC system with a gradient of 20% to 50% acetonitrile in water (v/v). UV wavelength of 214 nm was used to detect peptide peaks and the active fraction was subjected for the second time using the same column and conditions mentioned above to obtain the pure peptide. Well diffusion assay was performed to identify biologically active fractions using *Micrococcus luteus* ATCC®10240 and *Streptococcus pyogenes* ATCC®12344 as indicator targets. Pure lantibiotics were subjected to matrix-assisted laser desorption ionization time of flight mass spectrometry MALDI-TOF (MS) using 4800 *Plus* MALDI TOF-TOF™ Analyzer to determine the molecular weight of the lantibiotic.

3.11 Salivaricin B purification

S. salivarius K12 was grown for 18 hours in TSB at 37°C in a microaerophilic atmosphere (GasPak EZ CO_2 Container System, BD) before it was used to inoculate 150 plates containing M17YESUCa medium using sterile cotton swabs. The inoculated plates were incubated as mentioned above after which the whole cultures were scraped from the petri dishes, cut into small pieces and transferred into a 2000 mL beaker. This preparation was kept frozen at -40°C for overnight before it was allowed to thaw at 50°C in a water bath for 2 hours. The resultant extract was centrifuged at 10,000 × *g* for 30 minutes and the supernatant was passed through 0.22 µm pore size filter (Sartorius, Germany). The resultant liquid was passed through Amberlite XAD 16 column (Sigma, France) pre-equilibrated with methanol and water. The column was washed with 1 liter of distilled water followed by 500 mL of 50% methanol in water (v/v). Lantibiotic activity was eluted with 300 mL of 95% methanol adjusted to pH 2 using HCl. The methanol was evaporated using a rotary evaporator at 40°C under reduced pressure and the resultant lantibiotic-containing preparation was tested using a spot-on-lawn technique. Briefly, two-fold dilutions of the active fraction were tested by spotting 20µL on MHA and the spots were left to dry before the indicator strain (18 hours old *M. luteus* ATCC10240 adjusted to OD_{600} = 0.1 using PBS pH 7.2) was applied as a lawn on the top of the agar plate using a sterile cotton swab. Arbitrary units (AU) per milliliter were defined as the reciprocal of the highest dilution factor that showed inhibition of the indicator strain. Salivaricin B was further purified by HPLC (Waters) on Chromolith

SemiPrep RP-18e 100-10 mm column with a gradient of 20-50% acetonitrile in water (v/v) over 60 minutes at a flow rate of one mL per minute. The run was monitored using a UV detector at 214 nm. The fractions were collected manually every minute and the acetonitrile was evaporated using EYELA centrifugal evaporator CVE-2000 (Tokyo, Japan) equipped with a vacuum pump. The fractions were tested as mentioned above. After identifying the retention time of salivaricin B elution, several runs were performed and a consistent and reproducible chromatogram was achieved at the defined retention time. To further analyze salivaricin B purity, active salivaricin B eluted from the previous HPLC run was subjected to Aeris PEPTIDE 3.6u XB-C18 250x4.6mm column equilibrated with acetonitrile and water. Active fractions were subjected to 4800 *Plus* MALDI TOF/TOF Analyzer to determine the molecular weight.

3.12 Production of levansucrase

Strain YU10 was grown aerobically with 5% CO_2 for 18 hours at 37°C in one litre of M17 medium supplemented with 2% yeast extract, 2% sucrose and 0.1% $CaCO_3$ (M17YESUCa). The cells were collected by centrifugation at 18000 × *g* for 5 minutes and re-suspended in 200 mL of 95% methanol adjusted to pH = 2 using concentrated HCl and incubated at 4°C for 18 hours before the supernatant was collected by centrifugation at 18000 × *g* for 20 minutes. The methanol was evaporated using a rotary evaporator and the crude extract was lyophilized and kept at -20°C for further LC-MS/MS analysis.

3.13 Detection of levansucrase by LC/MS-MS

The lyophilized extract was re-hydrated using 500 µL of 0.1% formic acid and injected into Water Oasis HLB column equilibrated with 0.1% formic acid. The sample was eluted with 600 µL 50% acetonitrile in 0.1% formic acid. The eluted sample was separated by reverse phase chromatography. This was a Thermo Scientific EASY-nLC II system with a reverse phase pre-column Magic C18 AQ (100µm I.D., 2 cm length, 5 µm, 100Å. Michrom Bio Resources Inc, Auburn, located at University of Victoria, Canada) and attached to nano-analytical column Magic C18 (75µm I.D., 15 cm length, 5 µm, 100Å. Michrom Bio Resources Inc, Auburn, CA). The flow rate was set at 300 µl / minute. The system was coupled to an LTQ OrbitrapVelos mass spectrometer equipped with a Nanospray II source (Thermo Fisher Scientific). Mobile phases were A (2% acetonitrile in 0.1% formic acid) and B (90% acetonitrile in 0.1 formic acid). After a 249 bar (~ 5µL) pre-column equilibration and 249 bar (~ 8µL) nano-column equilibration, the sample was separated by 55 minutes gradient as follows: (5% solvent B: 0 minute, 40% solvent B: 60 minutes, 80% solvent B: 2 minutes and 80% solvent B: 8 minutes). The LTQ OrbitrapVelos (Thermo Fisher Scientific, Bremen, Germany) parameters were as follows: nano-electrospray ion source with spray voltage 2.2 kV, capillary temperature 225ºC, Survry MS1 scan *m/z* range from 400 to 2000 profile mode, resolution 60,000 at 400 *m/z* with AGC target 1E6 and one microscan with maximum inject time 200 ms. Lock mass Siloxane 445.120024 for internal calibration with preview mode for FTMS master scan: on, injection waveforms: on, monoisotopic precursor selection: on, rejection of charge state: 1. The sample was analysed with top-5 most intense ions charge state 2-4 exceeding 5000 counts were selected for CID FT-MSMS fragmentation and detection in centroid mode. Dynamic exclusion settings were: repeat count 2, repeat duration 15 seconds, exclusion list size 500, exclusion duration 60 seconds with a 10 ppm mass window. The CID activation isolation window was: 2 Da,

AGC target: 1E4, maximum inject time: 25 ms, activation time: 10 ms, activation Q: 0.250 and normalized collision energy 35%.

3.14 Parameters for Data analysis

Proteome Discoverer 1.3.0.339 software suite (Thermo Scientific) was used to analyze raw files. Parameters for the spectrum selection to generate peak lists of the CID spectra were as follows: activation type: CID, s/n cut-off: 1.5, total intensity threshold: 0, minimum peak count: 1, precursors mass: 350-5000 Da. The peak lists were submitted to an in-house mascot 2.2 against the Uniprot-Swissprot databases.

3.15 Antimicrobial peptides synthesis

The cecropins B1 and B2 were synthesized at Mimotopes Pty Ltd (Australia) using the 20 and 21 amino acid sequences namely KWKVFKKIEKMGRNIRNGIV (20 residues with a molecular weight of 2444.6 Da) and KWKVFKKIEKMGRNIRNGIVW (21 residues, with a molecular weight of 2630.6 Da), respectively. The purity of both peptides was checked by HPLC and was found to be ≥ 98%.

Table 3.2: Comparison between the cecropin B-like peptides used in this study and the natural cecropin B peptide.

Peptide	Amino acid sequence	Total hydrophobic ratio	Net charge	Molecular weight	Reference
Cecropin B	KWKVFKKIEKMGRNIRNGIVKAGPAIAVLGEAKAL	48 %	+7	3835.0	(van Hofsten *et al.*, 1985)
Cecropin B1	KWKVFKKIEKMGRNIRNGIV	40%	+6	2444.6	Current study
Cecropin B2	KWKVFKKIEKMGRNIRNGIVW	42%	+6	2630.6	Current study

3.16 Isolation of membrane vesicles of different bacterial cells

Cells from a 250 mL culture were resuspended in 7 mL of 10 mM HEPES buffer (pH 7.4) and lysed by sonication for 20 s and then left at 4˚C for 20 s. The step was repeated 4 times. The suspension was then centrifuged at 10,000 × *g* for 20 minutes at 4˚C to remove the cell debris. The membranes in the supernatant were collected by ultracentrifugation at 100,000 × *g* for 1 hour at 4˚C (Beckman Coulter, SW 41 Ti Ultracentrifuge Rotor Swinging Bucket). The pellets were resuspended in 2 mL of 10 mM HEPES at pH 7.4 and washed twice with 10 mL of 10 mM HEPES at pH 7.4. After final ultracentrifugation, the pellets were resuspended in 2 mL of 10 mM HEPES at pH 7.4 to concentrate the membranes.

3.17 Minimal inhibitory concentration (MIC) and bactericidal inhibitory concentration (MBC) of the synthetic peptides and other antibiotics

Minimal inhibitory concentration for Ampicillin, Penicillin G and Tetracyclin was performed according to Clinical and Laboratory Standards Institute (CLSI) using the broth microdilution method. MIC test of cationic antimicrobial peptides (synthetic cecropins B1 & B2 and nisin) was performed following the R.E.W. Hancock Laboratory protocol (Minimal Inhibitory Concentration Determination for Cationic Antimicrobial Peptides by Modified Microtitre Broth Dilution Method) with some modifications. Briefly, indicator strains namely clinical isolates *Pseudomonas aeruginosa* PA7, *Escherichia coli* UT181, *Staphylococcus aureus* RF122, *Corynebacterium spp* GH17 and *Bacillus cereus* ATCC14579 were subcultured on

MHA and incubated for 18 h at 37°C. Cultures were diluted with MHB to give 7×10^5 CFU/mL. 100 µL of each bacterial suspension was transferred to 10 polypropylene Eppendorf tubes and one tube was filled with MHB as a blank without bacteria. Serial dilutions of test cationic peptides were prepared at 10 times the required test concentrations in 0.01% (v/v) acetic acid, 0.2% (w/v) BSA in polypropylene Eppendorf tubes and 11 µL of 10× test peptide was added to the first nine bacterial tubes mentioned above. The tenth tube with bacterial growth was used as a control. The tubes were incubated for 18 h at 37°C. MIC was taken as the lowest concentration of the peptide that reduces growth by more than 50%.

3.18 Instrumentation for fluorescence measurements

Fluorescence spectra were recorded on a Shimadzu RF-5301 PC spectrofluorophotometer. Lifetime measurements were performed using a TimeMaster fluorescence lifetime spectrometer obtained from Photon Technology International. Excitation was done at 295 nm using a light-emitting diode and emission was detected using a Schott WG-320 nm cut-off filter. The instrument response function (IRF) was measured from the scattered light and estimated to be approximately 1.5 ns (full width at half-maximum). The measured transients were fitted to multiexponential functions convoluted with the system response function. The fit was judged by the value of the reduced chi-squared ($\chi 2$). The experimental time resolution (after deconvolution) was approximately 100 ps, using stroboscopic detection (James *et al.*, 1992). In all the experiments, samples were measured in a 1 cm path-length quartz cell at 23 ± 1 °C. The

concentration of all species (bacterial membrane, cecropins B1 and B2) was 0.05 mM in a phosphate buffer (10 mM) of pH 7.4. The reported values are the average of three measurements.

3.19 Minimal inhibitory concentration (MIC) and IC_{50} determination

MIC was determined using broth microdilution method. Two-fold dilutions of salivaricin B or nisin A lantibiotics were prepared in adequate media in polypropylene 96-well plate (Nunc) where each well contained 50 µL of each lantibiotic preparation. Overnight cultures of each test bacterium were diluted to 7×10^5 CFU.mL^{-1} using fresh medium and 50 µL of the diluted bacteria was added to every well. Wells with no bacteria added served as negative controls of no bacterial growth (blank). Wells containing bacterial culture only without any lantibiotic added served as a positive growth control. The plates were incubated at a suitable temperature and under suitable conditions. After 18 hours of incubation, the highest lantibiotic dilution which inhibited 90% of the bacterial growth was considered as the MIC. The IC_{50} values were measured as mentioned previously (Abts *et al.*, 2011) and the calculation was carried out using The IC50 Tool Kit (http://ic50.tk/).

3.20 Time killing assay

The mode of inhibitory activity of salivaricin B was determined by measuring the decrease in CFU over time for the targeted bacterial strains. Both *S. pyogenes* ATCC1234 and *M. luteus* ATCC10240 were used in this assay as sensitive strains. Ten hour old bacterial cultures were centrifuged at 2,000 × *g* for 5 minutes to pellet the cells

before washing with ice-cold 5 mM sodium phosphate buffer at pH 7.2. Each strain was washed twice and re-suspended in the same buffer to the original culture volume. The bacterial suspension was mixed at 1:1 ratio with either salivaricin B or nisin A lantibiotics at concentrations of 10× MIC and incubated at 37°C. Bacterial suspensions mixed with 5 mM sodium phosphate buffer pH 7.2 were served as a control. Survivors were determined at intervals by plating serial dilutions of the test and control mixtures on TSA and incubated for 24 hours at 37°C.

3.21 Pore formation assay

To investigate the ability of salivaricin B to generate pores into the targeted bacterial membranes, SYTOX Green probe was used as mentioned previously (Barbour *et al.*, 2013) with some modifications. *S. pyogenes* ATCC12344 was grown in GM17 under the same conditions mentioned above until mid-exponential phase was achieved (10^4-10^5 CFU.mL^{-1}). The bacterial culture was combined with SYTOX Green (final concentration 5µM) before ninety microliters of this suspension was transferred to MicroAmp Fast Optical 96-Well Reaction Plate (Applied Biosystems, Life Technologies, USA). The fluorescence was monitored for 10 minutes until the stable baseline was achieved. Ten microliters of pure salivaricin B (10× MIC) was added to this suspension and fluorescence signal from membrane-compromised bacteria labelled with SYTOX Green stain was detected with excitation and emission at 494 nm and 521 nm respectively using the Real-Time PCR as a fluorescence detection method as mentioned previously (Bourbon *et al.*, 2008). The same experiment was carried out

using *M. luteus* ATCC10240 grown in MHB until OD_{600} = 0.5 as the targeted strain. *M. luteus* was grown in MHB until OD_{600} = 0.5 before the cells were washed twice with sodium phosphate buffer pH 7.2 and re-suspended to the original culture volume using the same buffer. SYTOX Green labeling and detection was carried out as mentioned above. Nisin was also tested as a known pore-forming lantibiotic at 10× MIC. Sodium phosphate buffer was added to another sample instead of lantibiotic and served as a negative control. All the samples were performed with three biological replicates.

3.22 Estimation of membrane potential

Molecular dye 3,3-diethyloxacarbocyanine iodide DiOC2(3) was used to investigate the ability of salivaricin B to dissipate membrane potential of sensitive targeted strain. Cultures of *M. luteus* ATCC10240 were grown at 37°C for 6 hours with orbital shaking at 100 rpm in MHB medium and then diluted to OD_{600} = 0.1 using fresh MHB. Diluted cultures were combined with DiOC2(3) at a final concentration of 2 µM. Glucose (1 mM) and HEPES (1 mM) were added before the samples were incubated at room temperature for 30 minutes. Salivaricin B and nisin A were added at final concentrations of 10× MIC and samples were further incubated for 20 minutes. The protonophore Carbonyl cyanide m-chlorophenyl hydrazone (CCCP) was also tested as a positive control. Distilled water was added instead of antibiotic to the negative control sample. Changes in cell-associated DiOC2(3) fluorescence were measured with BD FACSCanto II flow cytometer (excitation at 488 nm) using argon laser.

3.23 Fluorescence measurements

Fluorescence spectra were recorded on a Shimadzu RF-5301 PC spectrofluorophotometer. In all the experiments, samples were measured in a 1 cm path-length quartz cell at 23 ± 1°C. The concentration of all species was 0.05 mM in a phosphate buffer (10 mM) of pH 7.4. The reported values are the average of three measurements. Bacterial membrane vesicles were prepared from cultures of *Bacillus cereus* and *E. coli* as described previously (Abou-Zied *et al.*, 2015). The fluorescence experiments were performed under the same concentration of Salivaricin B. This was achieved by dividing a buffer solution that contains Salivaricin B into three portions. One portion was used as a reference, or control, without any membrane. Membranes of *B. cereus* and *E. coli* were then each added to one of the other portions. In order to compensate for the amount added from each membrane, a similar volume of the buffer was added to the control solution. The buffer alone was checked and there was no fluorescence in the spectral region (295-550 nm) which rules out any contribution from the buffer to the observed signal. Since *B. cereus* and *E. coli* membranes showed fluorescence signal in the same spectral region, in two other sets of experiments, the control in each one was either membranes of *B. cereus* or *E. coli*.

3.24 Intracellular accumulation of the peptidoglycan cell wall precursor (UDP-MurNAc-pentapeptide)

The UDP-murnac-pentapeptide cytoplasmic pool was analyzed as described previously (Sass *et al.*, 2010; Schmitt *et al.*, 2010; Wilmes *et al.*, 2014) with some

modifications. *M. luteus* ATCC10240 cells were grown overnight in TSB on an orbital shaker at 150 rpm, 37°C. The culture was diluted 1/100 (v/v) using fresh medium and further incubated under same conditions mentioned above until OD_{600} reached 0.7. The culture was supplemented with chloramphenicol (final concentration 130 µg/mL) and further incubated for 15 minutes. Then the culture was divided into three equal samples in three 50 mL sterile tubes. Vancomycin was added to the first tube at 10× MIC. The second tube was supplemented with salivaricin B (10× MIC) and the third tube was served as a control without antibiotic addition. The three tubes were further incubated for an hour before samples were centrifuged at 3,000 × *g* for 30 minutes and the supernatant was discarded. The resultant cell pellets were extracted with boiled water for 20 minutes before centrifugation at 13,000 × *g* for 15 minutes. The supernatant was freeze-dried and the resultant powder was dissolved in 400 µL of 5 mM sodium phosphate buffer pH 5.2. Intracellular accumulation of UDP-MurNAc-pentapeptide was analyzed by RP-HPLC using 5 mM sodium phosphate buffer pH 5.2 as the mobile phase under isocratic conditions on Chromolith SemiPrep RP-18e 100-10 mm column. The run was monitored using UV detector at a wavelength of 260 nm at a flow rate of 1 $mL.min^{-1}$.

3.25 Transmission Electron Microscopy (TEM)

Overnight cultures of both *M. luteus* ATCC10240 and *S. pyogenes* ATCC1234 grown in TSB were centrifuged at 3,000 × *g* for 15 minutes. The cells were washed twice with ice-cold 10 mM phosphate buffer pH 7.2 before they were suspended in the

same buffer to the original culture volume. The bacterial suspensions were incubated with either salivaricin B or nisin A at 10× MICs for 30 or 120 minutes or for overnight. Control samples included bacterial suspension incubated with distilled water. The cells were fixed using 4% glutaraldehyde in 10 mM phosphate buffer pH 7.2 for overnight at 8°C. After 3 washes in cacodylate buffer, the pellets were incubated for 2 hours in OsO_4:cacodylate buffer (1:1) and then the samples were incubated with cacodylate buffer alone for overnight. After 3 washes in distilled water, the samples were washed with gradient concentrations of ethanol (35%, 50%, 70%, 95% and 3 times 100%) followed by two washes with propylene oxide, one wash with propylene oxide:epon mixture (1:1) and one wash with propylene oxide:epon mixture (1:3). After that, the samples were incubated with epon for overnight and embedded at 37°C for 5 hours followed by 60°C for overnight. After ultrathin sections were achieved (0.1 µm), the samples were coated on copper grids, stained with uranyl acetate and subjected to LEO-Libra 120 TEM (Carl Zeiss, Oberkochen, Germany).

CHAPTER 4: RESULTS

4.1 Screening of lantibiotic producing *S. salivarius* strains using deferred antagonism assay

The deferred antagonism assay was applied using different solid media to investigate lantibiotic production by *S. salivarius* isolates. Strain K12 (salivaricins A2 and B producer) used as a positive control gave the broadest antagonism spectrum against a number of selective indicators as shown in Table 4.1. BACa and TYECa appeared to be the best media for lantibiotics production with strain K12. When PTNYSMES medium was used, strain K12 failed to inhibit the growth of *Lactobacillus delbrueckii* subsp. *bulgaricus*. Strains YU10 and NU10 inhibited most of the streptococcal strains used in this study (but not *Streptococcus mutans*) while the levels of lantibiotics secreted by these strains were improved when PTNYSMES medium was used as the production medium. The inhibitory spectrum of both NU10 and YU10 included one *Listeria monocytogenes* strain (partial inhibition). When blood was used as a supplementary component in the production medium (BACa), strain GT2 expressed inhibitory activity towards *S. pyogenes*. Surprisingly, no anti-*Micrococcus luteus* inhibitory activity was detected when strain GT2 was grown on BACa plates indicating that the bacteriocin produced may not be a lantibiotic since *M. luteus* is known for its extreme susceptibility to lantibiotics. However, when GT2 was grown on PTNYSMES and TSYECa media, there was some inhibition towards *M. luteus*. In all media used with strain GT2 as a producer, most of the inhibitory activity was eliminated when the media were heated at 70°C for 30 minutes. This finding indicated that strain GT2 may be expressing a heat labile bacteriocin. It was noticed that strain YU10 did not exhibit self-immunity as it showed significant antagonistic activity towards itself when tested with self-immunity assay with different media. However, when PTNYSMES was used in this test, most of the producers showed a lack of self-immunity.

Table 4.1: Deferred antagonism assay of different lantibiotic producing *S. salivarius* strains using different production media.

Indicator microorganisms	**Antagonism activity of *S. salivarius* isolates towards indicator microorganisms using different production media**																			
	NU10					**YU10**					**GT2**					**K12**				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
A. naeslundii TG2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B. cereus ATCC14579	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Corynebacterium. spp GH17	+++	+++	+++	-	++	++	+++	+++	-	++	++	-	+++	-	-	+++	+++	+++	-	+++
E. faecium C1	(+)^H	(+)^H	-	-	(+)^H	-	-	-	-	-	-	-	-	-	-	++	++	++	-	++
H. parainfluenzae TONEJ11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(+)^H	-	-	-	-
L. bulgaricus M8	-	-	-	-	-	-	-	(+)^H	-	-	-	-	(+)^H	-	-	++	+	-	-	+
L. lactis ATCC11454	(+)^H	-	-	-	-	(+)^H	-	-	-	-	-	-	-	-	-	+++	+++	++	-	++
L. monocytogenes NCTC	+	++	++	-	++	-	-	(+)^H	-	-	-	-	-	-	-	+	+	+	-	++
M. luteus ATCC10240	+++	+++	+++	-	+++	++	+++	+++	-	+++	++	-	+++	-	+	+++	+++	+++	-	+++
S. aureus RF122	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	++	++	+	-	(+)^H
S. equisimilis ATCC12388	++	+++	+++	-	++	+	+++	+++	-	+++	++	+++	+	-	-	+++	+++	+++	-	+++
S. gordonii ST2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S. mutans GEJ11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	++	++	-	++
*S. pyogenes*ATCC12344	++	+++	+++	-	+++	+	+++	+++	-	+++	+	+++	++	+	-	+++	+++	+++	-	+++
S. pyogenes ATCC12348	++	+++	+++	-	+++	+	+++	+++	-	+++	+	+++	++	-	-	+++	+++	+++	-	+++
S. salivarius GT2	+	++	++	-	+	+	++	+	-	-	-	-	+	-	-	++	++	+	-	++
S. salivarius K12	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-	+	-	+	-	-
S. salivarius NU10	-	-	-	-	-	-	-	+	-	-	+	-	+	-	-	+	+	+	-	+
S. salivarius YU10	+	+	++	-	-	(+)^H	(+)^H	++	-	(+)^H	-	-	++	-	-	++	+++	+	-	++
S. sanguinis ATCC10556	-	(+)^H	+++	-	(+)^H	-	(+)^H	+++	-	(+)^H	-	(+)^H	+++	-	-	+++	++	+++	-	(+)^H
W. confusa A3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	+

+ Inhibition zone < 0.75 cm, ++ inhibition zone = 0.75 - 1 cm, +++ inhibition zone > 1 cm, - no inhibition. ^{H}: Hazy zone of inhibition.

1. TSYECa: Tryptic Soy agar supplemented with 2% Yeast extract and 0.1% CaCO3
2. BACa: Columbia agar base supplemented with 5% whole defibrinated sheep blood and 0.1% CaCO3
3. PTNYSMES medium supplemented with 1.5% bacteriological agar.
4. CAB+NBCSCa: Columbia agar base supplemented with 5% New born calf serum and 0.1% CaCO3
5. M17 agar supplemented with 2% Yeast extract, 1% Sucrose and 0.1% CaCO3

Additionally, some *S. salivarius* strains isolated from healthy children was shown to produce significant inhibitory activity especially against other streptococcal strains (Figure 4.1). However, out of 100 *S. salivarius* isolates, 70% showed to be lantibiotic-negative with no significant inhibitory activity such as strain AB20. The control lantibiotic-positive strain K12 was shown to inhibit all the streptococcal strains in this assay except for *S. mutans* GEJ11 where the inhibitory activity was insignificant (10 mm). On the other hand, no anti- *S. mutans GEJ11* activity was produced by any of the new isolates.

Strains K12, AB15, AB28, AB59 and AB85 were shown to produce significant inhibitory activity towards *S. pneumonia* ATCC6301.

In this study, *S. pyogenes* ATCC12344 and *S. pyogenes* ATCC12348 were the most sensitive streptococcal strains susceptible to inhibitory activities produced by *S. salivarius* isolates. Strain *S. equisimilis* ATCC12388 was also shown to be sensitive in this assay. Nevertheless, some resistant colonies were able to grow in the middle of the inhibition zone. It is likely that this strain requires a higher concentration of the lantibiotic produced for full inhibition. When the concentrated crude extract of the lantibiotic was tested in well diffusion or spot on lawn assays, no resistant colonies were formed and clear zones of inhibition were observed.

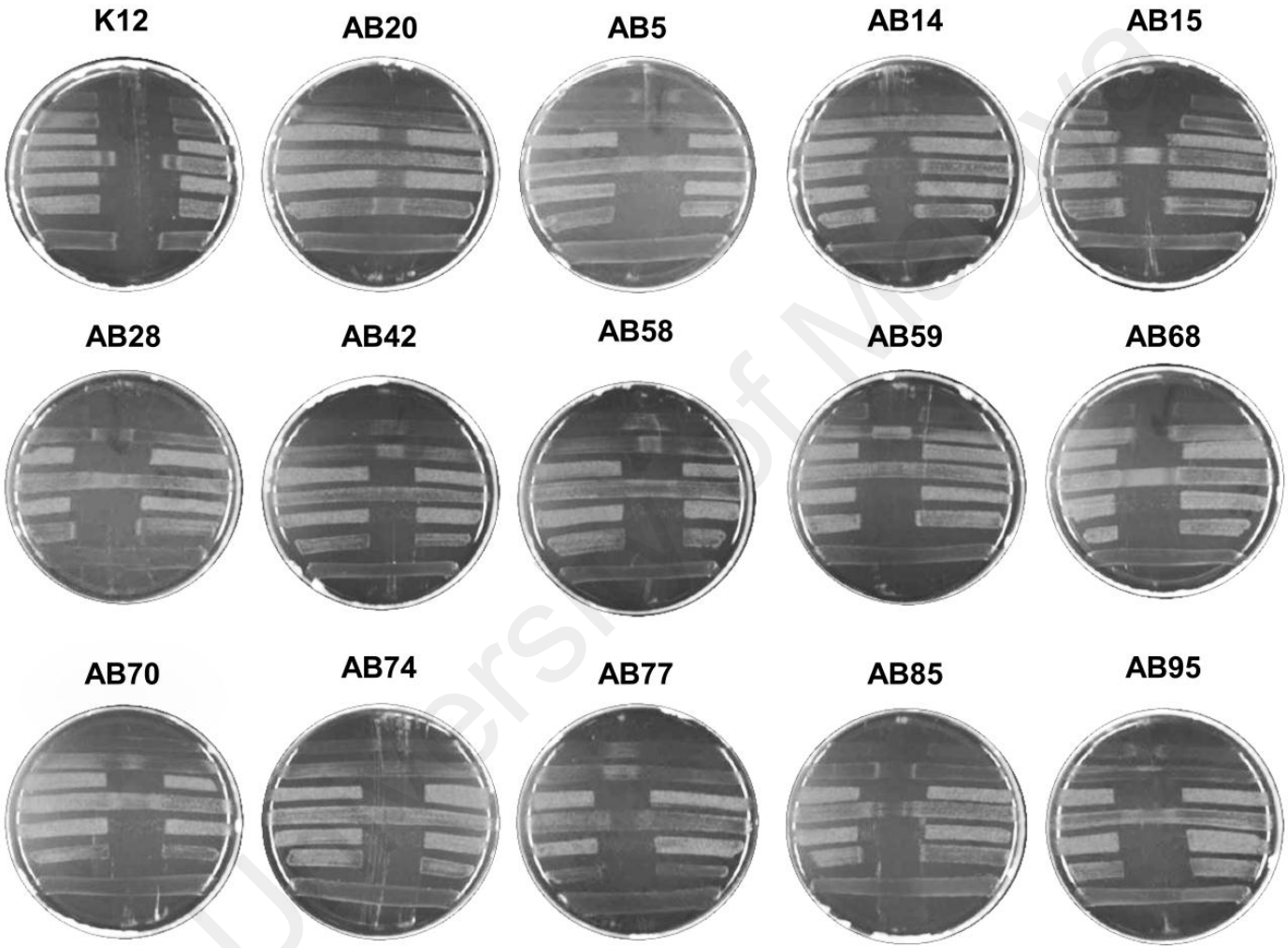

Figure 4.1: Deferred antagonism assay of lantibiotic-producing *S. salivarius* strains isolated from healthy Malaysian children. Strain K12 was used as a control. Indicator strains used are from the top to the bottom as follows: *S. pneumonia* ATCC6301, *S. constellatus* ATCC27823, *S. pyogenes* ATCC12348, *S. sanguinis* ATCC10556, *S. equisimilis* ATCC12388, *S. pyogenes* ATCC12344 *and S. mutans* GEJ11. In this assay BaCa was used as the production medium.

4.2 Genes encoding lantibiotic production in strains K12, NU10, YU10 and GT2

Strain K12 is known to harbour the structural genes *salA*, *sboA* and *MPS var* encoding salivaricins A, B and MPS variant production respectively while no *sivA*, *MPS* or *slnA* were detected within this strain based on K12 complete genome sequencing data (accession number ALIF00000000). In this study, strain YU10 was found to harbour structural genes encoding for streptin, salivaricin A3 and salivaricin G32 lantibiotics and the slnA1 lantibiotic-like protein (based on PCR results and bioinformatic analysis from the genome sequencing). However, salivaricin G32 was the only bioactive lantibiotic expressed and detected in strain YU10 and it seems that not all of these genes are likely to be expressed. Genome sequencing of strain YU10 confirmed the presence of genes mentioned above (Figures 4.9 & 4.11). It was found that salivaricin A is quite widely distributed in streptococcal species but the locus is often quite defective and so the inhibitory product is not expressed even though the salivaricin A immunity component may be functional. Strain GT2 was shown to harbour *MPS* and *MPS var* genes encoding the production of salivaricin MPS and MPS variant respectively. In addition to *salA*, strain NU10 was shown to harbour *sivA* locus encoding the biosynthesis of the lantibiotic salivaricin 9.

4.3 Prevalence of lantibiotic-producing *S. salivarius* among Malaysian children

Only 26% of *S. salivarius* isolates from Malaysian children aged 6-7 years were shown to harbour lantibiotic structural genes. The structural genes *siA* and *slnA* encoding lantibiotic precursors of salivaricins 9 and G32 were shown to be the most common lantibiotic determinants in this study (both found in 34% of lantibiotic-positive strains). Surprisingly, no *sboA* gene encoding salivaricin B was detected in this study indicating the rare occurrence of this lantibiotic among Malaysian subjects. *salA* gene encoding salivaricin A production was found in 26% of lantibiotic-positive strains in this study

(Figure 4.3). Table 4.2 shows the PCR screening of lantibiotics structural genes among *S. salivarius* isolates of Malaysian children.

Table 4.2: PCR screening of lantibiotics structural genes among *S. salivarius* isolates of Malaysian children.

Strain	Lantibiotics precursor genes					Strain source/reference
	salA	***sivA***	***slnA***	***srtA***	***sboA***	
K12	+	-	-	-	+	(Hyink *et al.*, 2007)
YU10	+	-	+	+	-	Current study
NU10	+	+	-	-	-	Current study
AB2	+	-	-	-	-	Current study
AB5	+	+	-	-	-	Current study
AB8	+	-	-	-	-	Current study
AB13	-	+	+	-	-	Current study
AB14	-	+	+	-	-	Current study
AB15	-	+	-	-	-	Current study
AB19	-	+	+	-	-	Current study
AB21	-	+	-	-	-	Current study
AB23	-	+	-	+	-	Current study
AB25	-	+	-	-	-	Current study
AB28	+	-	-	-	-	Current study
AB29	-	+	-	-	-	Current study
AB42	+	-	+	-	-	Current study
AB43	-	-	+	-	-	Current study
AB47	-	+	-	-	-	Current study
AB49	-	+	-	-	-	Current study
AB51	-	-	+	-	-	Current study
AB53	-	-	+	-	-	Current study
AB56	-	-	+	-	-	Current study
AB58	+	-	+	-	-	Current study
AB63	-	+	-	-	-	Current study
AB65	-	+	-	-	-	Current study
AB68	+	-	+	+	-	Current study
AB70	-	-	+	-	-	Current study
AB74	+	-	+	-	-	Current study
AB85	+	-	-	-	-	Current study
Total positive / Total tested*	9\26	12/26	12\26	2\26	0\26	

Interestingly, the structural genes encoding the lantibiotic precursors of salivaricins were found in other streptococci. Salivaricin A precursor was found in *S. salivarius*, *S. agalactiae*, *S. dysgalactiae* and *S. pyogenes*. Salivaricin B (SboA precursor) was found in *S. salivarius*, *S. mitis*, *S. infantis*, *S. sanguinis* and *S. parasanguinis*. Salivaricin 9 (SivA precursor) was found in *S. salivarius* and *S. suis*. Salivaricin G32 (SlnA precursor) was found in *S. salivarius*, *S. agalactiae*, *S. dysgalactiae*, *S. macedonicus*, *S. suis* and *S. pyogenes*. However, when these structural genes were found in streptococci rather than *S. salivarius* they showed some degree of mutations and changes in certain amino acids in different positions in both leader peptides and mature lantibiotic as shown in Figure 4.2.

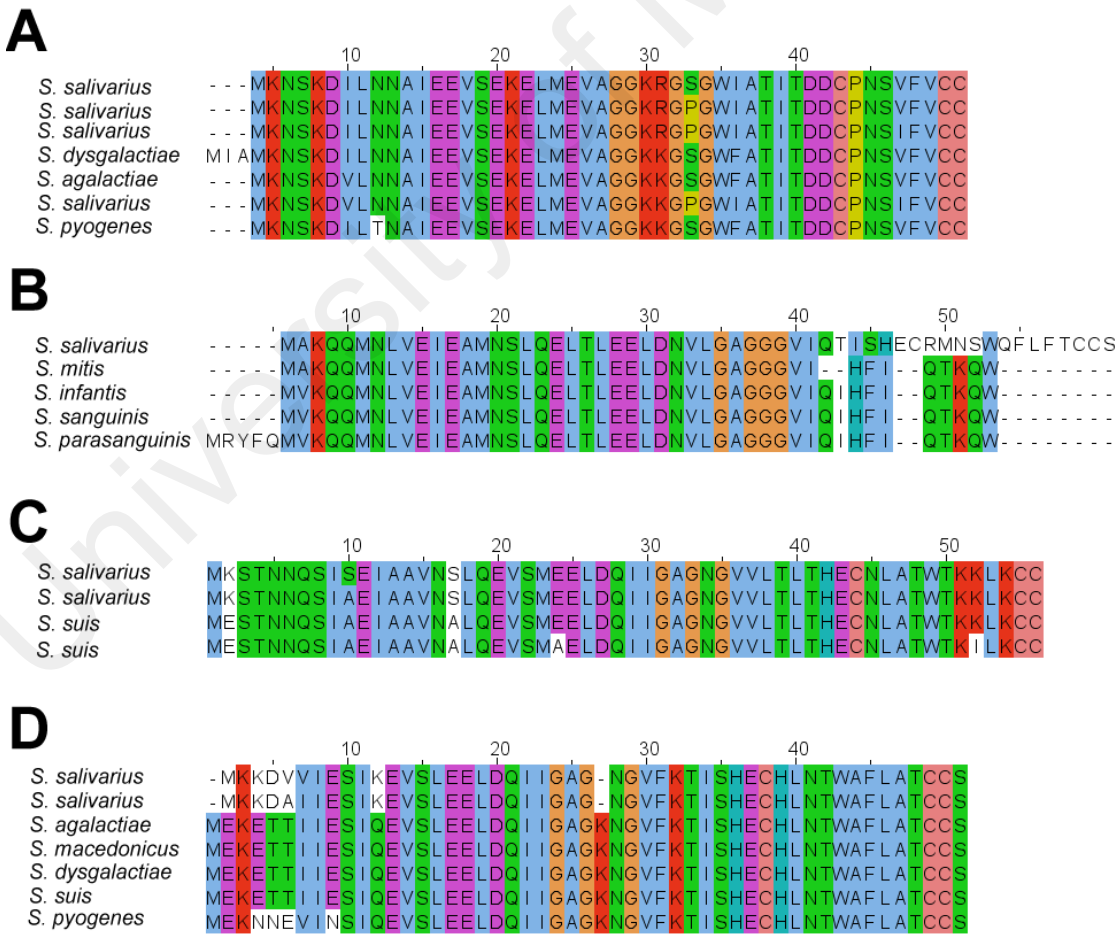

Figure 4.2: Alignments of salivaricins pre-pro-peptides found in different streptococcal species. A) salA, B) sboA, C) sivA and D) slnA. These alignments are developed using UniProt database.

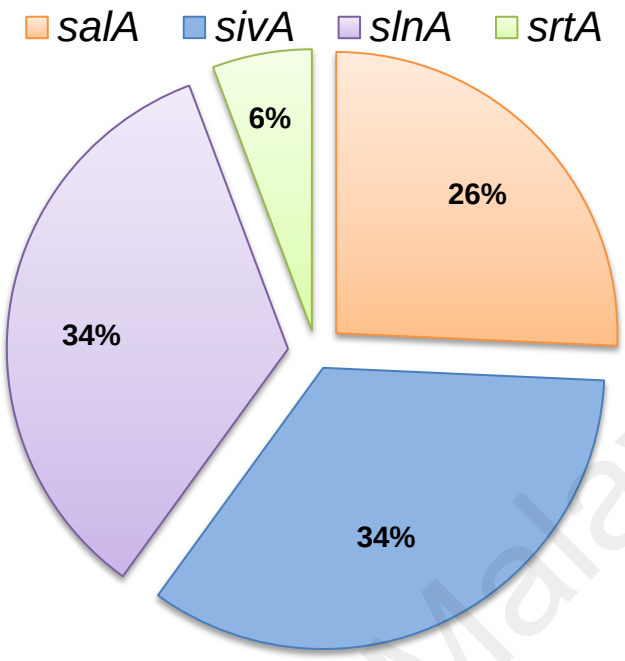

Figure 4.3: Distribution of lantibiotic precursor genes among lantibiotic-Positive *S. salivarius* strains isolated from Malaysian children.

4.4 Stability of the metabolic profiles and biochemical characteristics of *S. salivarius* strains K12, NU10, YU10 and GT2

The stability of the metabolic profiles of *S. salivarius* strains was carried out using API® kits. The biochemical characteristics of each strain provided valuable information on the needs and criteria of each isolate to achieve optimal growth. YU10 was the only strain tested in this study that showed D-sorbitol positive reaction. This information can also be used as a method for YU10 detection. Unlike NU10 and YU10, strains K12 and GT2 are able to use inulin or galactose as a carbon source. NU10 was the only strain with positive reaction for amygdalin while GT2 was the only strain that fermented melibiose. Strain K12 also differed with the other strains by fermenting D-tagatose. NU10 was the only strain that failed to ferment lactose and this is unusual for lactic acid bacteria. However, when lactose was used as a carbon source during media

development, strain NU10 was unable to grow adequately. YU10 was the only strain with trehalose negative reaction (Table 4.3).

Table 4.3: API 20 STREP and API 50 CHL reactions for *S. salivarius* isolates.

API 20 STREP	**K12**	**NU10**	**NU10$^{\Omega}$**	**YU10**	**YU10 $^{\Omega}$**	**GT2**	**GT2 $^{\Omega}$**
Acetoin production	+	+	+	+	+	+	+
Hydrolysis (HIP puric acid)	-	-	-	-	-	-	-
B-Glucosidase hydrolysis (Esculin)	+	+	+	+	+	+	+
Pyrrolidonyl arylamidase	-	-	-	-	-	-	-
α-Galactosidase	-	-	-	-	-	-	+
β-Glucuronidase	-	-	-	-	-	-	-
β-Galactosidase	-	-	-	-	-	-	-
Alkaline phosphatase	+	+	-	+	+	+	+
Leucine aminopeptidase	+	+	+	+	+	+	+
Arginine dihydrolase	-	-	-	-	-	-	-
D-Ribose	-	-	-	-	-	-	-
L-Arabinose	-	-	-	-	-	-	-
D-Mannitol	-	-	-	-	-	-	-
D-Sorbitol	-	-	-	+	+	-	-
D-Lactose	+	-	-	+	+	-	-
D-Trehalose	+	+	+	-	-	+	+
Inulin	+	-	-	-	-	-	+
D-Raffinose	+	+	+	+	+	+	+
starch	+	+	+	+	+	+	+
glycogen	-	-	-	-	-	-	-
API 50 CHL	**K12**	**NU10**	**NU10$^{\Omega}$**	**YU10**	**YU10 $^{\Omega}$**	**GT2**	**GT2 $^{\Omega}$**
L-Arabinose	-	-	-	-	-	+	+
Ribose	-	-	-	-	-	+	+
Galactose	+	-	-	-	-	+	+
D-Glucose	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	+
D-Mannose	+	+	+	+	+	+	+
Sorbitol	-	-	-	+	+	-	-
a-Methyl-D-glucoside	-	-	-	-	-	-	-
N-Acetyl glucosamine	+	+	+	+	+	-	-
Amygdalin	-	+	+	-	-	-	-
Arbutin	+	+	+	+	+	+	+
Esculin	+	+	+	+	+	+	+
Salicin	+	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+
Lactose	+	-	-	+	+	+	+
Melibiose	-	-	-	-	-	+	+
Saccharose	+	+	+	+	+	+	+
Trehalose	+	+	+	-	-	+	+
Inulin	+	+	+	+	+	+	+
Melezitose	-	-	-	-	-	-	-
D-Raffinose	+	+	+	+	+	+	+
ß-Gentiobiose	-	+	+	+	+	+	+
D-Tagatose	+	-	-	-	-	-	-

Ω: Strain was sub-cultured subsequently for 20 times before testing for the metabolic profile.

4.5 Stability of the antibiograms of lantibiotic producing strains

S. salivarius strains K12, NU10, YU10 and GT2 were assessed to be moderately resistant to gentamicin and this finding is similar to what was published for strain K12 and other *S. salivarius* isolates (Burton *et al.*, 2006). According to CLSI ~~breakpoints~~ (Vol. 32, No.3, Jan, 2012) breakpoints for ofloxacin, *S. salivarius* strains tested in this study were sensitive to this antibiotic (inhibition zone > 16 mm). Furthermore, *S. salivarius* strains tested in this study were susceptible to several routinely used antibiotics for the control of upper respiratory tract infections. It was noticed that strain YU10 showed intermediate susceptibility levels to erythromycin with 19.7 mm zone of inhibition. However, the other strains NU10, GT2 and K12 were susceptible to the same antibiotic. No significant differences regarding antibiotic susceptibility were observed after two years of storage indicating that the strains tested in this study are reasonably genetically stable. Results of antibiogram are listed in Table 4.4.

Table 4.4: Antibiotic disc sensitivities of *S. salivarius* isolates (*n*=3).

Antibiotic (discs)	Disc content	Inhibition zone size (mm) for *S. salivarius* strains							CLSI Breakpoints[a]		
		NU10[§]	**NU10[¥]**	**YU10[§]**	**YU10[¥]**	**GT2[§]**	**GT2[¥]**	**K12**	**S**	**I**	**R**
Penicillin G	10 µg	21	23	22	22	20	20	21.5	-	-	-
Penicillin V	10 µg	30.5	30	33.3	30.5	27	28	32	-	-	-
Ampicillin	10 µg	26	27	26	27	24	23	28.5	-	-	-
Amoxycillin	10 µg	30	32	31.5	29.5	25.6	27	30	-	-	-
Erythromycin	15 µg	30.5	30.2	19.7	19.7	32	29.5	30	≥ 21	16-20	≤ 15
Tetracyclin	30 µg	31	31	31.5	31.5	31.7	32	29	≥ 23	19-22	≤ 18
Gentamycin	10 µg	14.5	14.5	19	18	16	15.5	13.5	-	-	-
Clindamycin	2 µg	28.5	29	31.7	31	27	28	29	≥ 19	16-18	≤ 15
Ofloxacin	5 µg	21	21	20.5	20.5	20	19.5	18.3	≥ 16	13-15	≤ 12
Streptomycin	10 µg	12	11	13	13.5	10	11	9.5	-	-	-
Novobiocin	5 µg	13.5	14	13.5	12	13	13	12.5	-	-	-
Vancomycin	30 µg	20	20	24	22.5	22	21.5	21	≥ 17	-	-
Cloramphenicol	30 µg	26	25	26.5	26	27.5	25.5	27	≥ 21	18-20	≤ 17

[a] S: susceptible, I: intermediate, R: resistant.
[¥] Original isolate. [§] Routinely subcultured isolate.
The data is the average of three replicates and in all cases the error is insignificant.

4.6 Detection of known streptococcal virulence determinants

S. salivarius strains NU10 and YU10 showed PCR-negative reactions for virulence genes while *S. pyogenes* ATCC12344 was used as a control and was PCR-positive to all virulence determinants. This screening was carried out as a preliminary safety evaluation. However, *S. salivarius* strains were shown to have PCR products when *speB* primers were used for amplification and to ensure that these products are not *speB*-like genes, each product was sequenced and determined to have no homology to *speB*. However, genome sequencing of both NU10 and YU10 strains confirmed the absence of any streptococcal virulence determinants (Figure 4.4).

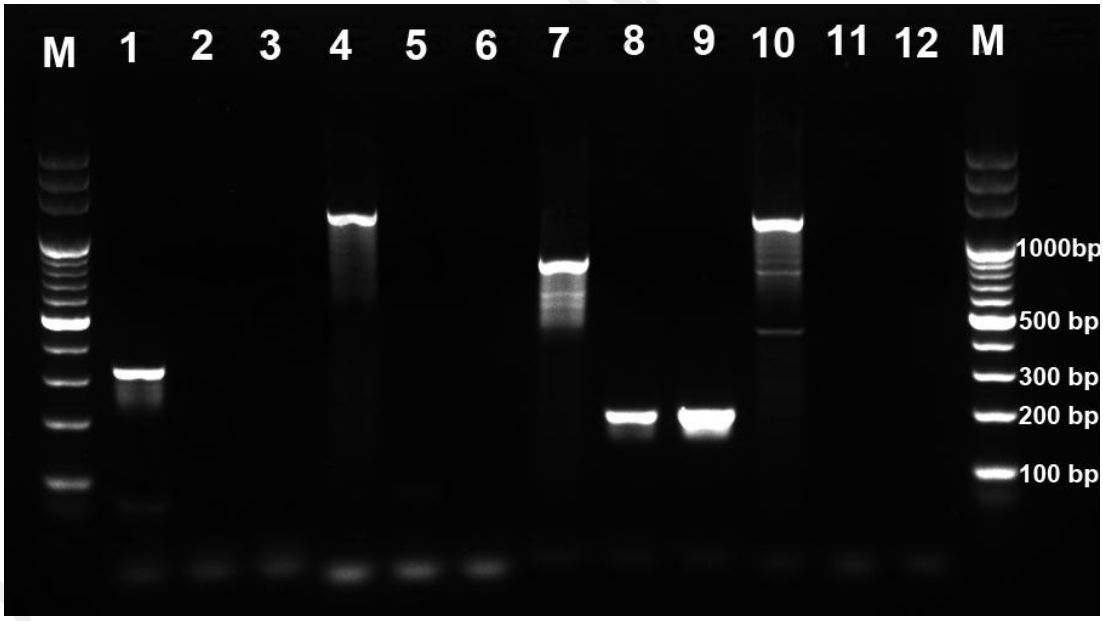

Figure 4.4: PCR screening of streptococcal virulence determinants. *S. salivarius* NU10 (lanes 2, 5, 8, 11 and 14). *S. salivarius* YU10 (lanes 3, 6, 9, 12 and 15). *S. pyogenes* ATCC12344 (lanes 1, 4, 7, 10 and 13) amplification products resulting from PCR using primers for *smez-2* (lanes 1, 2 and 3), *scpA* (lanes 4, 5 and 6), *speB* (lanes 7, 8 and 9), *sagA* (lanes 10, 11 and 12) and *emm* (lanes 13, 14 and 15). M, 100 bp Plus DNA Ladder (Genomics BioSci & Tech).

4.7 Genome sequencing of strains NU10 and YU10

Genome sequencing of strains NU10 and YU10 was performed with Illumina Miseq sequencer. Total of 2,344,494 and 2,345,259 pair-end reads were generated with contigs numbering 51 and 48 for NU10 and YU10, respectively. Genome annotation was performed using RAST (Aziz *et al.*, 2008). A total of 2146 coding sequences (CDSs) and 38 structural RNAs were predicted in strain NU10 while 2161 CDSs and 41 structural RNAs were predicted in strain YU10. Genes encoding for streptin, salivaricin A3, salivaricin G32 lantibiotics and slnA 1 lantibiotic-like protein were detected in the genome draft of strain YU10. However, only salivaricin A3, salivaricin G32 and slnA 1 lantibiotics showed 100% homology to genes present in strain YU10. The gene encoding for streptin present in YU10 genome has only 82% homology to streptin due to some mutations. Furthermore, strains YU10 and NU10 were shown to be free of any streptococcal pyrogenic exotoxins, streptococcal superantigen A (SSA), streptococcal mitogenic exotoxin Z (SmeZ) and streptodomase B. Other streptococcal virulence factors listed in Table 4.5 were also investigated and none of them were present in YU10 or NU10 genomes. Figure 4.5 shows subsystem feature counts of *S. salivarius* strains NU10 and YU10 detected by RAST. Under virulence, disease and defense category, we clearly observed no *Streptococcus pyogenes* virulence determinants, toxins or superantigens. Other gene factors related to bacteriocin production, protein synthesis and adhesion factors are not part of the toxin and virulence factors. This assessment suggests that both YU10 and NU10 are safe for future use as probiotics. Genomic mapping of both strains NU10 and YU10 is shown in Figure 4.6.

Table 4.5: Virulence assessment for *S. salivarius* strains YU10 and NU10.

Virulence determinant	**Gene designation**	***S. salivarius* strains**	
		YU10	NU10
M-protein	*emm*	-	-
Protein H	*sph*	-	-
Streptokinase	*Ska*	-	-
CAMP factor	*cfa*	-	-
Streptolysin S	*SagA*	-	-
Streptolysin O	*slo*	-	-
Hyaluronate lyase	*hyl*	-	-
Nicotin adenine dinuclutide glycohydrolase	*nga*	-	-
Streptococcal pyrogenic exotoxin A	*SpeA*	-	-
Streptococcal pyrogenic exotoxin B	*SpeB*	-	-
Streptococcal pyrogenic exotoxin C	*SpeC*	-	-
Streptococcal pyrogenic exotoxin G	*SpeG*	-	-
Streptococcal pyrogenic exotoxin H	*SpeH*	-	-
Streptococcal pyrogenic exotoxin I	*SpeI*	-	-
Streptococcal pyrogenic exotoxin J	*SpeJ*	-	-
Streptococcal pyrogenic exotoxin K	*SpeK*	-	-
Streptococcal pyrogenic exotoxin L	*SpeL*	-	-
Streptococcal pyrogenic exotoxin M	*SpeM*	-	-
Streptococcal superantigen A	*SSA*	-	-
Streptococcal metogenic exotoxin Z	*SmeZ*	-	-
Streptodornase B	*SdaB*	-	-
Fibrinogen binding protein	*fba*	-	-
Fibrotectin-binding protein (protein F)	*prtF*	-	-
Protein G-related alpha 2 macroglobulin binding protein	*grab*	-	-
Streptococcal inhibitor of complement	*SIC*	-	-
Immunoglobulin G-endopeptidase	*IdeS*	-	-
Secreted endo-β-N-acetylglucosaminidase	*ndoS*	-	-
C5a peptidase	*ScpA*	-	-
Fibronectin-binding protein	*FBP*	-	-
Serum opacity factor	*SOF*	-	-
C3 family ADP-ribosyltransferase	*SpyA*	-	-
Serine endopeptidase	*ScpC*	-	-
Hyaluronan synthase	*HasA*	-	-
Collagen-like surface protein	*SclB*	-	-

(-): absence of the virulence factor.

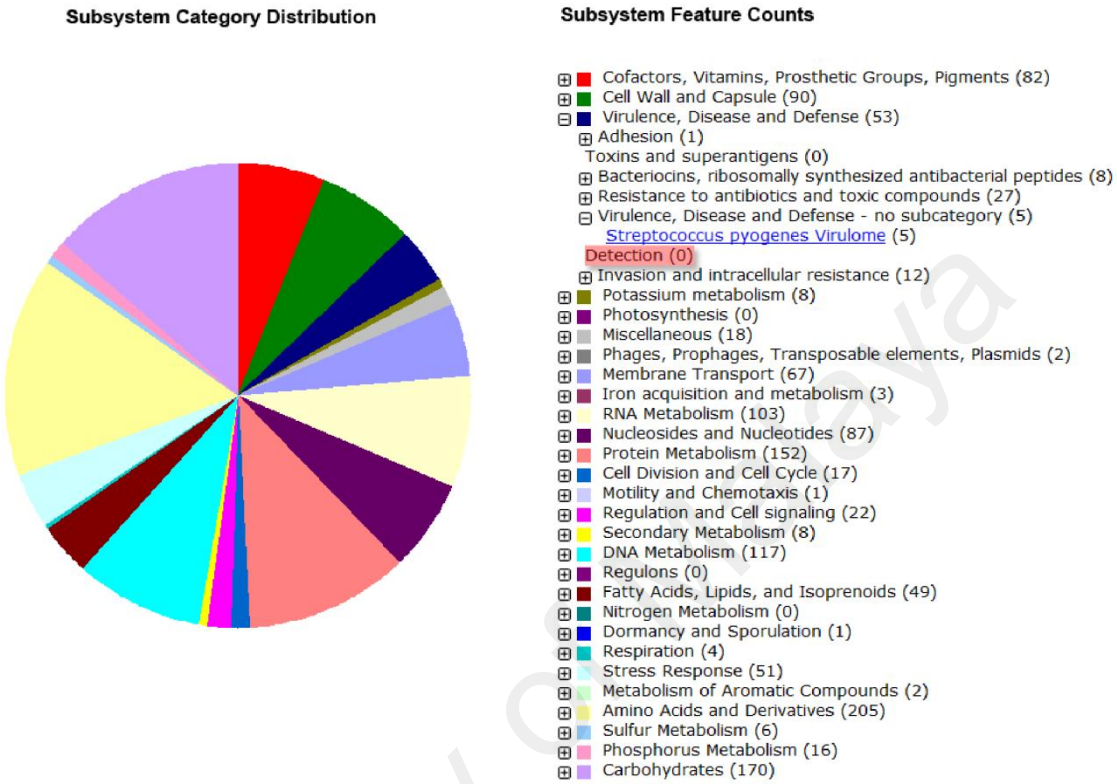

Figure 4.5: Subsystem feature counts of *S. salivarius* strains NU10 and YU10 detected by RAST. No *S. pyogenes* virulence determinants were detected.

The antibiotic resistant determinants have also been investigated in the genomes of strains NU10 and YU10. No significant antibiotic resistant genes were found either in NU10 or YU10 strains which make them potential candidates for probiotic development. However, like the commercial probiotic strains M18 and K12, both NU10 and YU10 were shown to harbour gene encoding PmrA (multidrug resistance efflux pump). Apparently, this is normal for *S. salivarius* strains which were shown in this study to also harbour genes encoding multi-antimicrobial extrusion protein (Na(+)/drug antiporter) of MATE family of MDR efflux pumps.

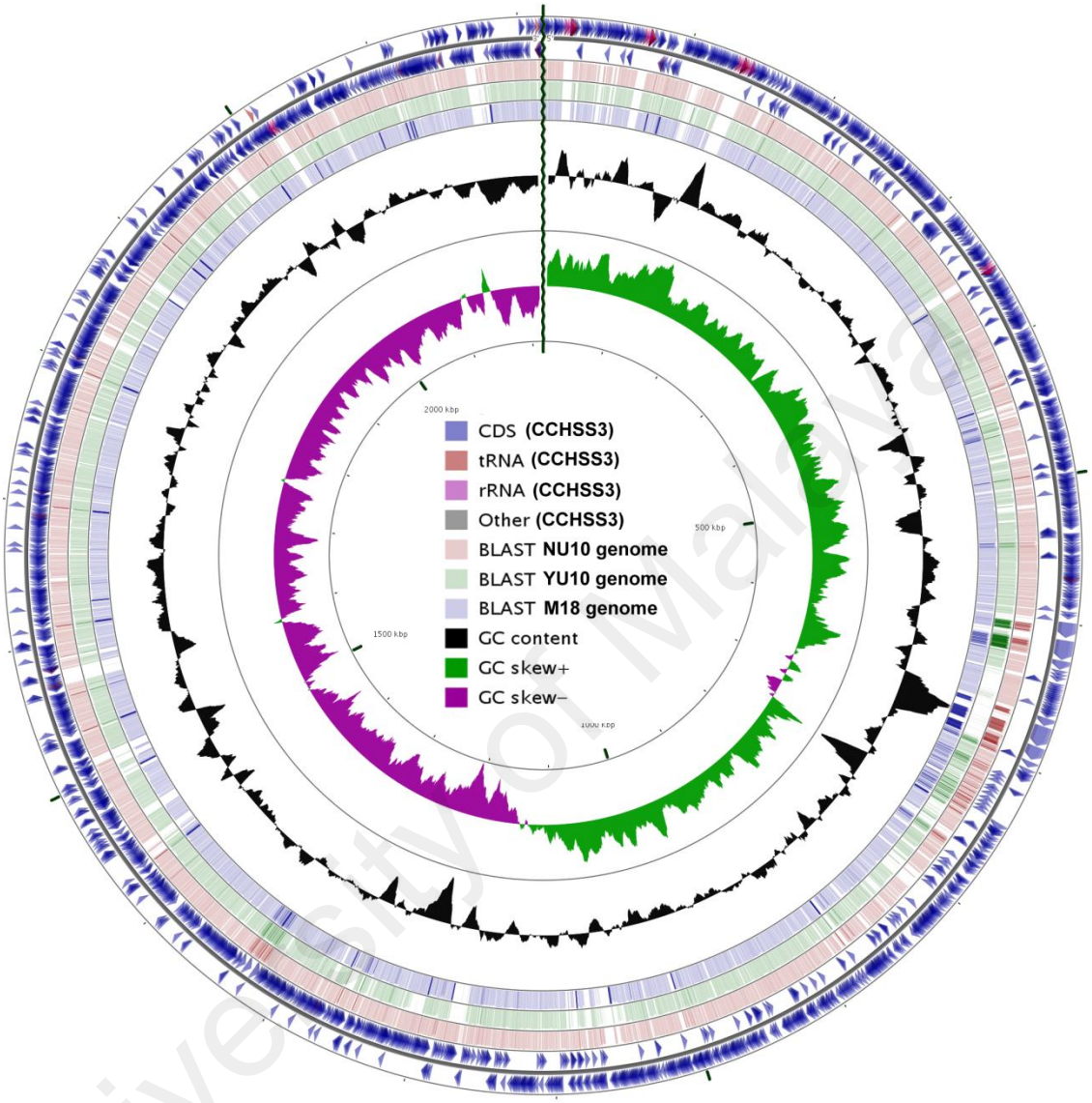

Figure 4.6: Genomic mapping of *S. salivarius* strains: the reference genome of strain CCHSS3 was used as a primary genome sequence, other *S. salivarius* genomes were used as BLAST templates. NU10 genome (light pink), YU10 genome (light green) and M18 genome (light blue). Genome maps were constructed using CGView Server V 1.0.

Table 4.6 Comparative genomic characteristics of related *S.salivarius* strains.

Strain	Genome size	# Coding Sequences	GC Content %	Number of RNAs	MegaPlasmid size	Lantibiotic loci	Reference
NU10	2,362,920 (bp)	2,181	39.3	50	190,254 (bp)	2	Current study
YU10	2,334,603 (bp)	2,158	39.4	51	148,112 (bp)	3	Current study
M18	2,325,981 (bp)	2,122	39.4	86	183,037 (bp)	3	(Heng *et al.*, 2011)
K12	2,241,913 (bp)	2,089	38.9	57	185,045 (bp)	2	(Barretto *et al.*, 2012)
57.1	2,138,805 (bp)	1,936	39.9	86	ND[†]	ND[†]	(Geng *et al.*, 2011)
JIM8777	2,210,574 (bp)	1,951	40.1	86	ND[†]	1	(Guédon *et al.*, 2011)
CCHSS3	2,217,184 (bp)	1,997	39.9	86	ND[†]	1	(Delorme *et al.*, 2011)

[†]Not detected.

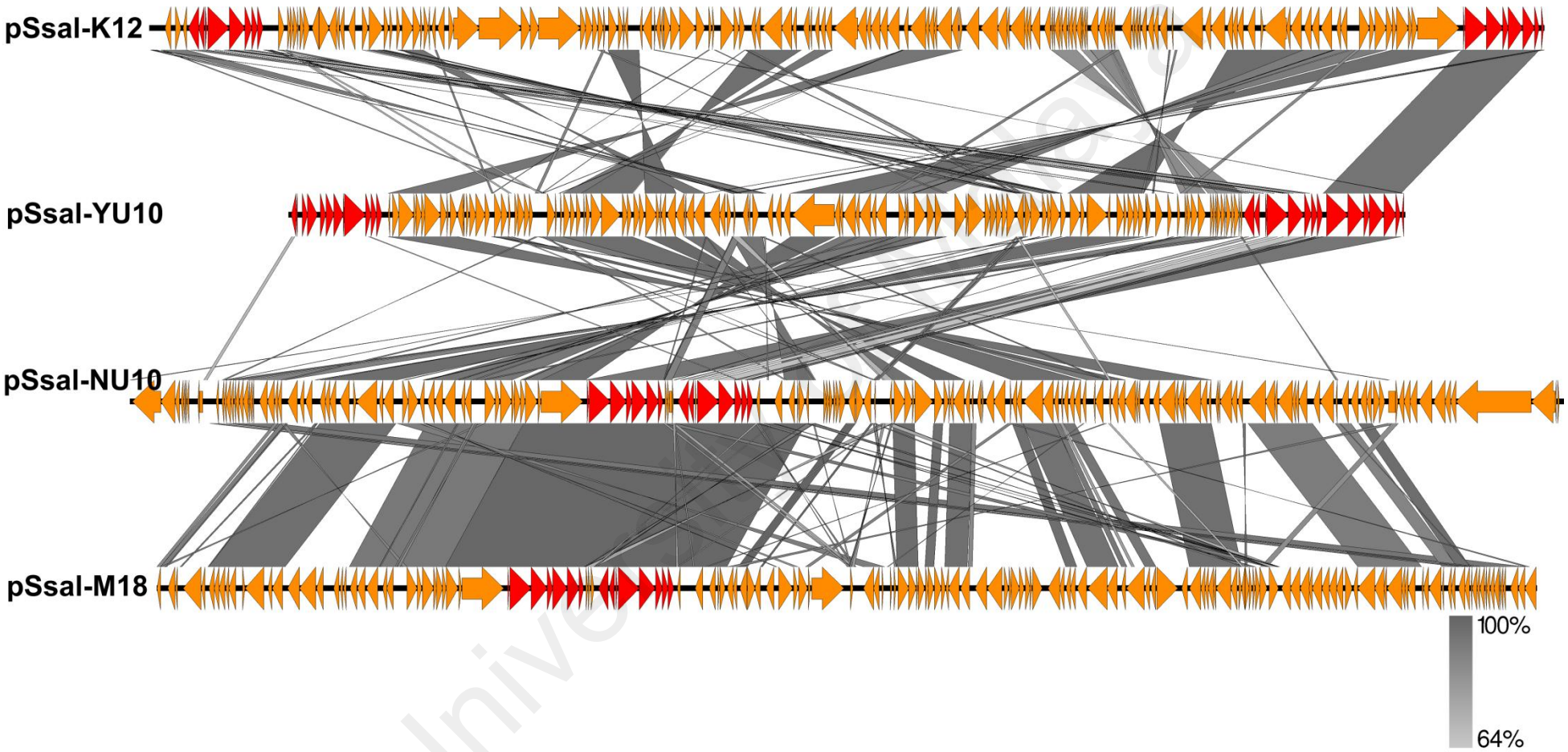

Figure 4.7: Genomic comparision of megaplasmids detected in *S. salivarius* strains. The loci encoding lantibiotics biosynthesis are indicated in red. The analysis was carried out using EasyFig software.

4.8 *S. salivarius* megaplasmids

It was reported previously that almost all lantibiotic-producing *S. salivarius* strains harbour megaplasmids. Hence, these plasmids may function as mobile repositories for lantibiotics loci. The genomic analysis of *S. salivarius* NU10 showed that this strain harbours 190,254 bp megaplasmid designated as pSsal-NU10 (Figure 4.8). In this study, pSsal-NU10 showed to harbour the biosynthesis loci of both salivaricin A and salivaricin 9. Additionally, pSsal-NU10 showed to contain genes encoding mobile element proteins, phage proteins, streptococcal extracellular nuclease 2, non-ribosomal peptide synthetase system, SNF2 protein, TraE-like plasmid transfer system and some adhesion factors like CspA and CspB.

Comparative genomics of the megaplasmids pSsal-K12, pSsal-NU10, pSsal-YU10 and pSsal-M18 is shown in Figure 4.7.

Genomic analysis of strain YU10 indicated the existence of 148,112 bp megaplasmid designated as pSsal-YU10. In addition to SalA locus, the pSsalYU10 showed to harbour the biosynthesis loci for SalG32 and streptin. Interestingly, pSsal-YU10 showed to harbour the gene encoding salivaricin MPS (large heat sensitive-non lantibiotic bacteriocin) (Figure 4.9). The lantibiotic-associated genes found in both strains in NU10 and YU10 are listed in Table 4.7 and Figure 4.11.

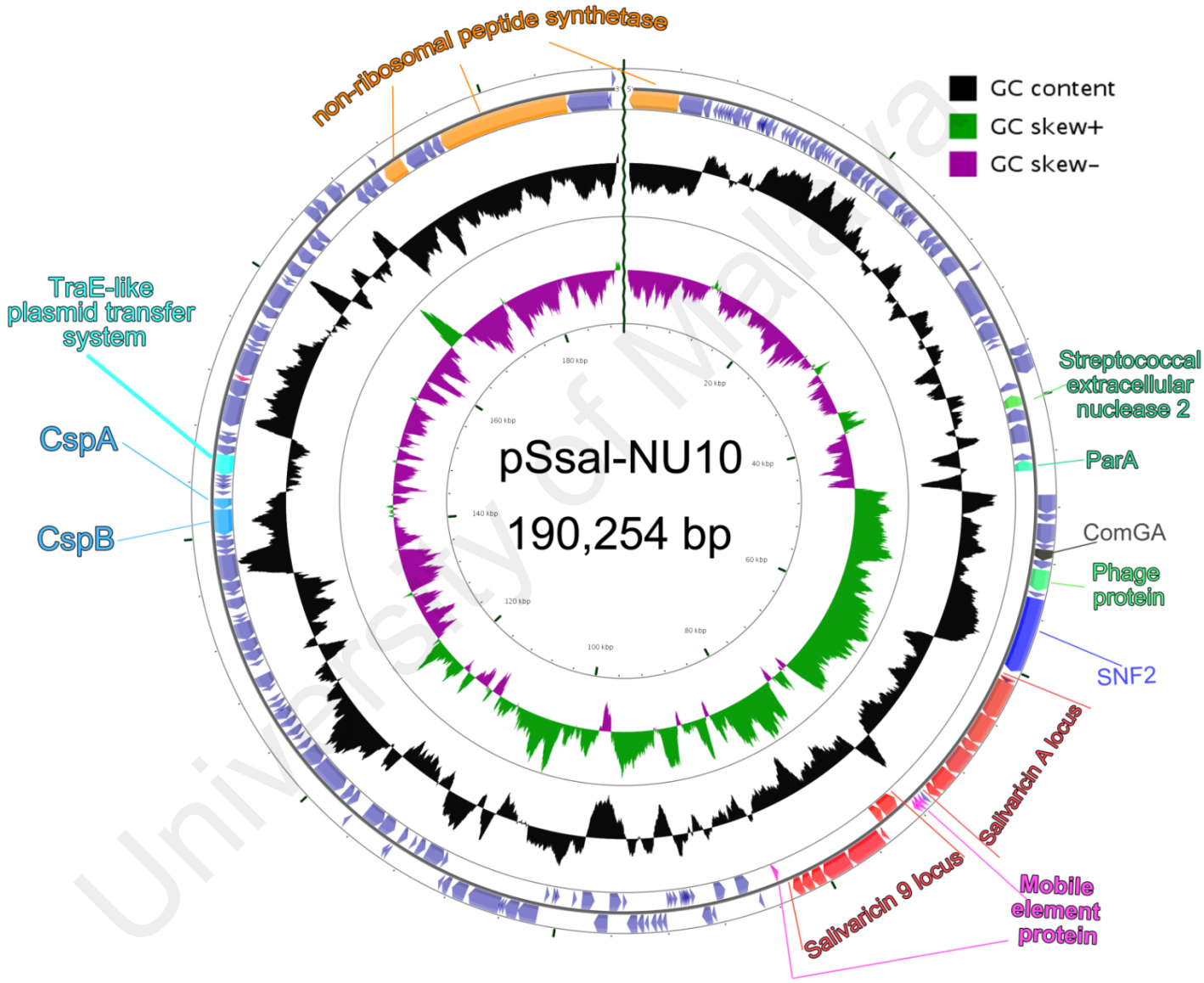

Figure 4.8: Megaplasmid of *S. salivarius* NU10 (pSsal-NU10) containing SalA and Sal9 loci (figure generated using CGView Server)

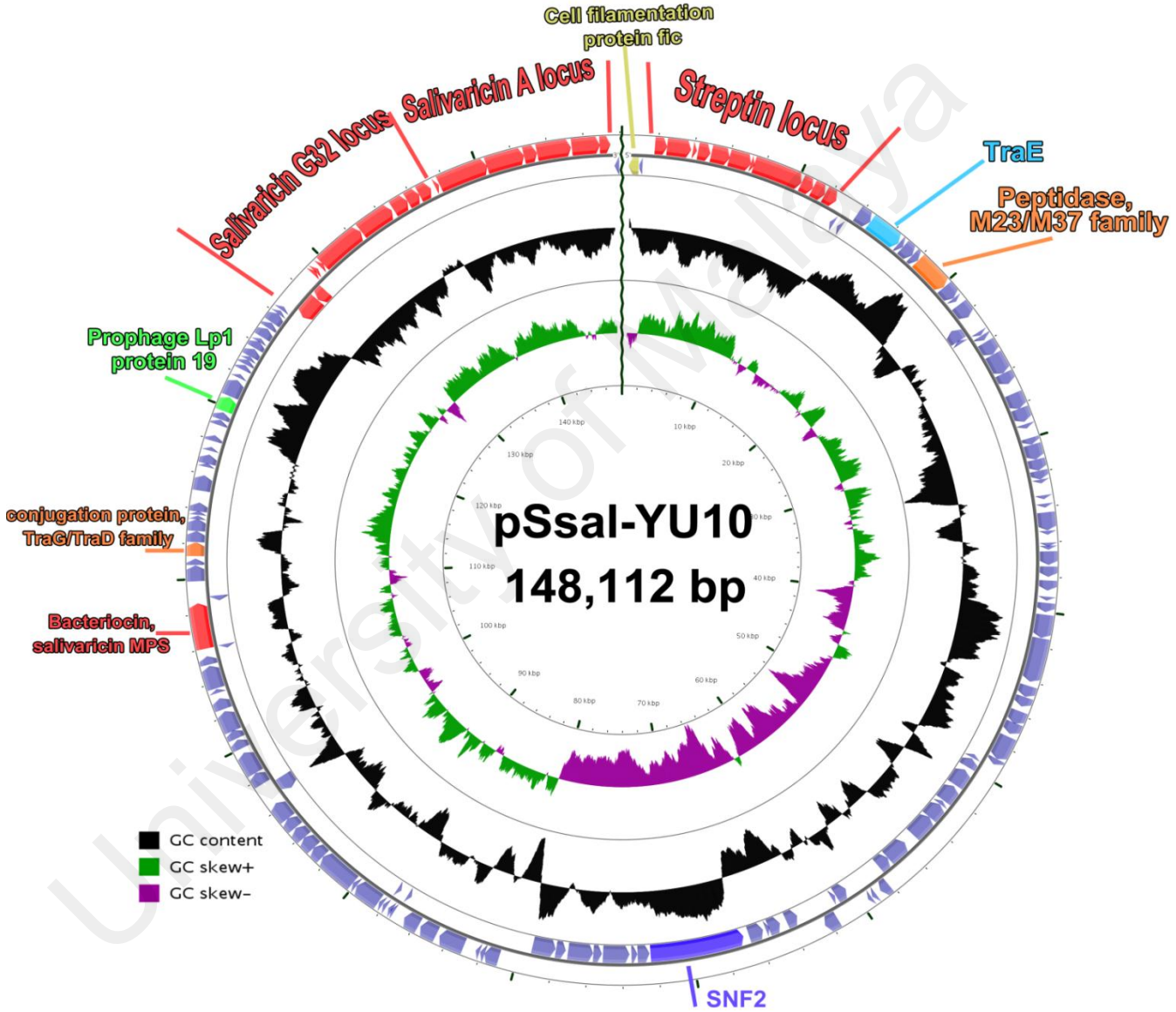

Figure 4.9: Megaplasmid of *S. salivarius* YU10 (pSsal-YU10) containing SalA, SalG32 and streptin loci (figure generated using CGView Server)

Table 4.7: Lantibiotic-associated genes found in the genomes of strains NU10 and YU10.

Strain	Gene	Function
NU10		
Salivaricin A biosynthesis locus	*salA*	Salivaricin A precursor
	salM	Lantibiotic modification enzyme
	salT	Lantibiotic transporter
	salX	Salivaricin A immunity
	salY	Salivaricin A immunity
	salK	Histidine sensor kinase
	salR	Response regulator
NU10		
Salivaricin 9 biosynthesis locus	*sivA*	Salivaricin 9 precursor
	sivM	Lantibiotic modifin enzyme
	sivT	ABC Lantibiotic transporter
	sivFEG	Lantibiotic immunity and self-protection
	sivK	Histidine sensor kinase
	sivR	Response regulator
YU10		
Salivaricin A biosynthesis locus	*salA*	Salivaricin A precursor
	salM	Lantibiotic modification enzyme
	salT	ABC Lantibiotic transporter
	salX	Salivaricin A immunity
	salY	Salivaricin A immunity
	salK	Histidine sensor kinase
	salR	Response regulator
YU10		
Salivaricin G32 biosynthesis locus	*slnA*	Salivaricin G32 precursor
	slnA'	Salivaricin G32 precursor
	slnA1	Salivaricin G32 precursor
	slnM	Lantibiotic modification enzyme
	slnT	ABC Lantibiotic transporter
	slnFEG	Lantibiotic immunity and self-protection
	slnK	Histidine sensor kinase
	slnR	Response regulator
YU10		
Streptin biosynthesis locus	*srtA*	Streptin precursor
	srtM	Lantibiotic modification enzyme
	srtT	ABC Lantibiotic transporter
	srtFEG	Lantibiotic immunity and self-protection
	srtK	Histidine sensor kinase
	srtR	Response regulator

Comparative genomics of the lantibiotic loci of different members of lacticin 481 group showed that biosynthesis loci of salivaricins G32, B, and 9 are closely related to that of macedocin and streptococcin A-FF22. However, salivaricin A locus showed minimum similarity and homogeneity to the other members of the group. Interestingly, the immunity gene *lctE* (in lacticin locus) showed similarity to *nukE* (in nukacin locus) but no homogeneity at all to *macE*, *scnE*, *slnE*, *sboE* or *sivE* suggesting possible scuseptibility of the lantibiotic-producing *S. salivarius* strains which lack this immunity gene to lacticin 481 or nukacin (Figure 4.10).

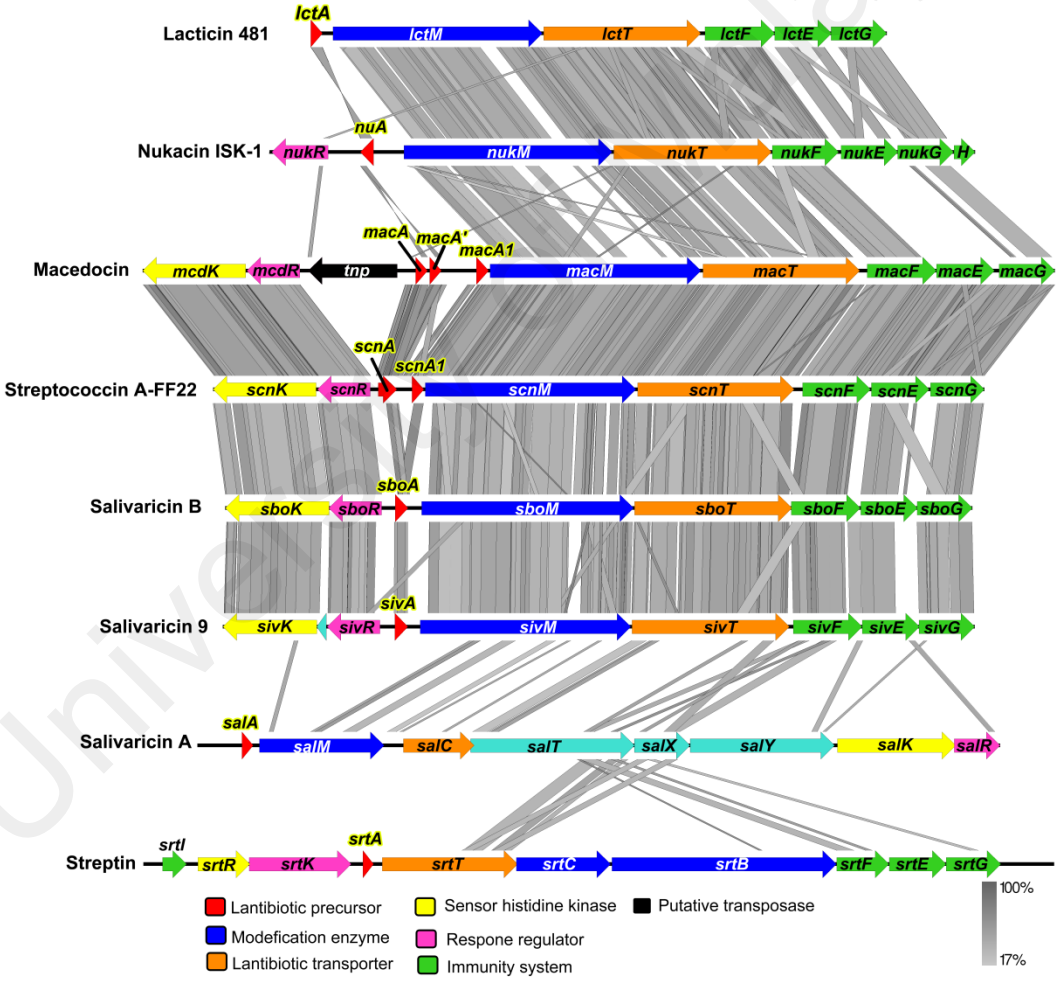

Figure 4.10: Genomic comparison of the biosynthesis loci of different members of lacticin 481 group (class AII lantibiotics). Streptin locus (class AI lantibiotic) found in the genome of strain YU10 was also included for comparison. This figure was generated using Easyfig software.

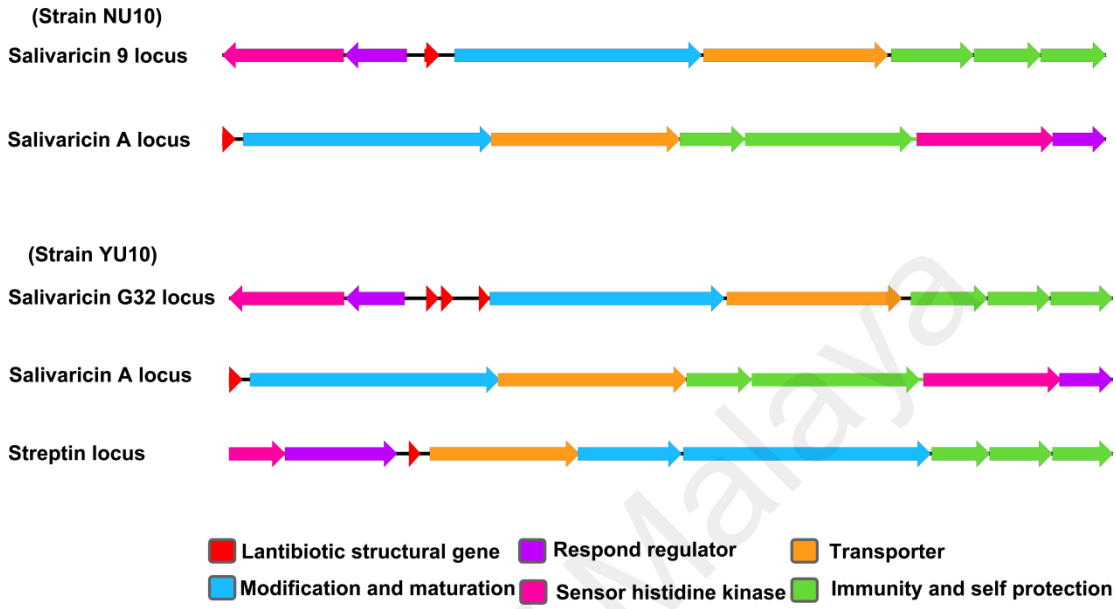

Figure 4.11: Lantibiotic biosynthesis loci revealed in this current study through whole genome sequencing in *S. salivarius* strains NU10 and YU10.

4.9 The biosynthetic locus of salivaricin G32 lantibiotic

In this study, the full sequence of the salG32 locus was revealed and analysed. The *slnA* gene encoding salivaricin G32 precursor in strain YU10 was found to be identical to that of strain G32 reported previously (Wescombe *et al.*, 2012). The structural gene *slnA* was shown to be present in strain YU10 as two almost identical copies. The full sequence of the *slnM* gene encoding salivaricin G32 modification enzyme was reported in this study. Blasting the sequence against the UniProt database (http://www.uniprot.org/) showed that McdM (the modification enzyme of the lantibiotic macedocin) is SlnM's closest homologue with 75% identity as shown in

Figure **4.12**. This suggests that SlnM belongs to LanM family of modification enzymes. However, *slnM*-like genes were found in other streptococcal genomes e.g. *S. mutans*, *S. mitis*, *S. pyogenes*, *S. agalactiae* and *S. dysagalactiae* (Figure 4.13).

Figure 4.12: Sequence alignment of the novel salivaricin G32 modification enzyme (SlnM) and its closest homologue McdM (the modification enzyme of the lantibiotic macedocin produced by *Streptococcus macedonicus*). Identical residues are highlighted in blue.

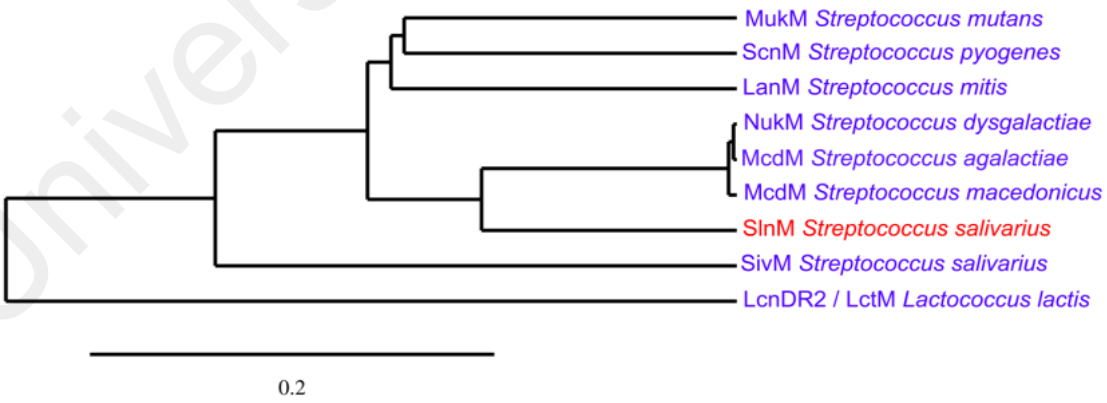

Figure 4.13: Phylogenetic tree of LanM enzymes. The modification enzymes presented here were predicted to be involved in the maturation of lacticin 481 group lantibiotics. SlnM of *Streptococcus salivarius* strain YU10 is indicated in red and it is shown to be most relative to the macedocin modification enzyme (McdM). The figure was produced using TreeDyn http://www.phylogeny.fr/.

The putative ABC transporter of the lantibiotic salivaricin G32 was shown to be 81.9% similar to peptidase C39 of *S. agalactiae* strain DK-B-USS-146, accession number: A0A0H1ZCH9, 81.4% to McdT the putative ABC transporter of the lantibiotic macedocin, accession number: H2A7D3, 75.7% to MukT the putative ABC transporter of the lantibiotic mutacin K8, accession number: A0SXQ4 and 73.3% to ScnT the putative ABC transporter of the lantibiotic streptococcin A-FF22, accession number: O31053.

The self-protection and immunity system *slnFEG* of salivaricin G32 was sequenced and reported in this study for the first time. The gene *slnF* was shown to be 97.9% identical to *bcrA* gene encoding ABC transporter, ATP-binding protein in *Streptococcus vestibularis* F0396 accession number: E3CSL6. However, the *slnF* gene was shown to be 80.1% similar to a *mcdF* gene encoding McdF immunity protein of macedocin, accession number: A6MER5. The second gene in salivaricin G32 immunity system (*slnE*) was shown to have 99.6% homogeneity with gene designated as HMPREF9192_0192 in *Streptococcus vestibularis* F0396, accession number: E3CSL5. The *slnG* gene was found to be 98.8% similar to gene designated as HMPREF9192_0191 in *Streptococcus vestibularis* F0396, accession number: E3CSL4. This indicates that *Streptococcus vestibularis* may harbour salivaricin G32-like locus which was not identified before for this streptococcal species. An extensive bioinformatics analysis was applied to assess the transmembrane capacity of the salivaricin G32 immunity proteins using the SPLIT 4.0 SERVER (http://split4.pmfst.hr/) and TMHMM server (http://www.cbs.dtu.dk/services/TMHMM/). The topology prediction of the immunity proteins showed that the N-terminus of both SlnE and SlnG are inside the cytoplasm and both proteins were predicted to have six transmembrane regions. However, SlnF was shown to be a non-transmembrane protein (Figure 4.14 and Figure 4.15).

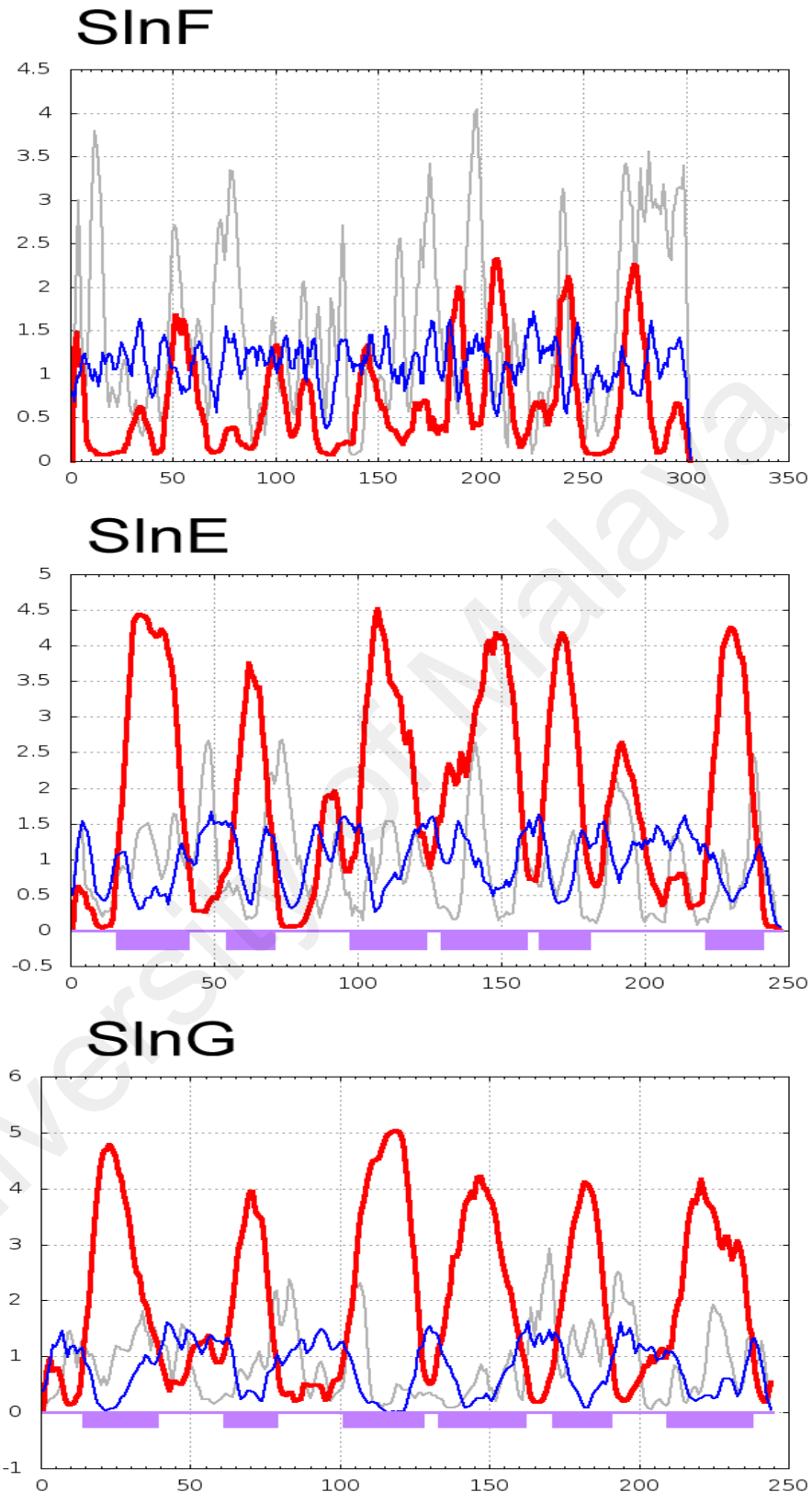

Figure 4.14: Topology predictions of salivaricin G32 immunity proteins using SPLIT 4.0. Red line: Transmembrane helix preference. Blue line: Beta preference. Gray line: Modified hydrophobic moment index. Violet boxes (below abscisa): Predicted transmembrane helix position.

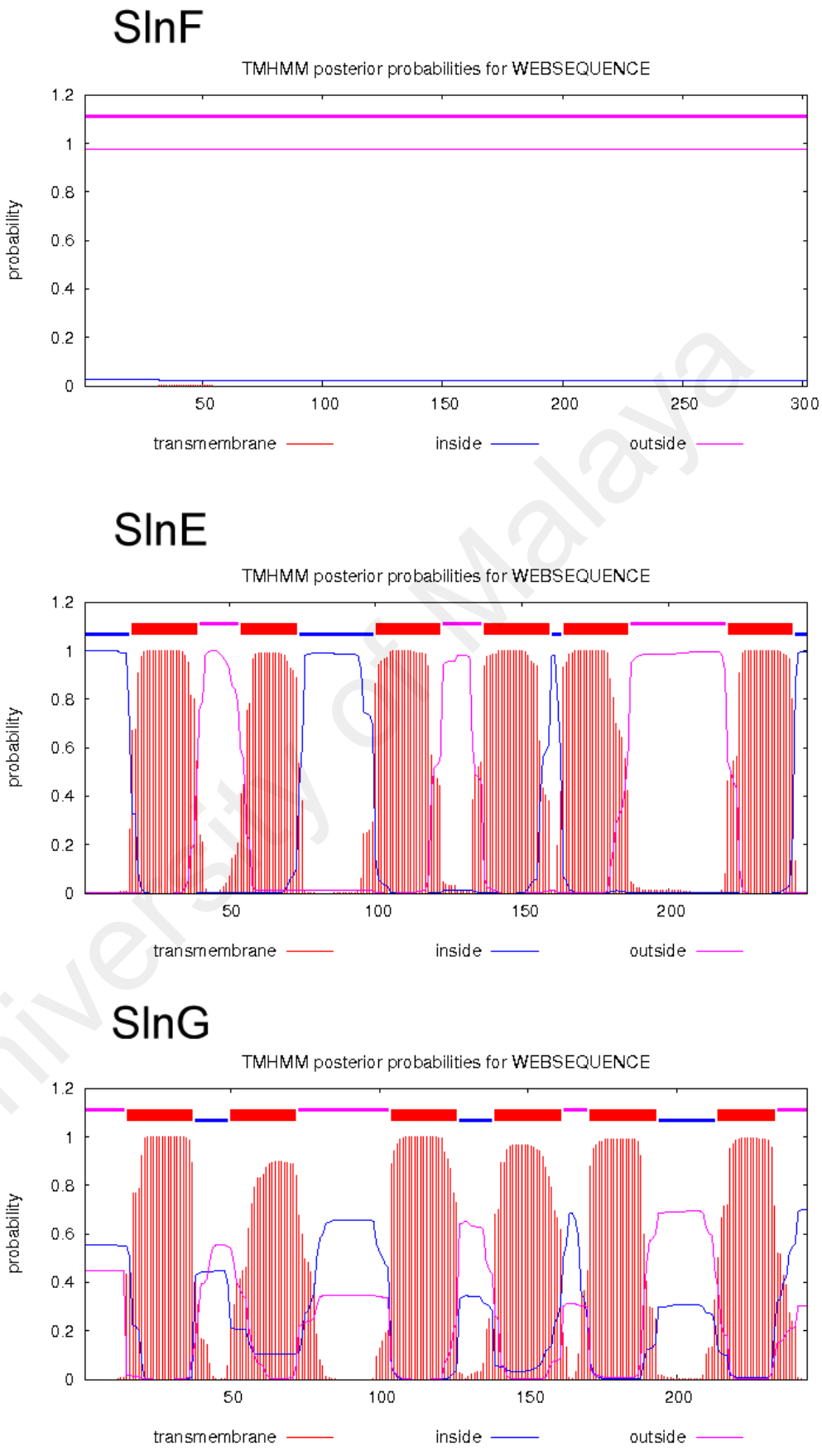

Figure 4.15: Topology predictions of salivaricin G32 immunity proteins using TMHMM server. Red: amino acids predicted to be in the membrane, Blue: amino acids inside. Pink: amino acids outside.

The salivaricin G32 respond regulator (SlnR) was shown to share 99.1% similarity with response regulator receiver domain protein of *Streptococcus vestibularis* strain F0396, accession number: E3CSL7, 89.2% with McdR of *Streptococcus macedonicus*, accession number: H2A7D9 and 85.3% with ScnR of *Streptococcus pyogenes*, accession number: O31050 (Figure 4.16).

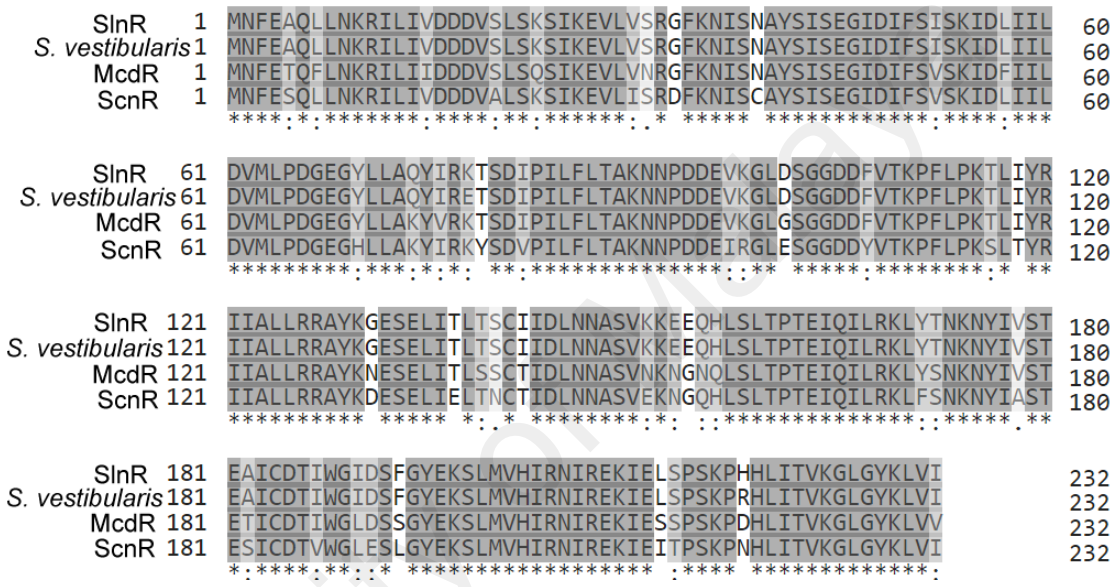

Figure 4.16: Sequence alignment of SlnR of *S. salivarius* YU10, SlnR-like of *S. vestibularis* F0396, McdR of *S. macedonicus* and ScnR of *S. pyogenes*. UniProt was used for alignment and to derive this graphic.

The sensor histidine kinase SlnK of salivaricin G32 showed in this study to be 79.7% similar to histidine kinase protein of *Streptococcus vestibularis* ATCC 49124, accession number: E8KX40, 77.4% to McdK of *Streptococcus macedonicus*, accession number: H2A7E0 and 75.4% to ScnK of *Streptococcus pyogenes*, accession number: O31049 (Figure 4.17).

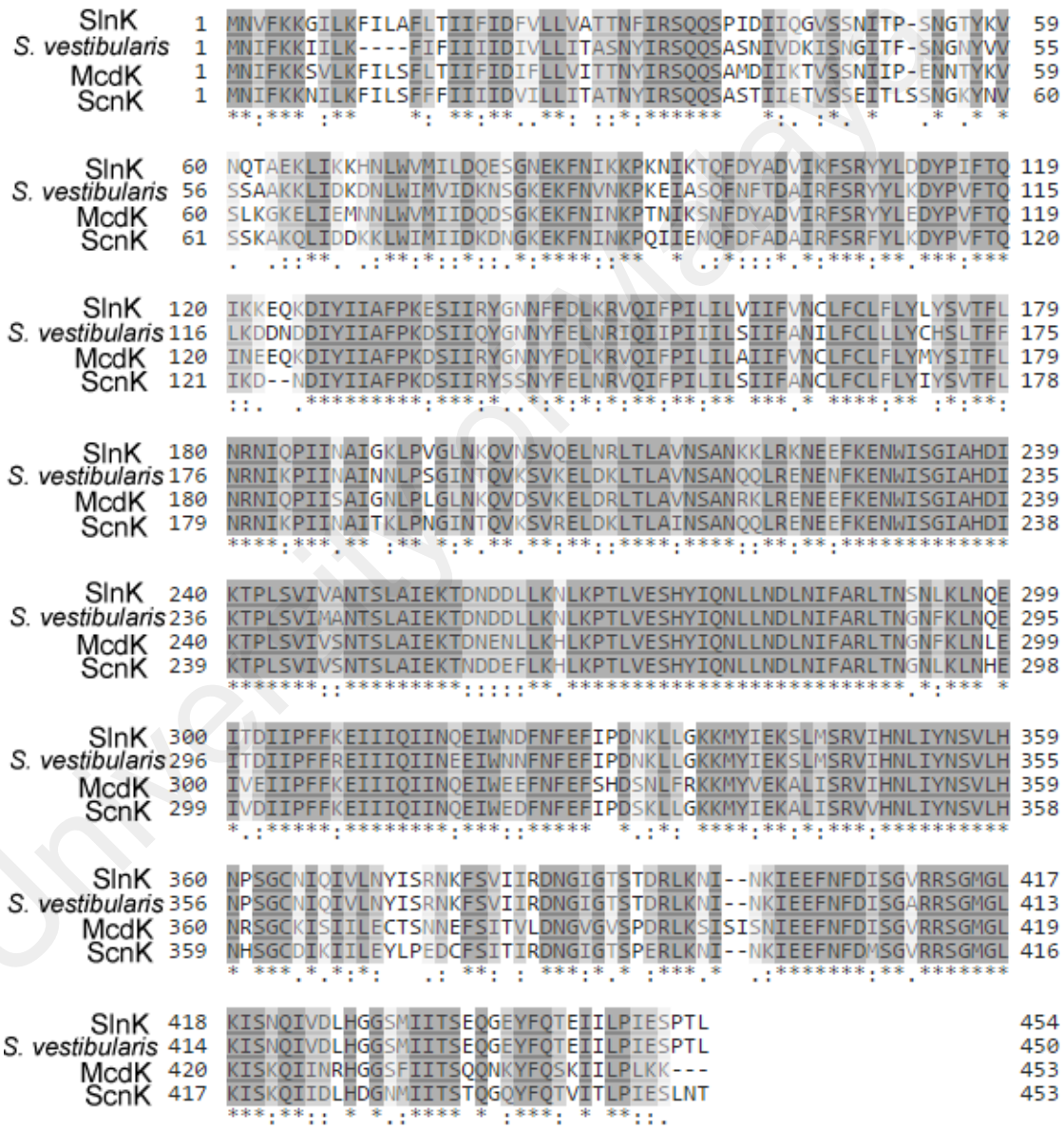

Figure 4.17: Sequence alignment of SlnK of *S. salivarius* YU10, SlnK-like of *S. vestibularis* ATCC 49124, McdK of *S. macedonicus* and ScnK of *S. pyogenes*. UniProt was used for alignment and to derive this graphic.

4.10 Developing new lantibiotic-production medium

The newly developed medium used in this study helped to increase the biomass of *S. salivarius* cultures grown aerobically. The carbon source (sucrose) used within this medium was adequate to grow all the strains as some of them like NU10 cannot process lactose. Usually, *S. salivarius* requires CO_2 enriched atmosphere to grow adequately and thereby produce lantibiotic molecules. However, in this study, enriched medium was developed to cultivate *S. salivarius* aerobically without any supplemented CO_2. However, strain NU10 showed some growth limitations to different levels of CO_2 (3-5%). It also did not grow in M17 medium (Merck) supplemented originally with lactose as a carbon source (Figure 4.20). This finding confirmed the metabolic profile of strain NU10 which was unable to uptake lactose. Strain GT2 also showed weak growth in M17 medium used in this study and this event cannot be linked to carbon source since this strain showed a positive reaction to lactose test. When THB or BHI media were used, some lytic activities were observed after 20 hours of bacterial growth as the OD_{600} values started to decrease (Figure 4.18 and Figure 4.19). Although it is designed for lactic acid bacteria, MRS medium failed to grow *S. salivarius*. However, strain GT2 showed better growth (but still weak, OD_{600} = 0.4) when cultivated in this medium as compared with other *S. salivarius* strains (Figure 4.21). YNS medium showed better bacterial growth compared to other commercial media especially for strains GT2 and K12 (Figure 4.22). However, the newly developed PTNYSMES was the best medium tested for *S. salivarius* growth in this study and showed a significant increase in the optical density of all the isolates. Compositions of all media used are listed in Table 4.8.

The differences in pH values before and after 22 hours of fermentation for each medium are listed in Table 4.9. All isolates reached the stationary phase of growth in just 10 hours and showed no autolytic activities even after 24 hours. OD_{600} = 1 was

achieved with strains K12, NU10 and GT2 while strain YU10 also showed good biomass accumulation with OD_{600} value of 0.9 (Figure 4.23).

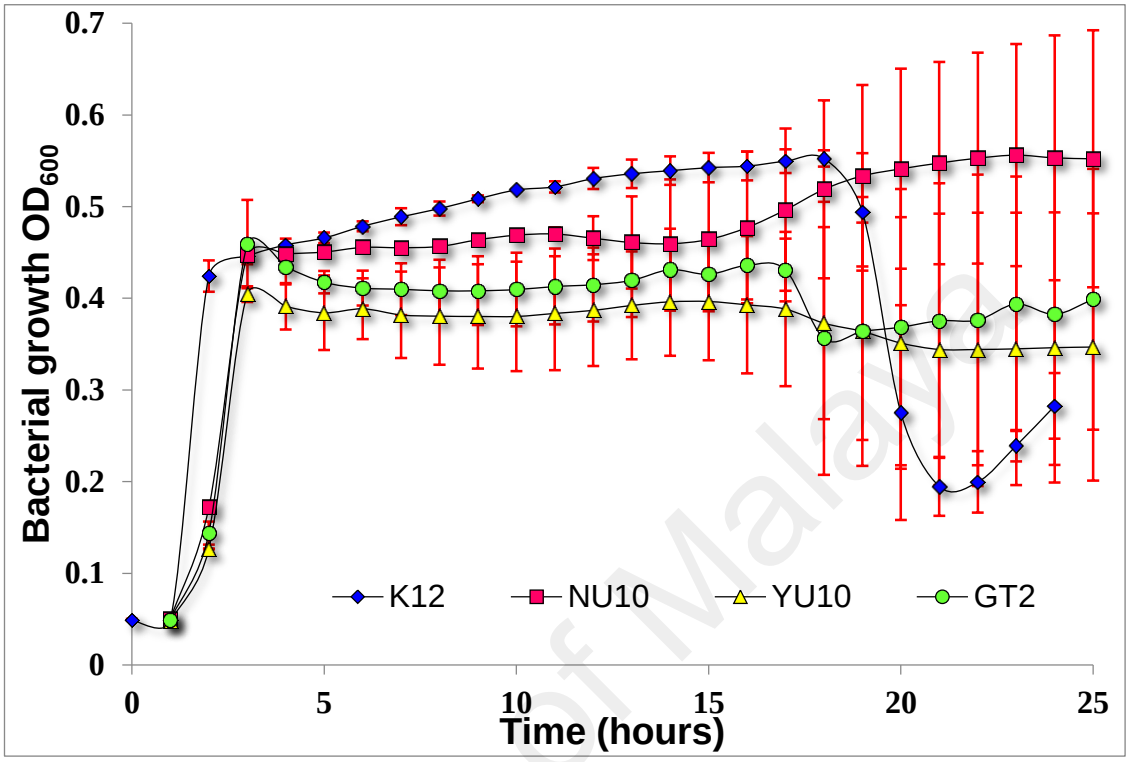

Figure 4.18: Growth kinetics of *S. salivarius* strains grown aerobically in THB medium. Where error bar is not visible, then the error is smaller than the symbol used. (n=3, P<0.05).

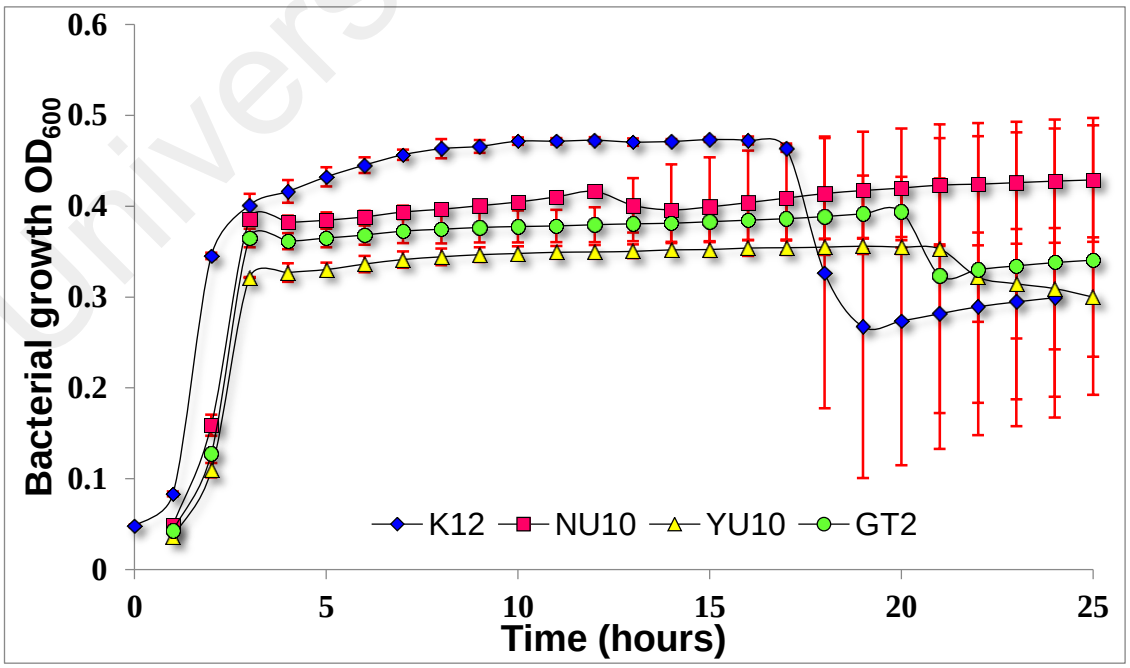

Figure 4.19: Growth kinetics of *S. salivarius* strains grown aerobically in BHI medium. Where error bar is not visible, then the error is smaller than the symbol used. (n=3, P<0.05).

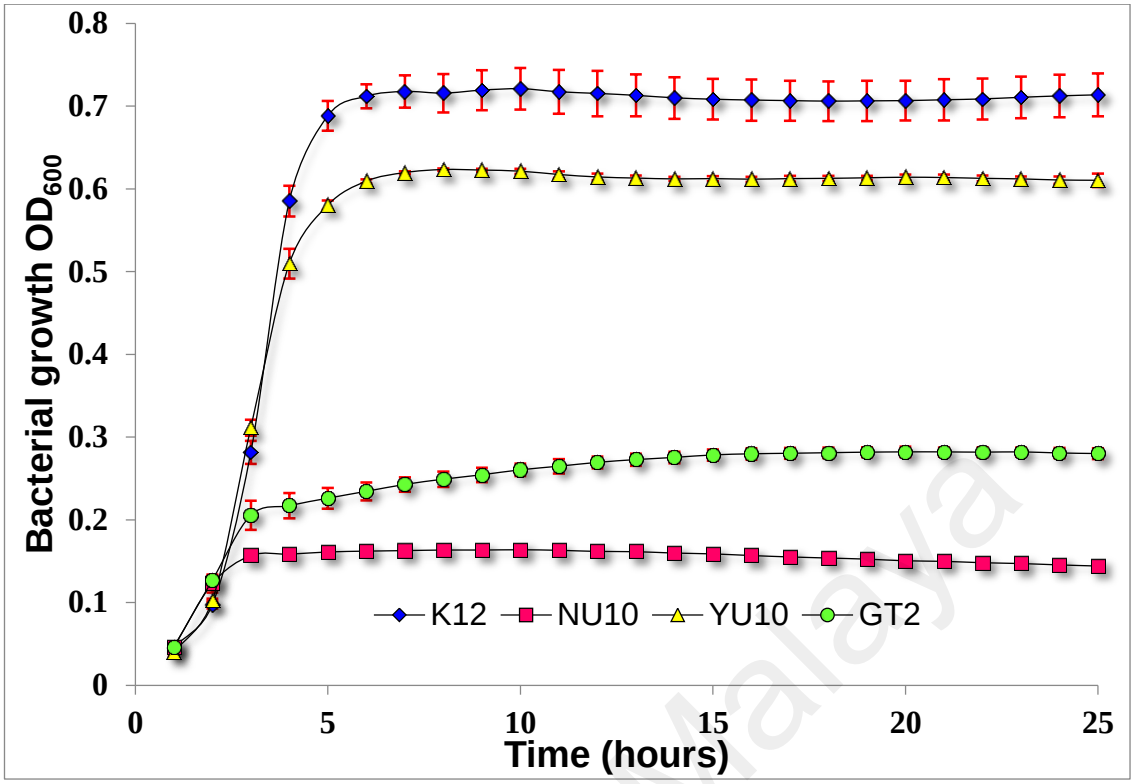

Figure 4.20: Growth kinetics of *S. salivarius* strains grown aerobically in M17 medium (Merck). Where error bar is not visible, then the error is smaller than the symbol used. (n=3, P<0.05).

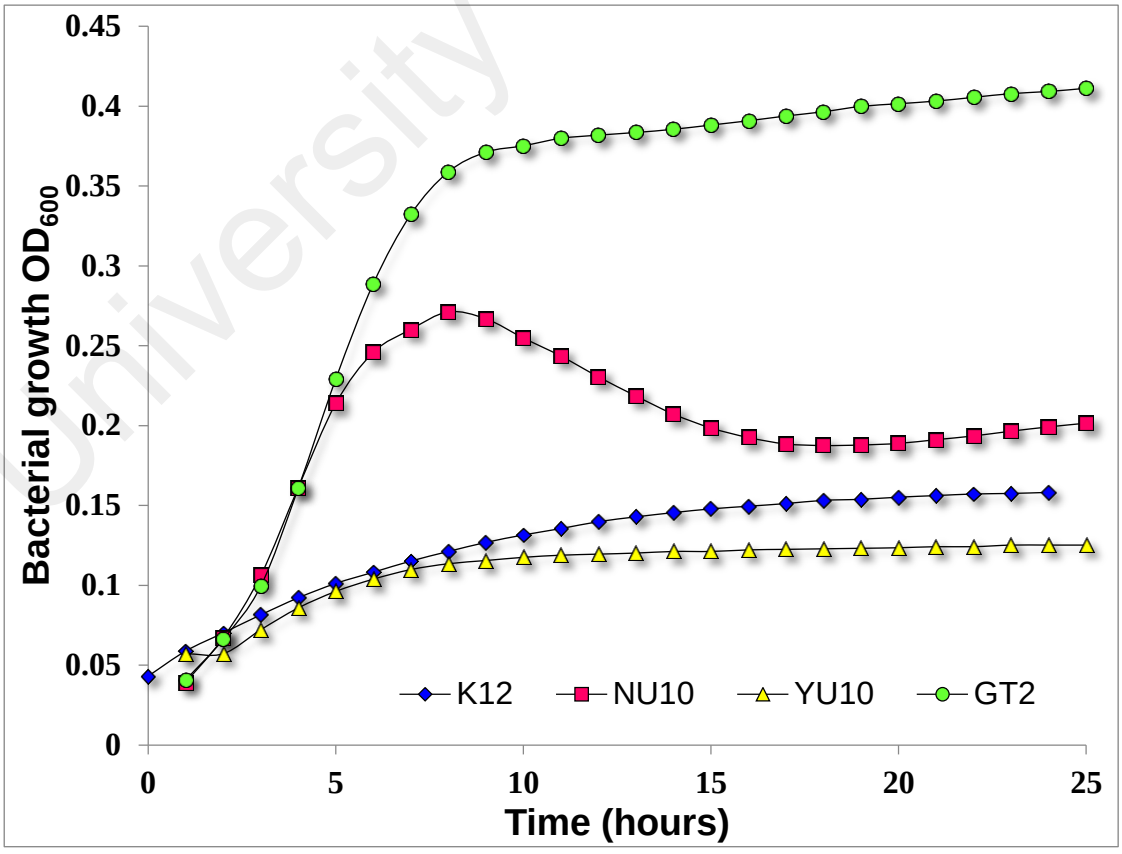

Figure 4.21: Growth kinetics of *S. salivarius* strains grown aerobically in MRS medium (Merck). Where error bar is not visible, then the error is smaller than the symbol used. (n=3, P<0.05).

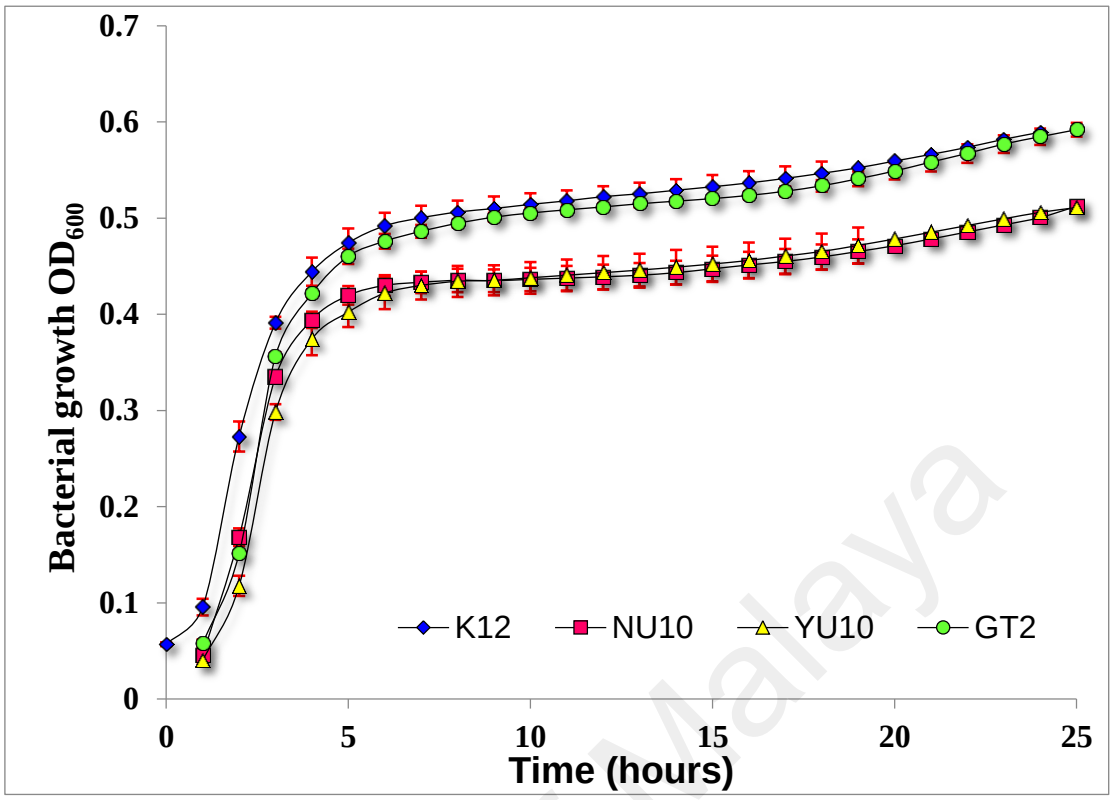

Figure 4.22: Growth kinetics of *S. salivarius* strains grown aerobically in YNS medium. Where error bar is not visible, then the error is smaller than the symbol used. (n=3, P<0.05).

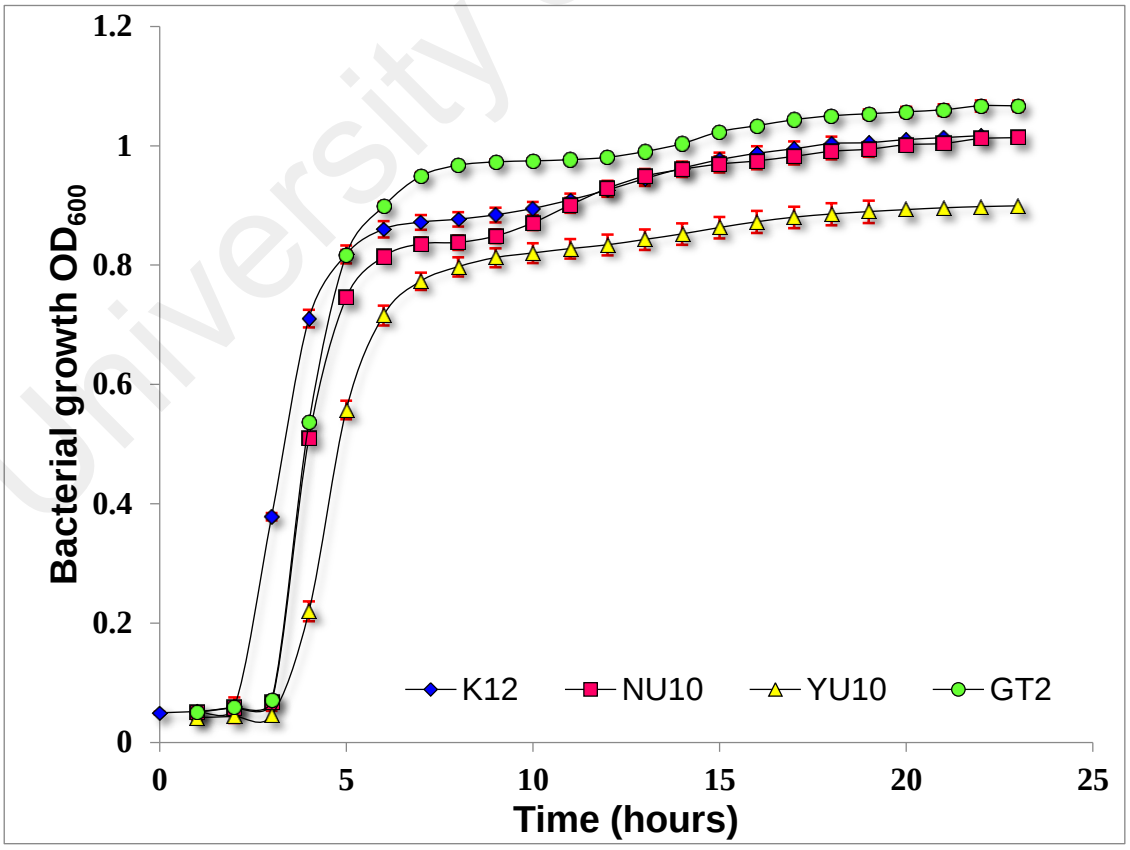

Figure 4.23: Growth kinetics of *S. salivarius* strains grown aerobically in PTNYSMES medium. Where error bar is not visible, then the error is smaller than the symbol used. (n=3, P<0.05).

Table 4.8: Typical compositions for media used to cultivate *S. salivarius.*

Typical Composition	Media used for *S. salivarius* growth						
	PTNYSMES	**YNS**	**M17**	**MRS**	**BHI**	**THB**	**BruB¥**
Nitrogen source							
Peptone	✓		✓	✓	✓		✓
Tryptone	✓						
Neopeptone	✓	✓				✓	
Yeast extract	✓	✓	✓	✓			✓
Meat extract			✓	✓			
Heart/Brain infusion					✓	✓	
Digest of animal tissue							✓
Carbon source							
Dextrose				✓	✓	✓	✓
Lactose			✓				
Sucrose	✓	✓					
Vitamins							
Ascorbic acids	✓		✓				
Salts							
Sodium chloride	✓				✓	✓	✓
Disodium phosphate					✓	✓	
Sodium carbonate							
Magnesium sulfate			✓	✓			
Manganese sulfate	✓			✓			
Sodium bisulfite				✓			✓
Sodium acetate	✓			✓			
Sodium carbonate						✓	
Buffers							
MES	✓						
di-potassium hydrogen phosphate				✓			
Na-β-Glycerophosphate			✓				

¥: Brucella broth (Difco)

Table 4.9: Variation of the pH values of *S. salivarius* cultures grown in different media after 22 hours of growth (*n*=3).

Medium	Initial pH of the medium / final pH of the culture after 22 h fermentation			
	K12	**GT2**	**NU10**	**YU10**
THB	7.8 / 5.00	7.8 / 5.08	7.8 / 5.16	7.8 / 5.23
BHI	7.01 / 5.16	7.01 / 5.38	7.01 / 5.33	7.01 / 5.64
M17	7.13 / 5.57	7.13 / 6.55	7.13 / 6.82	7.13 / 5.74
MRS	5.59 / 4.90	5.59 / 5.00	5.59 / 5.03	5.59 / 5.59
Brucella Broth	6.53 / 5.09	6.53 / 5.14	6.53 / 5.08	6.53 / 5.11
YNS	6.71 / 3.83	6.71 / 3.78	6.71 / 3.84	6.71 / 3.91
PTNYSMES	6.51 / 4.31	6.51 / 4.30	6.51 / 4.27	6.51 / 4.33

4.11 Production of lantibiotics using PTNYSMES

Attempts to recover lantibiotics from *S. salivarius* cells grown in PTNYSMES medium were successful. Both strains NU10 and YU10 were grown for 24 hours in this medium and the lantibiotics were subsequently recovered by cell extraction followed by further chromatography techniques for lantibiotic─purification (Figure 4.24 and Figure 4.25). MALDI-TOF (MS) analysis showed that like in a previous report (Barbour *et al.*, 2013), salivaricin 9 (2560 Da) was produced by strain NU10 using PTNYSMES medium in this current study. Furthermore, salivaricin G32 (2667 Da) (Figure 4.26) was the only detectable and known lantibiotic produced by this strain when grown in the new medium. Salivaricin A was not produced or detected by strains YU10 or NU10 using this medium even though the strains harbour the structural gene encoding this lantibiotic. The production experiment was repeated without adjusting the pH of the medium after fermentation (without adsorption) to calculate levels of lantibiotics attached to the producer cells. However, attempts to recover lantibiotics from the cell-free supernatant of this preparation using 80% ammonium sulphate saturation, as described previously (Tagg and Skjold, 1984), showed that 60-70% of lantibiotic produced by NU10, YU10 and K12 strains presented in this study is cell-wall associated peptide (Table 4.10). The bacteriocin units (arbitrary units) were calculated as mentioned previously (Barbour *et al.*, 2013).

Table 4.10: Inhibitory activity recovered from cell extracts and cell-free supernatants of *S. salivarius* cultures (*n*=3).

Inhibitory activity recovery (From 1 L culture) [§]	Lantibiotic-producing *S. salivarius* strains		
	NU10	**YU10**	**K12**
Total activity	4.8×10^4 AU (100%)	1.32×10^4 AU (100%)	1.02×10^5 AU (100%)
Cell extract activity	3.2×10^4 AU (66.6%)	9.6×10^3 AU (72.7%)	6.4×10^4 AU (62.5%)
Cell-free supernatant activity	1.6×10^4 AU (33.4%)	3.6×10^3 AU (27.2%)	3.84×10^4 AU (37.5%)

[§] Strains were grown in PTNYSMES medium. AU: arbitrary unit.

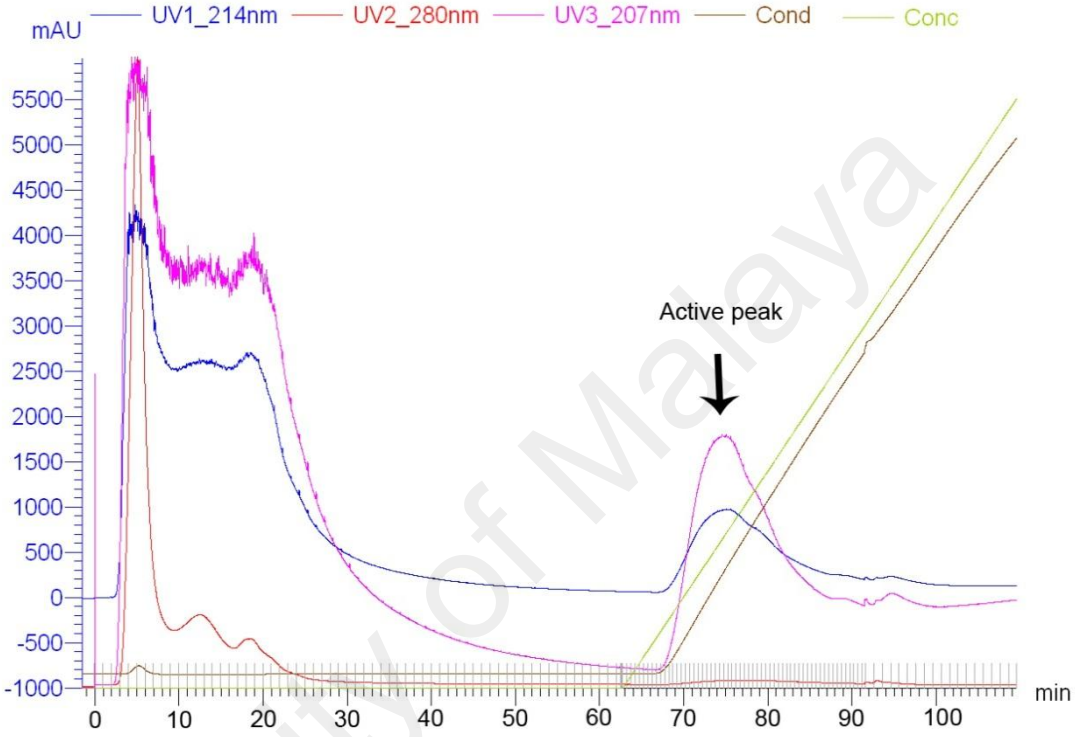

Figure 4.24: Purification and detection of salivaricin G32 produced by strain YU10 (step 1). Cell extract of strain YU10 was subjected to cation exchange chromatography.

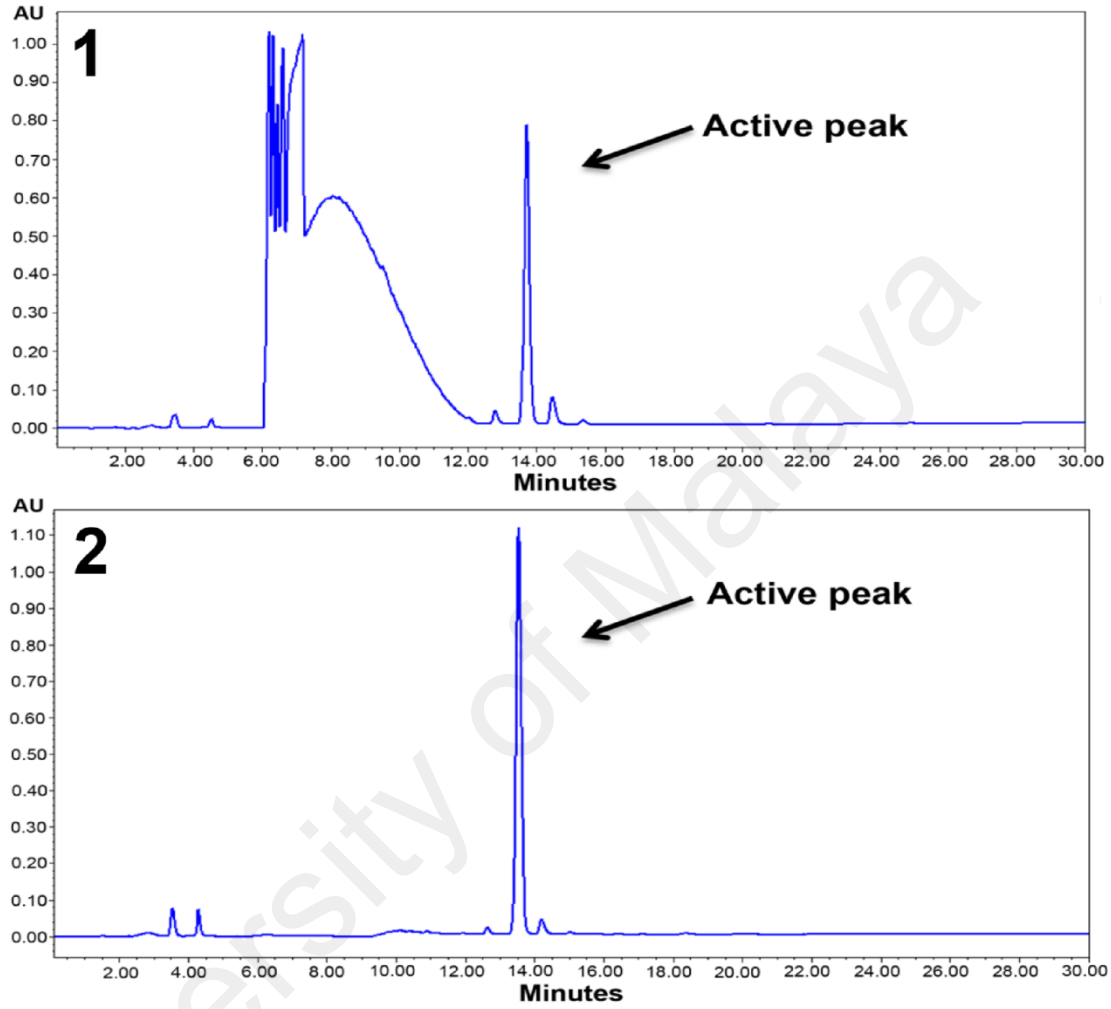

Figure 4.25: RP-HPLC purification of the lantibiotic salivaricin G32 produced by *S. salivarius* YU10. This is the second step after the cation exchange chromatography described above.

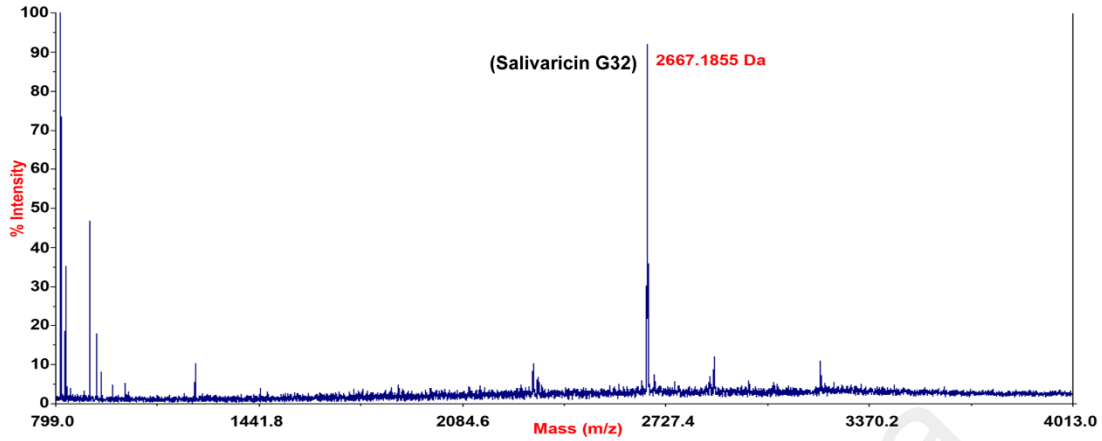

Figure 4.26: MALDI TOF (MS) analysis of the lantibiotic salivaricin G32 produced by strain YU10

Freeze-thaw extraction of K12 cultures grown on M17-agarose followed by hydrophobic interaction chromatography yielded crude lantibiotic preparations containing both salivaricin A2 and salivaricin B. High performance liquid chromatography (HPLC) on a C18 semi-preparative column helped to separate the two lantibiotics at retention times of 50 minutes for salivaricin A2 and 55 minutes for salivaricin B (Figure 4.27). The detection of active fractions was carried out by a spot on lawn assay Figure 4.28. High resolution MALDI-TOF (MS) analysis confirmed the predicted molecular weights. Salivaricin B mass spectrum showed an exact mass of 2732.3867 Da and an average mass of 2733.3899 Da. Salivaricin A2 showed an exact mass of 2366.1946 Da and an average mass of 2367.1975 Da (Figure 4.30). Both lantibiotics can be seen as single peaks resolution as shown in Figure 4.29.

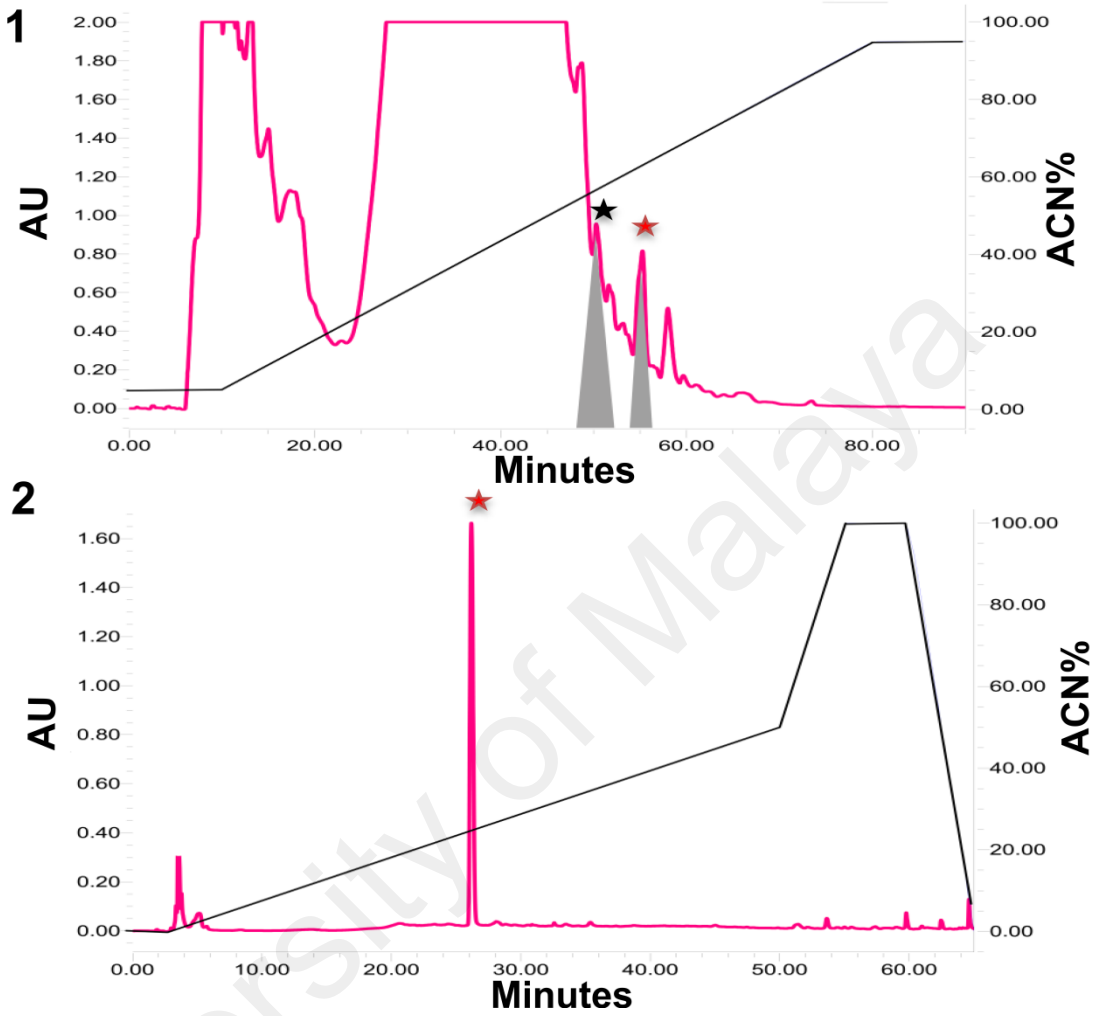

Figure 4.27: Purification of the lantibiotics salivaricins A & B recovered from freeze-thawed cultures of *S. salivarius* K12 grown on agar plates. In (1) the crude extract obtained by HIC on XAD-2 column was subjected to RP-HPLC. Fractions with inhibitory activity were shaded, (2) purity check of salivaricin B using Aeris PEPTIDE column.

Figure 4.28: Spot on lawn assay of salivaricins A2 and B eluted during RP-HPLC fractionation. The MALDI-TOF (MS) analysis showed that the inhibitory activity of fractions 51 and 52 correspond to salivaricin A2 and fractions 54, 55, 56, 57 and 58 correspond to salivaricin B. HPLC fractions were concentrated under reduced pressure to evaporate the acetonitrile before 20µL of each fraction was spotted on MHA plate. The plate was exposed for aeration after which molten soft agar containing 10^5 CFU of *M. luteus* was overplayed the plate.

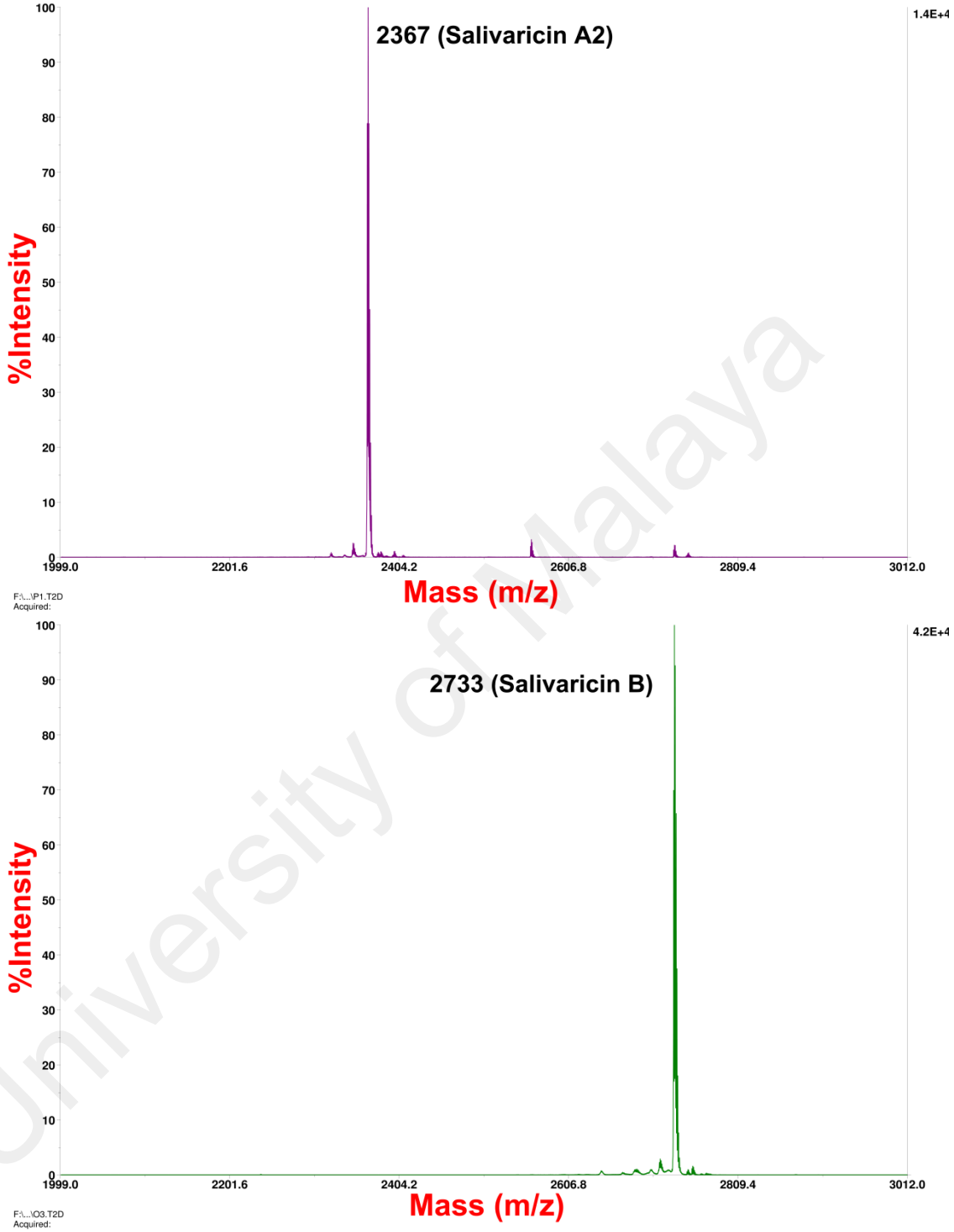

Figure 4.29: Single peak resolution MALDI-TOF (MS) of the lantibiotics salivaricin A2 and salivaricin B.

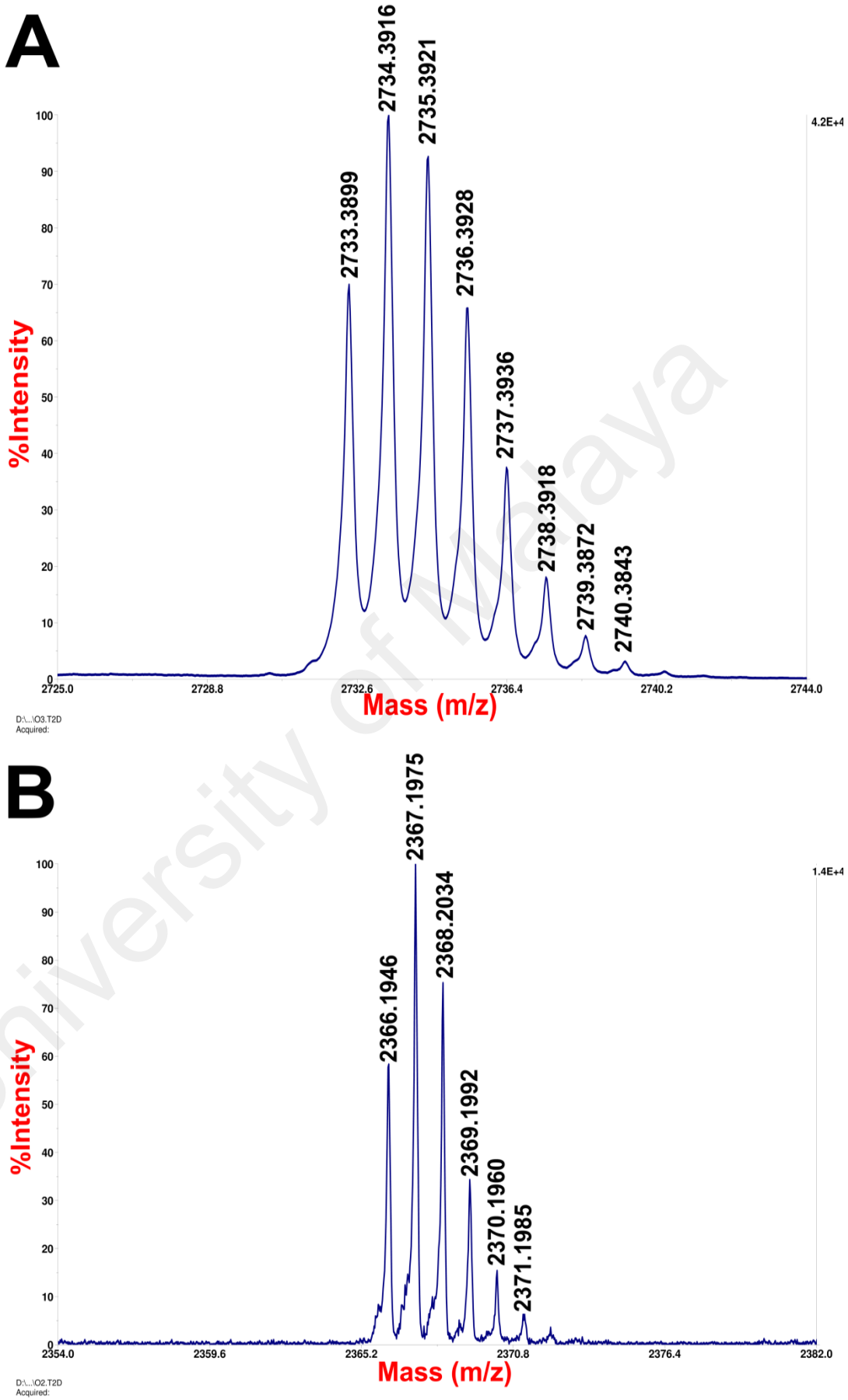

Figure 4.30: High resolution MALDI-TOF (MS) analysis of lantibiotics salivaricin B (A) and salivaricin A2 (B).

4.12 Levan-sucrase Detection and Characterization

Cell-associated levan-sucrase was extracted from *S. salivarius* cells of strain YU10. Advanced LC-MS/MS method was developed for direct detection of this unique enzyme from the cell extract using reverse phase chromatography. The peptide which matched the levansucrase enzyme (accession: Q55242) contains 14 residues (VGTLAFLGATQVKA). The match was considered significant by the search algorithm with a score of 78.88 and coverage of 1.44. This defines matches with ion score of 51 for identity and charge of 2. Retention time for levansucrase was 38.71 minutes with MH+ [Da]=1375.79582. Genome sequencing of strain YU10 revealed the structural gene encoding for levansucrase or fructosyltransferase (FTF) production. Full characterisation of the gene (*ftf*) with *in silico* protein translation was achieved in this study. The *ftf* region of strain YU10 was compared to *ftf* region in the commercial probiotic strain M18 genome and both regions were almost identical. In addition to fructosyltransferase, this region included gene encoding for levanase production (Figure 4.31).

A

Detected amino acids sequence: VGTLAFLGATQVKA Description: Levansucrase OS=Streptococcus Accession: Q55242

MW [kDa]: 103.9 MH+ [Da]: 1375.79582

B

```
MDSTVNSQSNTVAPKQAECKKMRYSIRKVATVGATSALVGTLAFLGATQVKADQVTETAP  60
AVATATATPETSTASLTVASEAATSVATSEAVESSVAHSEVATTPVTETQPSNTTPSVVE  120
EKVSSTVVTSSSDATTPSATVAAVSAPAHTSEAAVEAPTSTASSETADTHTEVALKPTEN  180
SAANANLSKLNGRIKSIVEDNMTSDQIVALTEEEIKALNKVDFSDDAIKGTGTSLTYRNL  240
KDIVASFLKQDSKLAVPYFKADTIINMPAFNTVDAQTMKKEEIDVWDSWPVQDAESGVVS  300
NWNGYQLVISMAGAPNKNSNHIYLLYSKYGDNDFTHWKNAGPIFGYNALEDDQQWSGSAT  360
VNSDGSIQLYYTKNDTSGGKLNWQQLASATLNLAVENDEVVIKSVENDHILFGGDNYHYQ  420
SYPKFMSTFNDDHNHDGNPDRTDNYCLRDPHIIEDNGSRYLIFESNTGDENYQGEKQIYN  480
WSNYGGDDAFNLKSFLNIVNNKHLYNLASWANGSIGILKLDDNEKNPSVAELYTPLVTSH  540
MVTDEVERPSVVKMGNKYYLFTASRINKSTDAEGTVAAREAVGDDVVMLGFVSDSLRGEY  600
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LKPHTYPTVDGSPGVSEARGVLTVTVKDGEDKKSDKSETPVNPTEGNHSVDDKSNKPDTP  780
SKPADNNQPSTNKEDKPATPTNPDSPVRNPFPYFTDRPSNDNNSSDDHHVEVPAKPSTES  840
SVGDRRPVAQATEIASPVPEAIVATGPTVSTTPVKEESVTETEAPKPAKSEEEVQSHGVA  900
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LVTGKIHLPKK  971
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C

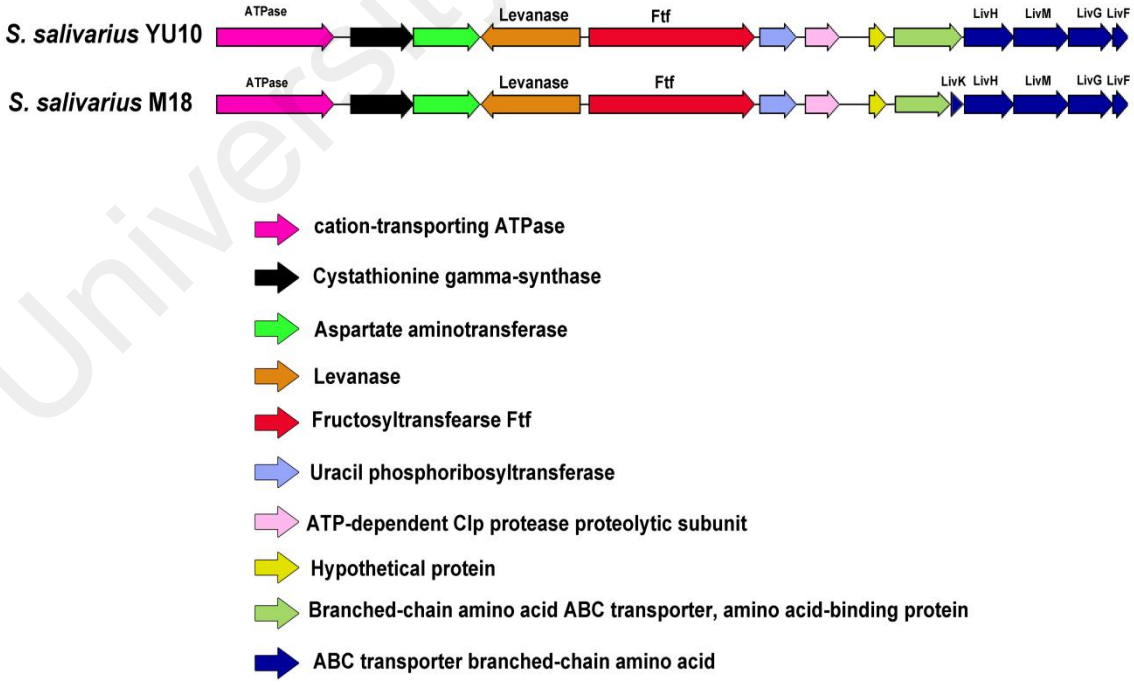

Figure 4.31: Identification of levan-sucrase produced by *S. salivarius* strain YU10. A) LC-Ms/Ms characterisation of levan-sucrase. B) The full sequence of levan-sucrase enzyme derived from the genome sequencing of *S. salivarius* YU10. C) Comparison of regions for genes encoding levan-sucrase (fructosyltransferase) enzyme in *S. salivarius* YU10 and *S. salivarius* M18.

4.13 Developing peptide-membrane binding model to elucidate pore forming mechanism

To study the mechanism by which small antimicrobial peptides penetrate the membrane vesicles of targeted bacterial strains, a model based on the small cationic peptide cecropin B was developed. In order to understand the mechanism of peptide-membrane binding, the original peptide (cecropin B1: KWKVFKKIEKMGRNIRNGIV) was modified by attaching a terminal tryptophan residue (cecropin B2: KWKVFKKIEKMGRNIRNGIVW). Both peptides showed a significant inhibitory effect against a wide range of bacteria compared to naturally occurring peptides (Table 4.11 and Table 4.12). The fluorescence results showed an enhancement in the peak intensity of cecropin B1 upon mixing with the membrane accompanied by a blue shift. For cecropin B2, a blue shift was observed upon mixing with the *Pseudomonas aeruginosa* (PA) membrane but no enhancement in intensity was observed. The results indicated perpendicular penetration of cecropins B1 and B2 from the Lys side where the Trp residue of cecropin B1 is immersed in the PA membrane (Figure 4.32). Partial quenching of the Trp fluorescence by acrylamide was observed and the values of the Stern-Volmer constants (Ksv) indicated that the Trp molecule penetrated into the membrane but resided close to the interface region (Figure 4.33). Two fluorescence lifetimes were measured for the cecropin B1-PA complex containing two rotamers of Trp (Figure 4.34). The results pointed to a degree of flexibility of the local environment around the Trp molecule. A mechanism of membrane disruption is proposed in which the cecropin peptide created cracks through the negatively charged membrane of *P. aeruginosa*.

Table 4.11: Inhibitory activity of synthetic antimicrobial peptides and other antimicrobial agents (*n*=3).

Antimicrobial	MIC values (µg/ml)				
	P. aeruginosa	***E. coli***	***S. aureus***	***Corynebacterium* spp**	***B. cereus***
Synthetic cecropin B 1	0.75 µg	0.75 µg	3 µg	0.09375 µg	1.5 µg
Synthetic cecropin B 2	0.828 µg	0.207 µg	1.656 µg	0.103 µg	1.656 µg
Ampicillin	1000 µg	≤15.62 µg	≤15.62 µg	≤15.62 µg	2000 µg
Penicillin G	250 µg	3.90 µg	≤ 0.9 µg	≤ 0.9 µg	125 µg
Nisin A	NI*	NI*	0.734 µg	≤0.367 µg	2.937 µg
Tetracyclin	≤1.56 µg	≤1.56 µg	≤1.56 µg	≤1.56 µg	≤1.56 µg

*No Inhibition.

Table 4.12: Minimal bactericidal concentration of synthetic cecropins B1 and B2 (*n*=3).

Antimicrobial	MBC values				
	P. aeruginosa	***E. coli***	***S. aureus***	***Corynebacterium* spp**	***B. cereus***
Synthetic cecropin B 1	1.5 µg	1.5 µg	6 µg	0.09375 µg	6 µg
Synthetic cecropin B 2	1.656 µg	0.414 µg	3.321 µg	0.09375 µg	3.312 µg

*No inhibition.

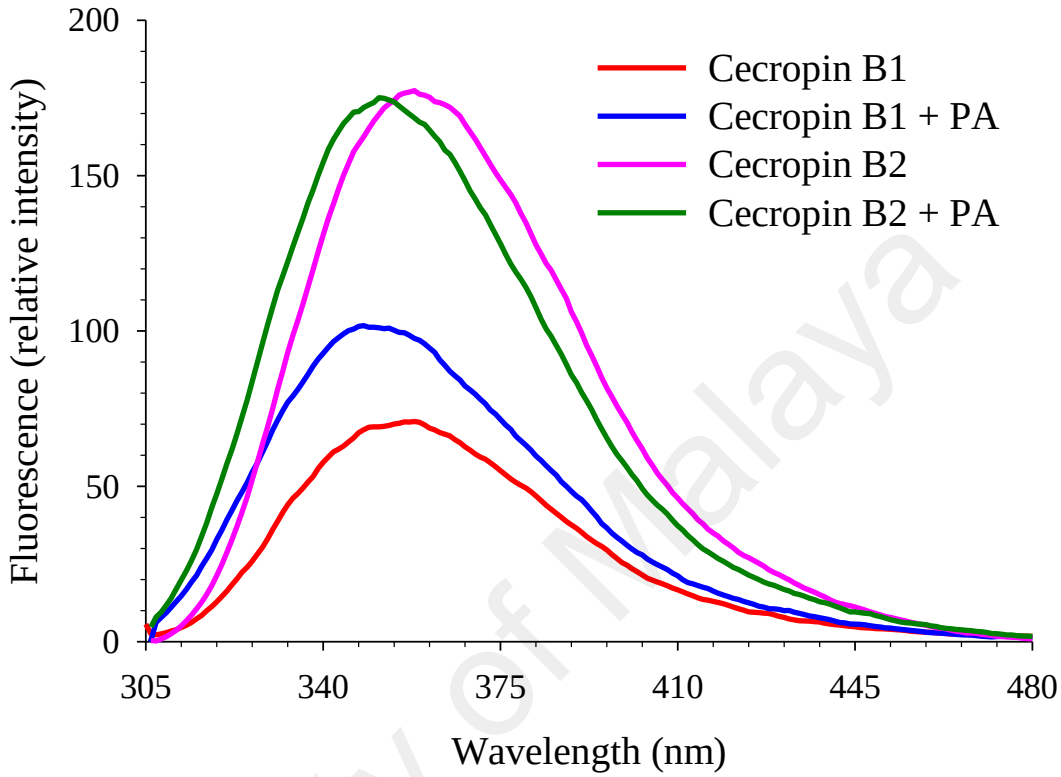

Figure 4.32: Fluorescence spectra of the synthetic peptides cecropins B1 and B2 in the absence and present of the PA membrane (1:1 molar ratio). The solvent was 10 mM HEPES buffer of pH 7.4. λex = 295 nm. The concentration of all species was 0.05 mM. (n=3, P<0.05).

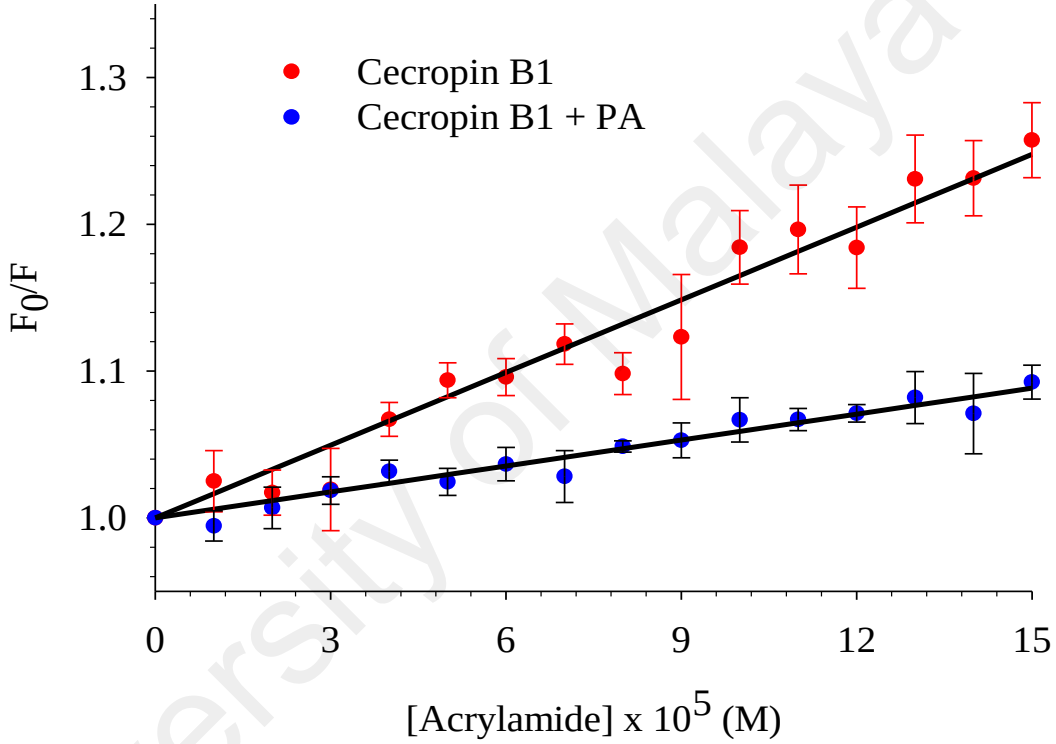

Figure 4.33: Stern-Volmar plots for the fluorescence quenching of tryptophan in cecropin B1 by acrylamide in the absence and presence of the PA membrane. The concentration of PA and cecropin B1 was 0.05 mM (1:1 molar ratio). λex = 295 nm. (n=3, P<0.05).

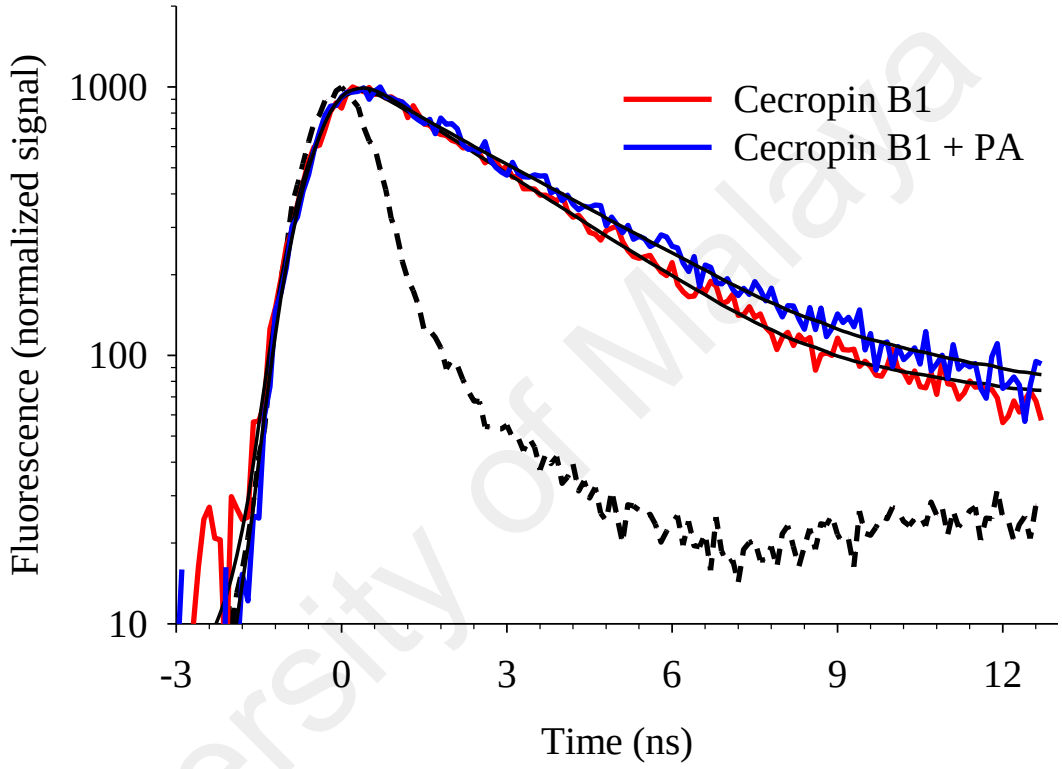

Figure 4.34: Fluorescence decay transients of cecropin B1 in the absence and presence of the PA membrane. λ_{ex} = 295 nm. Fluorescence was detected using a Schott WG-320 nm cut-off filter. IRF is shown by a dashed line. The best fits are shown in black solid lines. (n=3, P<0.05).

The same developed strategy was applied on the lantibiotic salivaricin B. Interaction of the salivaricin B peptide with bacterial membranes was investigated by measuring the fluorescence change for tryptophan. The fluorescence peak position of tryptophan reflected the medium polarity and can be used to predict the local environment around the tryptophan residue in a biological system (Lakowicz, 2006; Zahid *et al.*, 2011). The lowest energy is shown at the fluorescence peak maximum of ~ 355 nm and this is usually taken as an indication of the exposure of tryptophan to the aqueous solution.

Figure 4.35 shows the fluorescence spectra of the aqueous-buffer solution of salivaricin B, *B. cereus* membrane, *E. coli* membrane and the mixtures of salivaricin B with the membranes. The peak location of the salivaricin B peptide is at ~ 355 nm while the spectral locations of the two membranes are at 348 and 336 nm for *B. cereus* and *E. coli* membranes, respectively. The latter values indicated the presence of partially buried tryptophan(s) in the two membranes whereas in salivaricin B the peak at 355 nm was indicative of exposed tryptophan(s) to buffer. Upon mixing the peptide with the membranes, there was a slight increase in intensity relative to that of the membranes alone which indicated the presence of an interaction between the peptide and the two target membranes. On the other hand, the spectral location of the fluorescence peak in each mixture remained the same, within the measurements of uncertainty, as that of the corresponding membrane alone.

It is always difficult to correlate the change in fluorescence to the actual mode of peptide-membrane attack when more than one tryptophan residue is present (Nichols *et al.*, 2013). Nevertheless, comparing the spectral change in the absence and presence of the peptide indicated that the mode of binding between the peptide and the membrane was not vertical (penetration) and more likely to be parallel. If the attack was a perpendicular penetration, a blue shift is usually expected in the fluorescence peak of

tryptophan, compared to that of the peptide alone observed with cecropin B (Abou-Zied *et al.*, 2015).

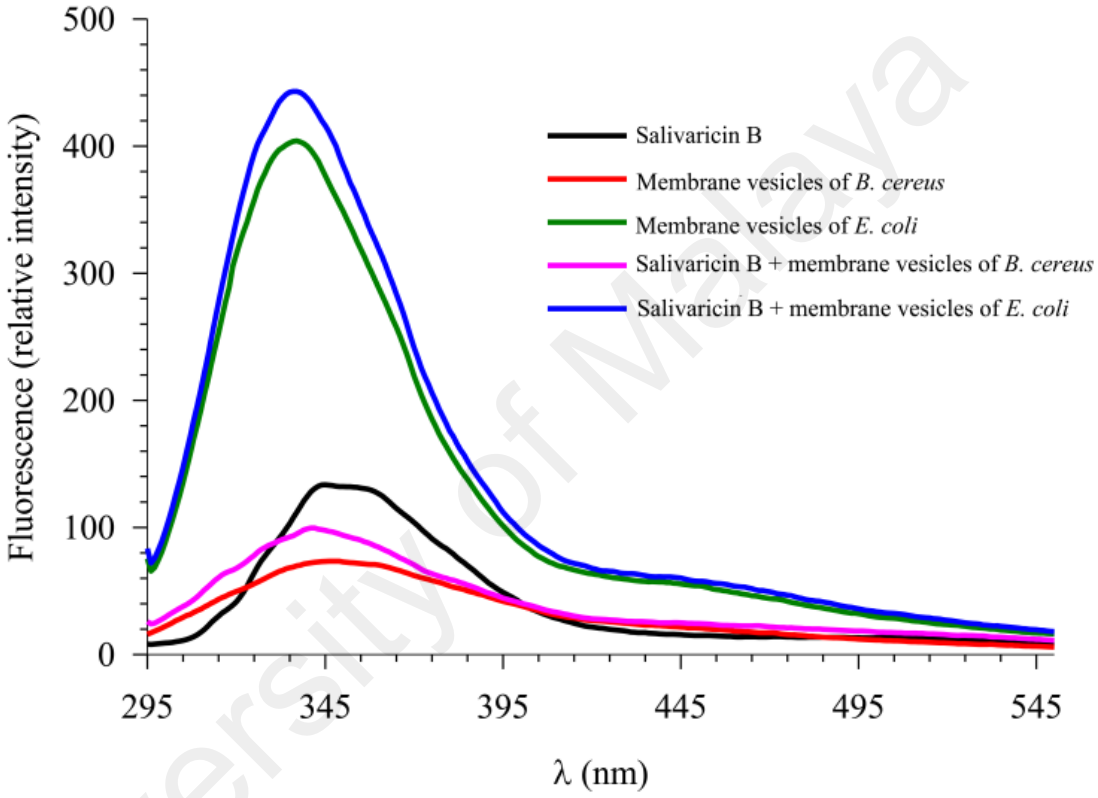

Figure 4.35: Fluorescence spectra of salivaricin B in the absence and presence of different bacterial membrane vesicles. The concentration of membrane vesicles and peptides was 0.05 mM in 10 mM phosphate buffer pH 7.4. (n=3, P<0.05).

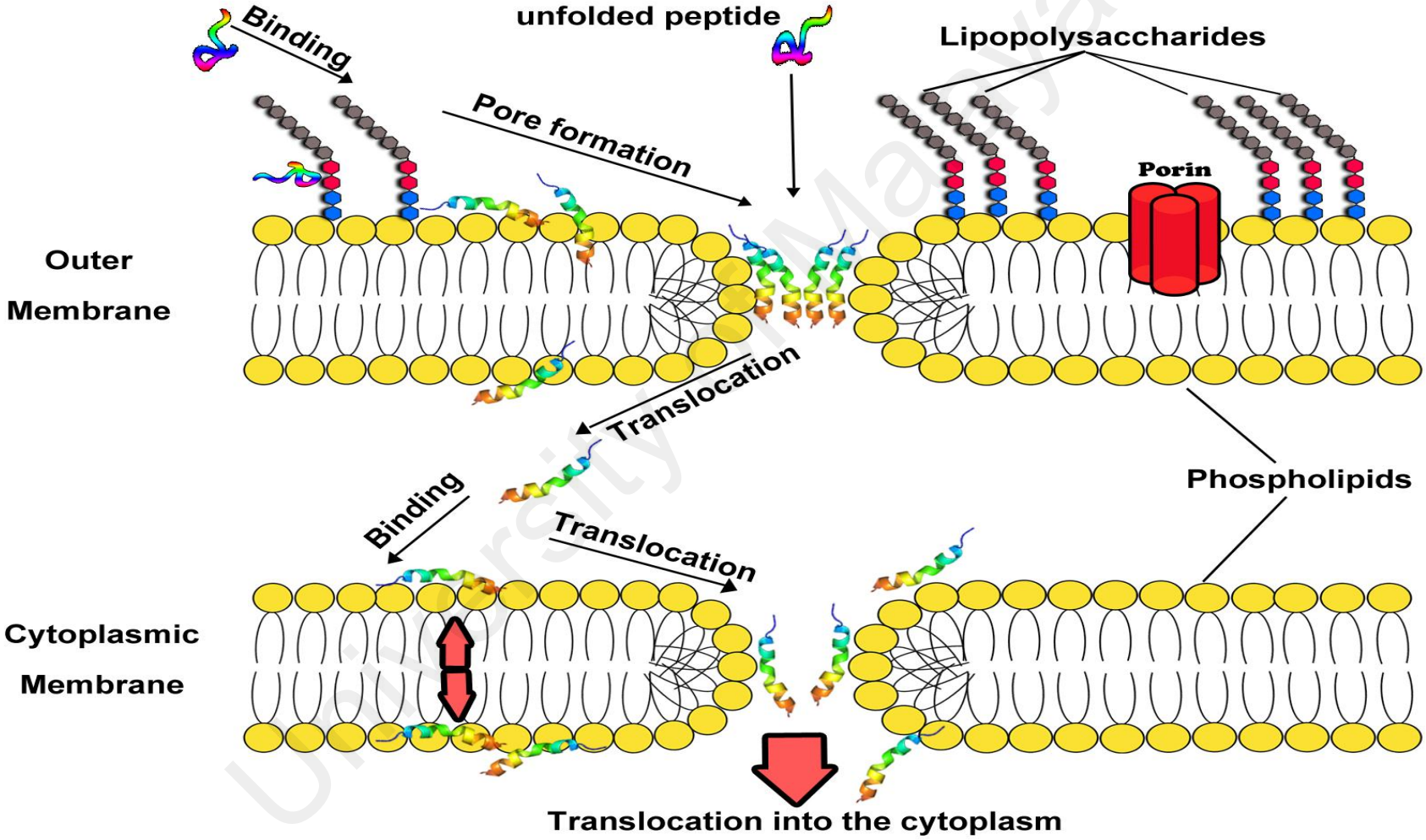

Figure 4.36: Proposed mechanism of interaction of cecropin-like peptides with the cell envelope of *P. aeruginosa*.

4.14 Salivaricin B: minimal inhibitory concentration (MIC), IC_{50} and time killing assay

Agar well diffusion assays were initially performed to obtain preliminary qualitative data concerning the relative susceptibility of various Gram-positive bacteria to salivaricin B and nisin A. Bacterial strains showing susceptibility towards both lantibiotics in this assay were then subjected to growth inhibition assays in liquid media from which quantitative MIC values were determined. Both salivaricin B and nisin A failed to inhibit Gram-negative bacteria. The diverse specific concentrations of salivaricin B and nisin A required to inhibit individual strains of Gram-positive bacteria indicated that the activity of both lantibiotics is strain-dependent. *Lactococcus lactis* HP has been shown previously to be highly susceptible to nisin A and various other lantibiotics. In this study, nisin A was shown to have a very low nano-molar MIC value for this strain (39 nM) when compared with the relatively modest potency of salivaricin B (MIC=1080 nM). On the other hand, the nisin A producer *L. lactis* ATCC11454 was more sensitive to salivaricin B than to nisin A. This study showed that salivaricin B is bactericidal for most streptococci in a range of 2160-4320 nM. However, some streptococci such as *S. mutans* exhibited resistance to ≥ 8 μM of salivaricin B. The list of MIC values obtained in this study can be seen in Table 4.13 below. IC50 values for the same strains are analyzed and stated in Table 4.14.

Table 4.13: Minimal inhibitory concentrations of salivaricin B and nisin A (*n*=3).

Indicator strain	**Salivaricin B (nM)**	**Nisin A (nM)**
Corynebacterium spp GH17	269	39
Lactococcus lactis subsp. *cremoris* HP	1080	39
Lactococcus lactis ATCC11454	2160	2500
Micrococcus luteus ATCC10240	540	312.5
Staphylococcus aureus RF122	ND[†]	617.5
Streptococcus constellatus ATCC27823	1080	154.3
Streptococcus equisimilis ATCC12388	2160	154.3
Streptococcus mutans GEJ11	ND[†]	2500
Streptococcus pneumonia ATCC6301	2160	154.3
Streptococcus pyogenes ATCC12344	2160	617.5
Streptococcus pyogenes ATCC12348	2160	617.5
Streptococcus salivarius K12	ND[†]	78
Streptococcus salivarius NU10	2160	78
Streptococcus salivarius YU10	2160	312.5
Streptococcus sanguinis ATCC10556	4320	154.3

[†]Not determined, strain resistant to 8640 nM of salivaricin B.

Table 4.14: IC_{50} values of salivaricin B and nisin A (*n*=3).

Indicator strain	Salivaricin B (nM)	Nisin A (nM)
Corynebacterium spp GH17	140.557 ±7.067	16.4117 ± 0.567
Lactococcus lactis subsp. *cremoris* HP	407 ± 34	17.7 ± 0.9
Lactococcus lactis ATCC11454	891.7 ± 29.69	523.5 ± 152.6
Micrococcus luteus ATCC10240	269.548 ± 41.33	128.641 ± 19
Staphylococcus aureus RF122	ND†	240 ± 35
Streptococcus constellatus ATCC27823	451.4 ± 85	66.8 ± 6.859
Streptococcus equisimilis ATCC12388	1177 ± 223.4	74.25 ± 7
Streptococcus mutans GEJ11	ND†	903.9 ± 96.49
Streptococcus pneumonia ATCC6301	810.242 ± 45.26	57.9 ± 2.27
Streptococcus pyogenes ATCC12344	1435.48 ± 326	219.146 ± 41.79
Streptococcus pyogenes ATCC12348	1263.53 ± 596	232.152 ± 51.8
Streptococcus salivarius K12	ND†	34.658 ± 3.408
Streptococcus salivarius NU10	972.01 ± 218.7	23.4701 ± 1.825
Streptococcus salivarius YU10	912.285 ± 72.11	136.315 ± 8.895
Streptococcus sanguinis ATCC10556	1590.27 ±110.8	57.753 ± 5.71

†Not determined, strain resistant to 8640 nM of salivaricin B.

Salivaricin B was bactericidal for both *S. pyogenes* and *M. luteus*. After 30 minutes of salivaricin B exposure, more than 40% of *S. pyogenes* cells were killed indicating a rapid killing activity. Moreover, salivaricin B (10× MIC) killed more than 90% of *S. pyogenes* in less than 3 hours. Similar activity was detected against *M. luteus* but with even stronger potency (Figure 4.37). No significant lysis occurred when either *S. pyogenes* or *M. luteus* were treated with 10× MIC levels of salivaricin B.

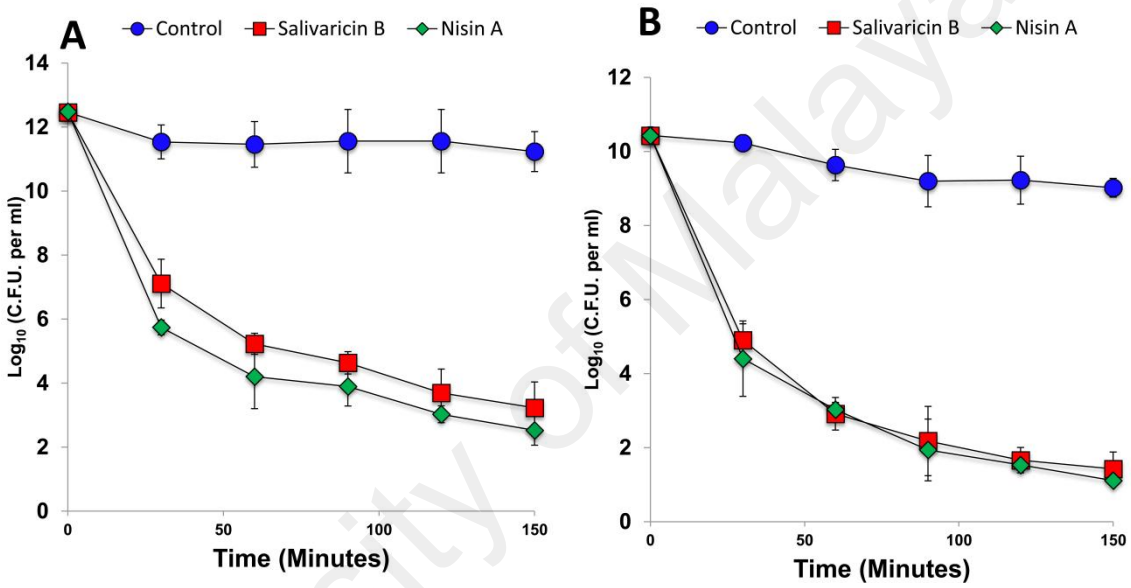

Figure 4.37: Time killing assay of salivaricin B against *S. pyogenes* (a) and *M. luteus* (b). Error bars represent the SD of the mean from three independent tests (n=3, P<0.05).

4.15 Salivaricin B microplate growth inhibition assays

Treating susceptible *S. pyogenes* cells with different concentrations of salivaricin B revealed that the bactericidal effect is concentration-dependent. Cells treated with 2.5 µM salivaricin B failed to propagate whereas 90% growth inhibition was obtained in cells treated with 2 µM salivaricin B and 50% of population growth of *S. pyogenes* was inhibited by 1.0 µM salivaricin B. Growth was not affected significantly in cells treated with 0.5 µM salivaricin B (Figure 4.38A below). This assay was also carried out against

M. luteus cells which were shown to be more susceptible to salivaricin B. *M. luteus* was grown in MHB at 37°C and monitored for 10 hours, which was the required incubation time for *M. luteus* cells to reach OD_{600} = 0.4 in the microplate growth inhibition assay. Complete inhibition of *M. luteus* growth was achieved with only 0.5 µM of salivaricin B and 0.125 µM affected 70% killing (Figure 4.38).

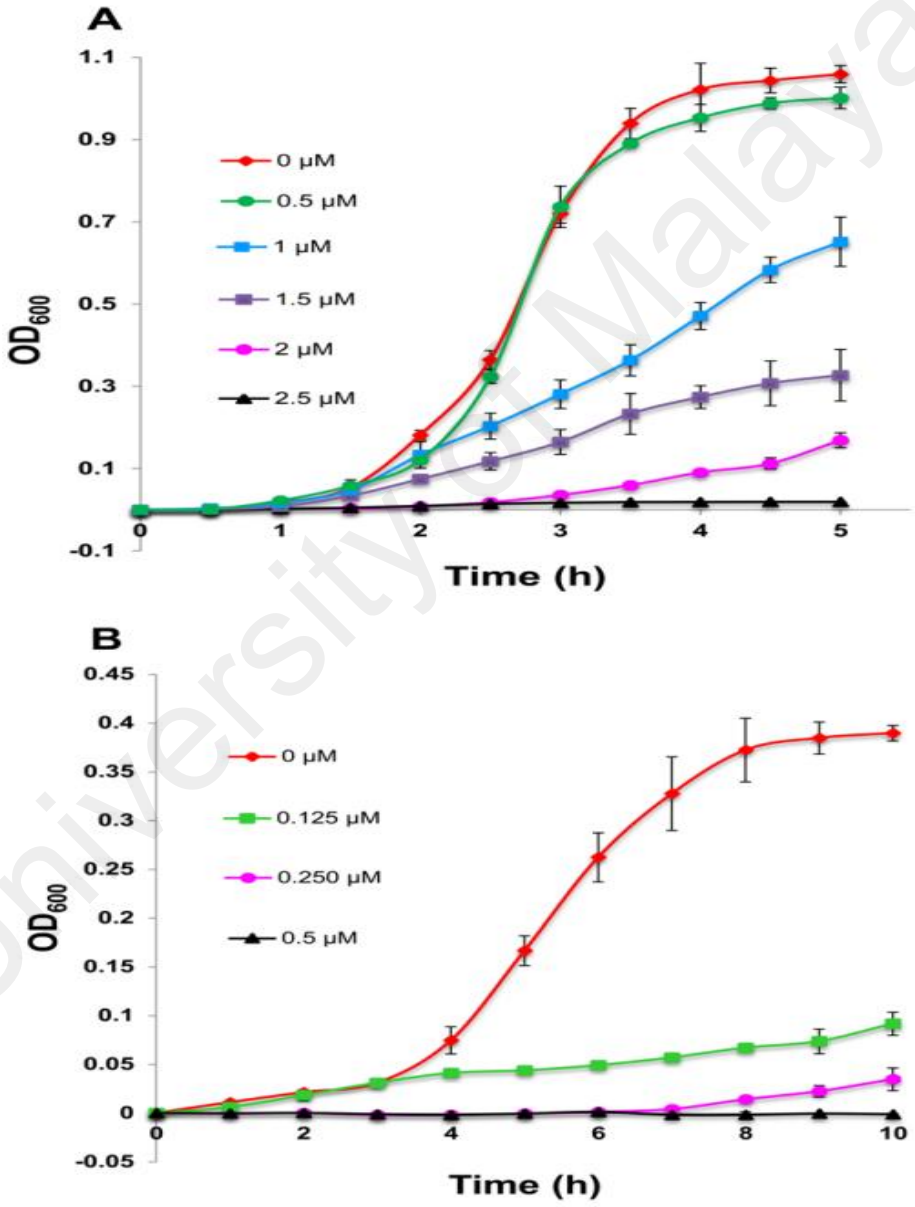

Figure 4.38: Inhibitory activity of salivaricin B in microplate growth inhibition assay against *S. pyogenes* ATCC1234 (A) and *M. luteus* ATCC (B). Error bars represent the SD of the mean from three separate tests. Where error bar is invisible, the error is smaller than the symbol used. (n=3, P<0.05).

4.16 Assessment of pore formation using SYTOX Green-labeled cells

SYTOX Green probe is a high-affinity DNA stain which is impermeable to cells with intact membranes. However, when pores are formed in the targeted membranes by a pore-forming agent, the probe enters the cells and interacts with the genomic DNA to generate increased fluorescence intensity. In this study, we probed potential membrane permeabilization by salivaricin B in *S. pyogenes* ATCC1234 using SYTOX Green. Nisin A, when used at 5-fold MIC, gave a significant increase in cell-associated fluorescence intensity (FI) consistent with loss of membrane integrity and pore formation. In contrast, salivaricin B was unable to increase FI above control levels at concentrations up to 10-fold of the MIC value. It was noticed that the FI of nisin-treated cells increased gradually with time indicating disruption of membrane integrity of more cells during an extended incubation period whereas no significant increment of the FI of salivaricin B-treated cells was observed even after 30 minutes of incubation (Figure 4.39). Similarly, unlike nisin A, salivaricin B (10× MIC) did not induce pore formation in *M. luteus* cell membranes in this study even with extended incubation (30 minutes). The results provided clear evidence that salivaricin B is not able to induce pore formation in sensitive cells.

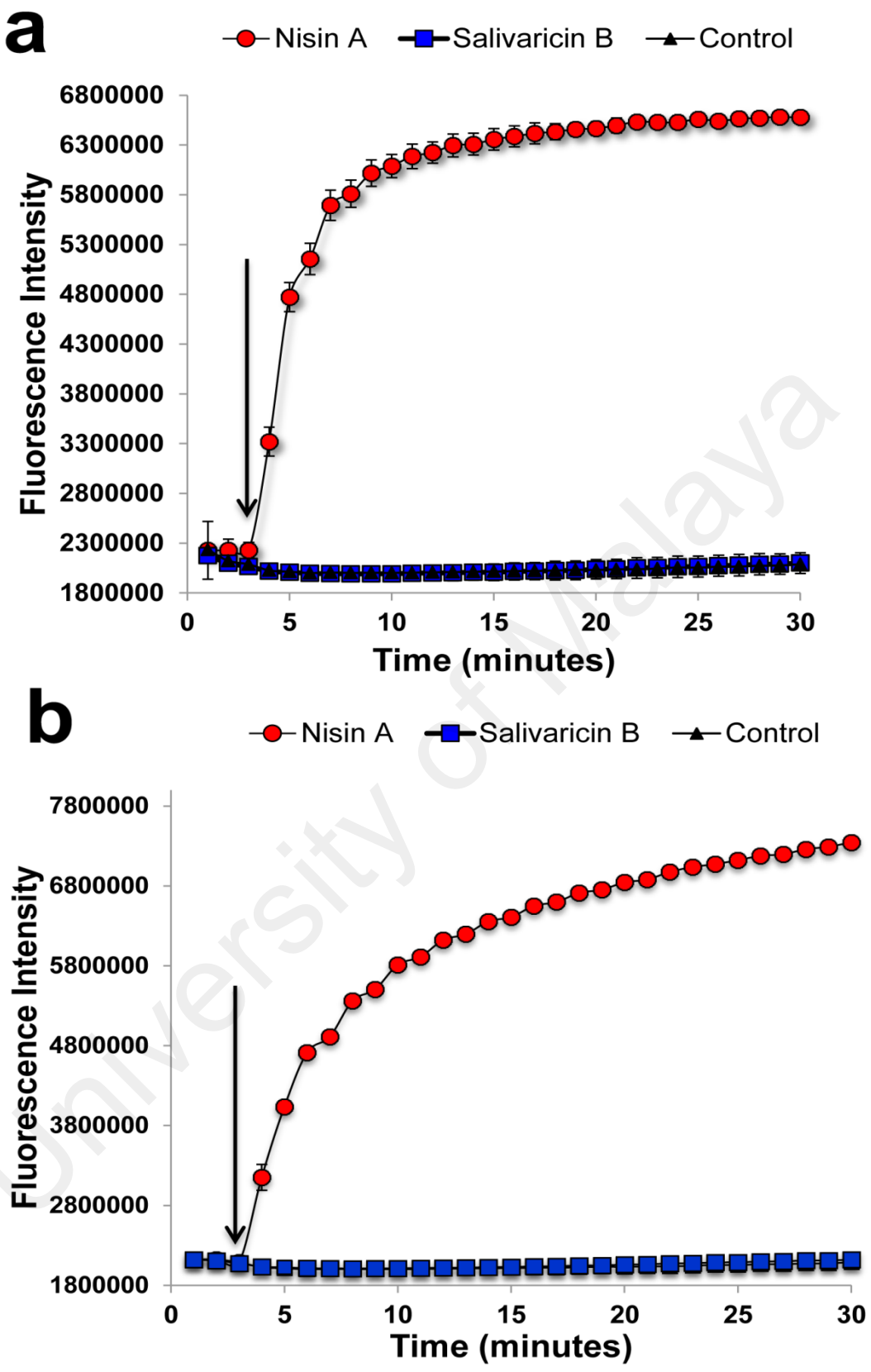

Figure 4.39: Pore formation assay. Unlike nisin A, salivaricin B did not induce pore formation as no detectable SYTOX green fluorescence could be observed for cells exposed to salivaricin B in both *M. luteus* (a) and *S. pyogenes* (b). Arrows indicate the time of lantibiotic addition. Error bars represent the SD of the mean from three separate tests. Where error bar is invisible, the error is smaller than the symbol used (n=3, P<0.05).

4.17 Dissipation of membrane potential

The ability of salivaricin B to dissipate the membrane potential of the targeted bacterial cells was investigated in this study using DiOC2(3) (3,3-diethyloxacarbocyanine iodide). This molecular probe exhibits green fluorescence in all bacterial cells, but the fluorescence shifts towards red emission as the dye molecules self-associate at the higher cytosolic concentrations caused by higher membrane potentials. Analysis of DiOC2(3)-labeled *M. luteus* ATCC10240 cells was carried out using scatter plots of green versus red fluorescence. Previous reports of the depolarization ability of nisin A showed similar results to the present flow cytometric study in that *M. luteus* cells were significantly depolarized by nisin A. Figure 4.40 shows both intact and depolarized population due to nisin pore formation activity. However, in the case of exposure to salivaricin B (10-fold MIC), no changes in the population location could be distinguished from intact cells indicating no membrane potential dissipation occurred. These findings provided strong evidence that salivaricin B is not able to damage the membrane integrity of salivaricin B-susceptible bacterial cells.

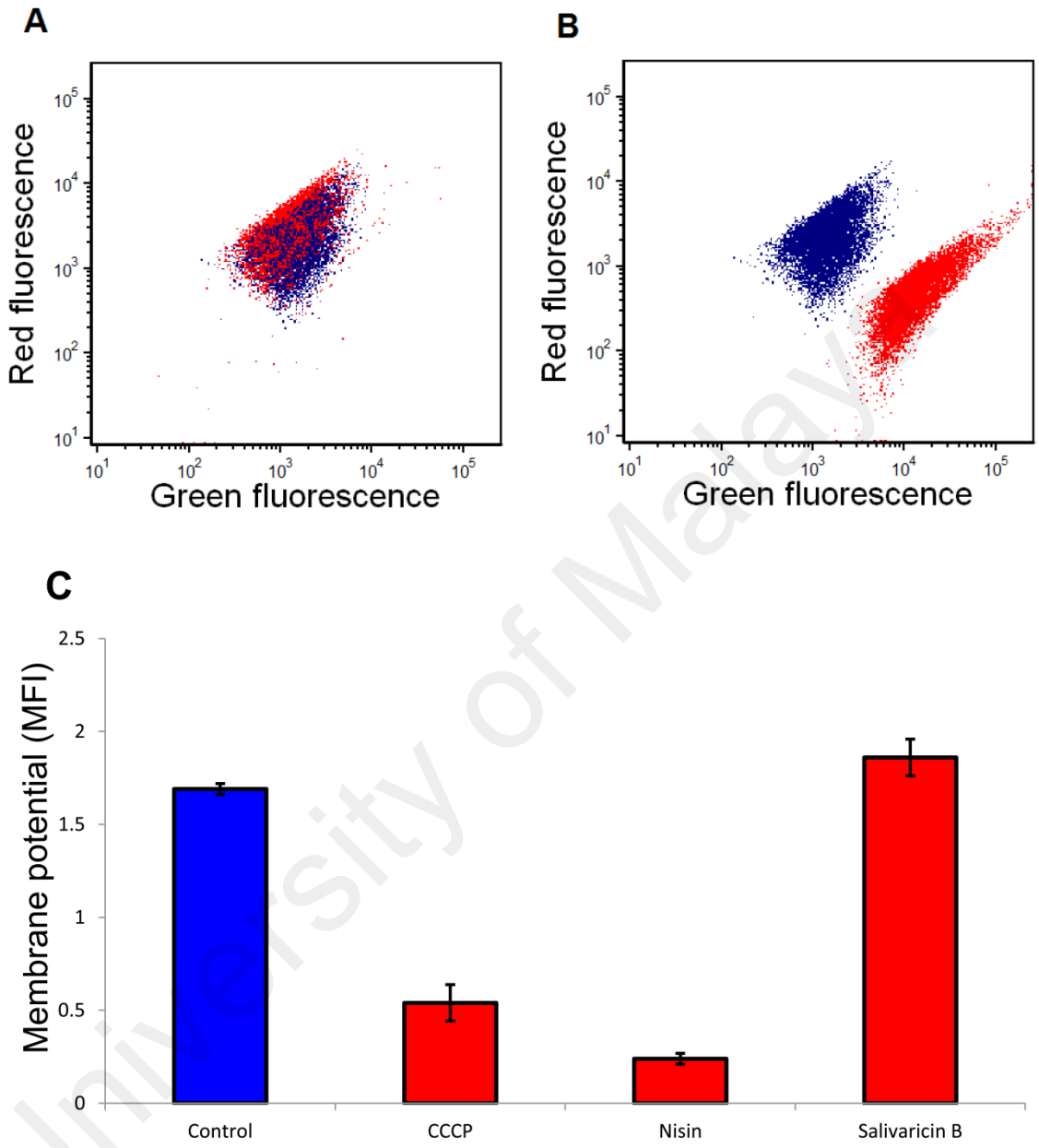

Figure 4.40: Cytometric profiles of *M. luteus* cells labeled with DiOC2(3). Blue dots represent healthy cells with no lantibiotic added. Red dots represent lantibiotic-treated cells. Comparison between healthy and salivaricin B treated cells (a). Comparison between healthy and nisin treated cells (b). Geometric means of fluorescence intensity of DiOC2(3) labeled *M. luteus* cells treated with different antimicrobials (c). Error bars represent the SD of the mean from three separate tests (n=3, P<0.05).

4.18 Accumulation of the final soluble cell wall precursor UDP-MurNAc-pp

In order to establish whether salivaricin B interferes with peptidoglycan biosynthesis, the cytoplasmic level of the cell wall precursor UDP-MurNAc-pentapeptide in *M. luteus*-treated cells was determined. Usually, accumulation of this cell wall precursor is induced by antibiotics such as vancomycin, which inhibit the late membrane-bond steps of cell wall biosynthesis. In this study, salivaricin B induced accumulation of cell wall precursors when compared to non-treated cells as revealed by reverse phase high performance liquid chromatography (RP-HPLC). Extracts of vancomycin-treated cells served as a positive control for this test. This study showed that like vancomycin, salivaricin B induces the accumulation of the final soluble cell wall precursor UDP-MurNAc-pentapeptide and ultimately interferes with peptidoglycan biosynthesis in susceptible cells as illustrated in Figure 4.41. The accumulated peak of the cell wall precursor was subjected to Orbitrap mass spectrometer for identification and showed to have a mass of 1148.31704 Da which is the typical mass of UDP-MurNAc-pentapeptide (Figure 4.42). It was noticed that in Figure 4.41C additional peaks (8-10 min) were present when the salivaricin B-treated cells were extracted with boiling water. This could be additional cytoplasmic materials accumulated when cells were exposed to salivaricin B. It was also noticed that when salivaricin B-treated *M. luteus* cells were extracted with water at boiling temperature after which there was a slight change in the biomass colour from yellow (typical of *M. luteus* cells) to light brown. Possibly excess peptide became embedded in the cell envelopes of those cells that had been exposed to the boiling water. As a result, the additional peaks (at 8-10 min) in Figure 4.41C could be fragments of denatured peptides extracted with the cells.

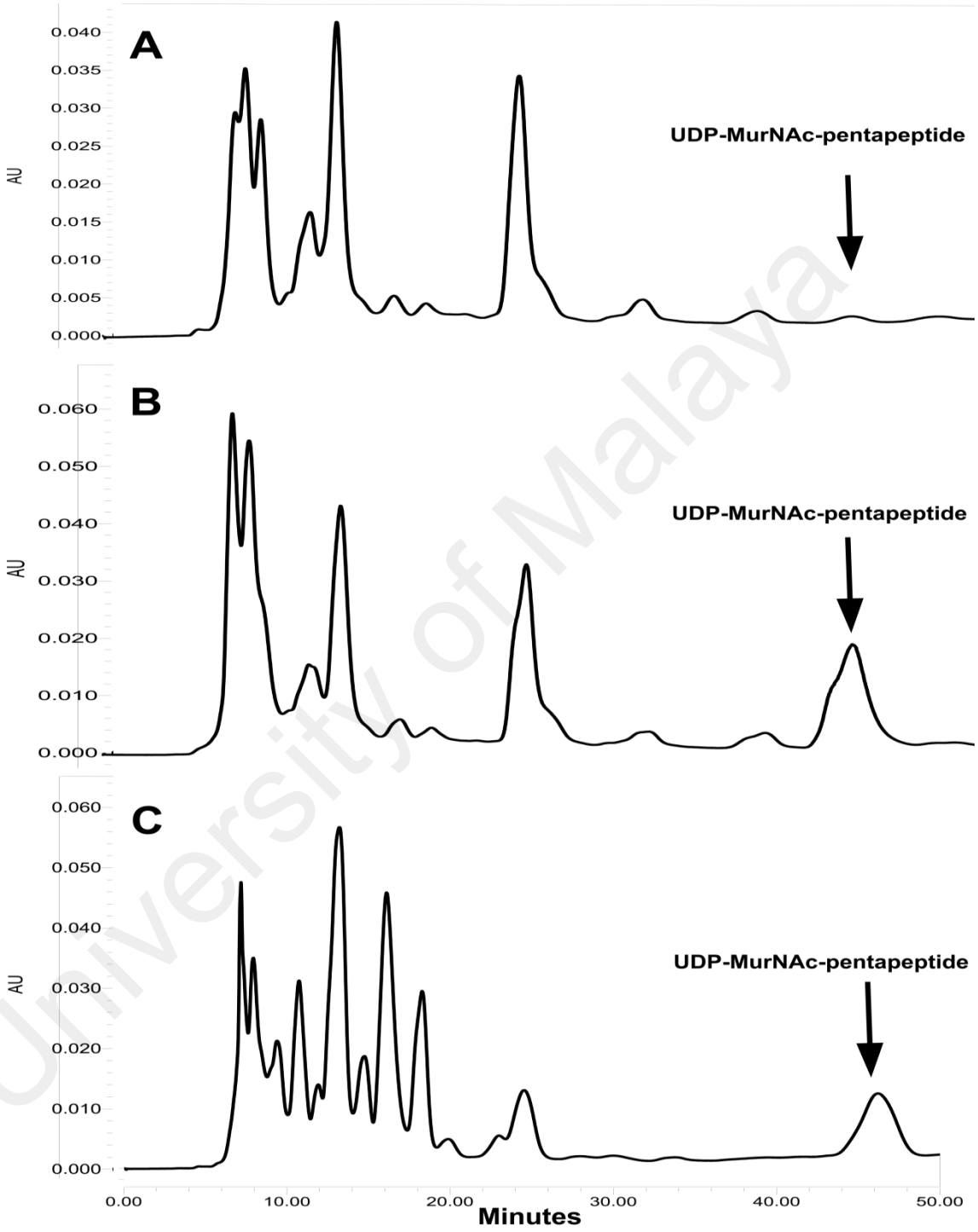

Figure 4.41: Intracellular accumulation of the final soluble cell wall precursor UDP-MurNAc-pentapeptide in *M. luteus* ATCC10240 exposed to salivaricin B. Cells were treated with vancomycin (10× MIC) (B) or salivaricin B (C), incubated for 60 min, and subsequently extracted with boiling water. The cytoplasmic pool of UDP-linked cell wall precursors was analysed by RP-HPLC. Bacterial culture with no antibiotic added served as a control (A).

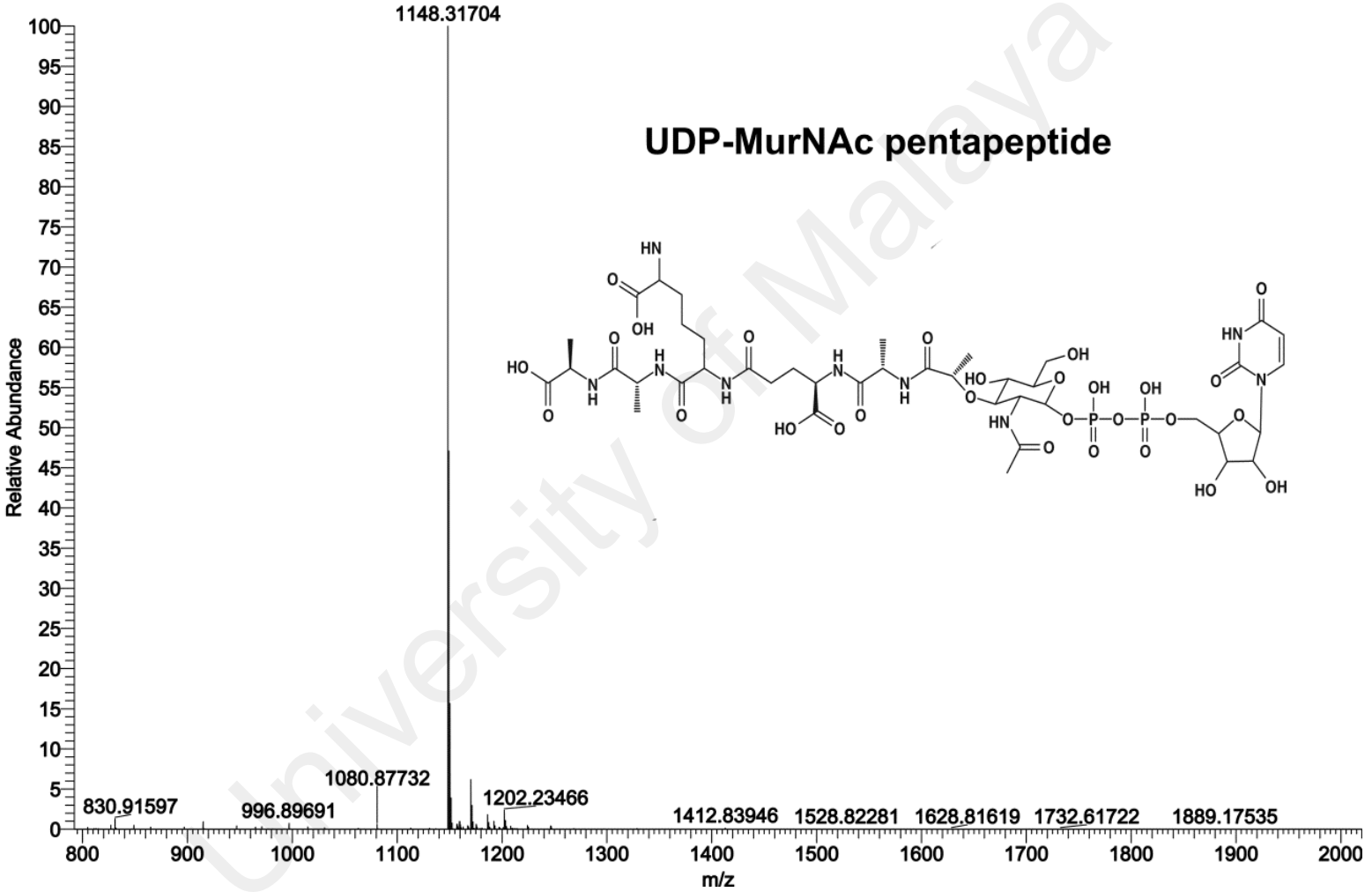

Figure 4.42: Identification of the accumulated UDP-MurNAc pentapeptide cell wall precursor with a molecular mass of 1148.31704 Da using Orbitrap mass spectrometer.

4.19 Ultrastructural modification and inhibition of cell wall biosynthesis

After 30 minutes of salivaricin B exposure, most of the *S. pyogenes* ATCC12344 cells did not show significant damage or ultrastructural changes when compared to untreated control cells except for some modifications in the polar sides of the cells which developed a sharper shape when compared to the smooth spherical shape of the control cells. However, less than 5% of the cells showed additional and aberrant dividing septa after 120 minutes of salivaricin B treatment. No sign of membrane damage or detachment of the cell wall from the cytoplasmic membrane was observed even after 120 minutes of salivaricin B treatment. However, some *S. pyogenes* cells showed thinning cell wall and partial lysis (Figure 4.43). Another indicator strain *M. luteus* ATCC10240 was tested and shown to be more susceptible to salivaricin B than *S. pyogenes*. *M. luteus* cells treated with salivaricin B showed aberrant division whereby the cells divided into many sections and expanded without losing membrane integrity, although the cell walls were drastically reduced in thickness (Figure 4.44). Compared with nisin A-treated cells, salivaricin B-treated *M. luteus* cells did not show any sign of membrane damage. On the contrary, immediately after the addition of nisin A, *M. luteus* cells started to get depolarized membranes due to the pore formation mechanism exhibited by nisin A as its principal mode of action (Christ *et al.*, 2007; Wiedemann *et al.*, 2004) (Figure 4.44). It was very clear that the inner cytoplasmic material of nisin A-treated cells oozed out of the cells when observed by TEM.

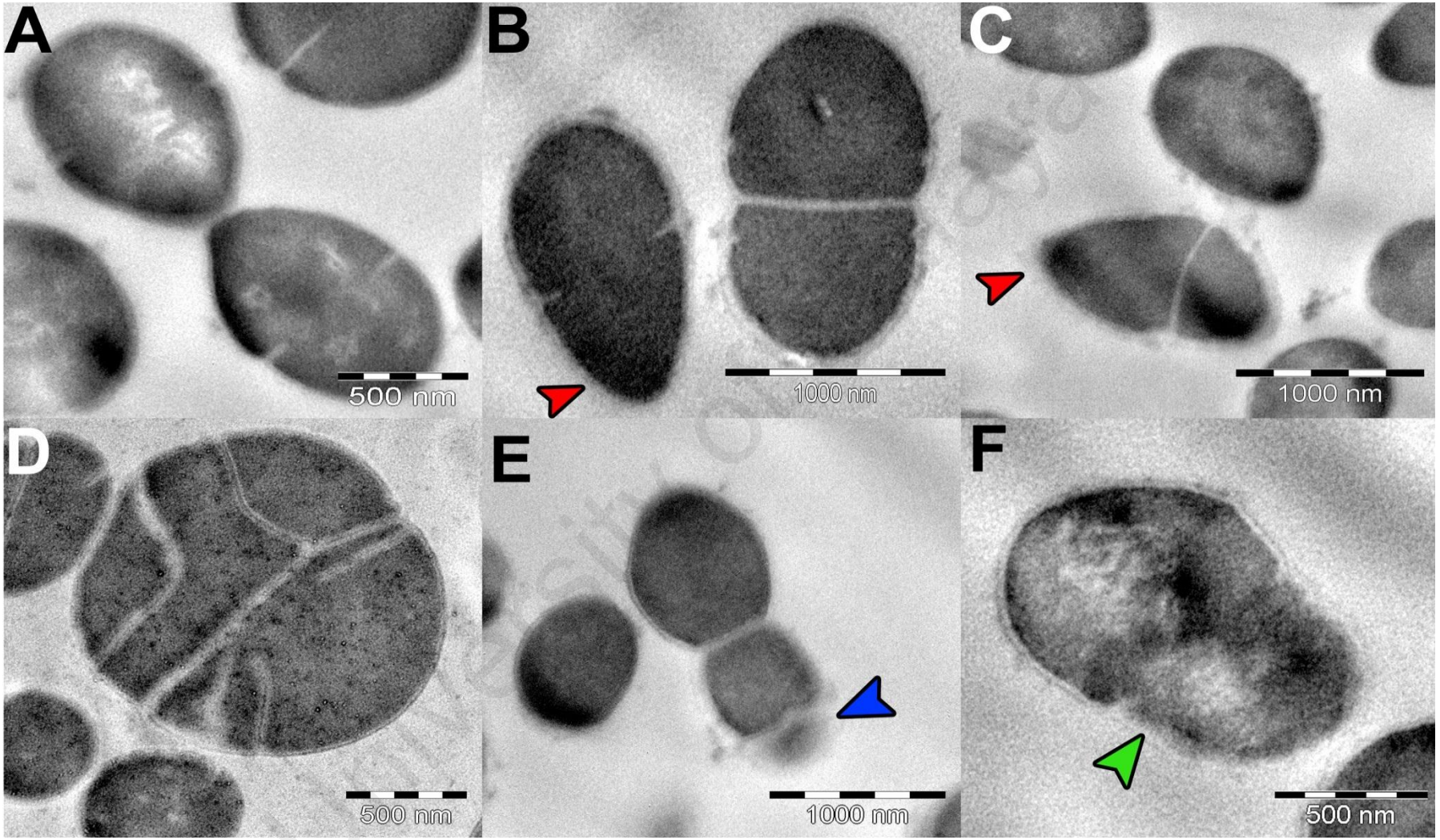

Figure 4.43: Ultrastructural effect of salivaricin B towards *S. pyogenes* cells. a: Control (untreated), b, c and d: Salivaricin B treated cells (30 minutes), e: salivaricin B treated cells (120 minutes), f: Nisin A treated cells. Red arrows indicate change in the typical spherical shape of *S. pyogenes* cells. Blue arrow indicates partial lysis of the cell. Green arrow indicates depolarized membrane due to nisin activity.

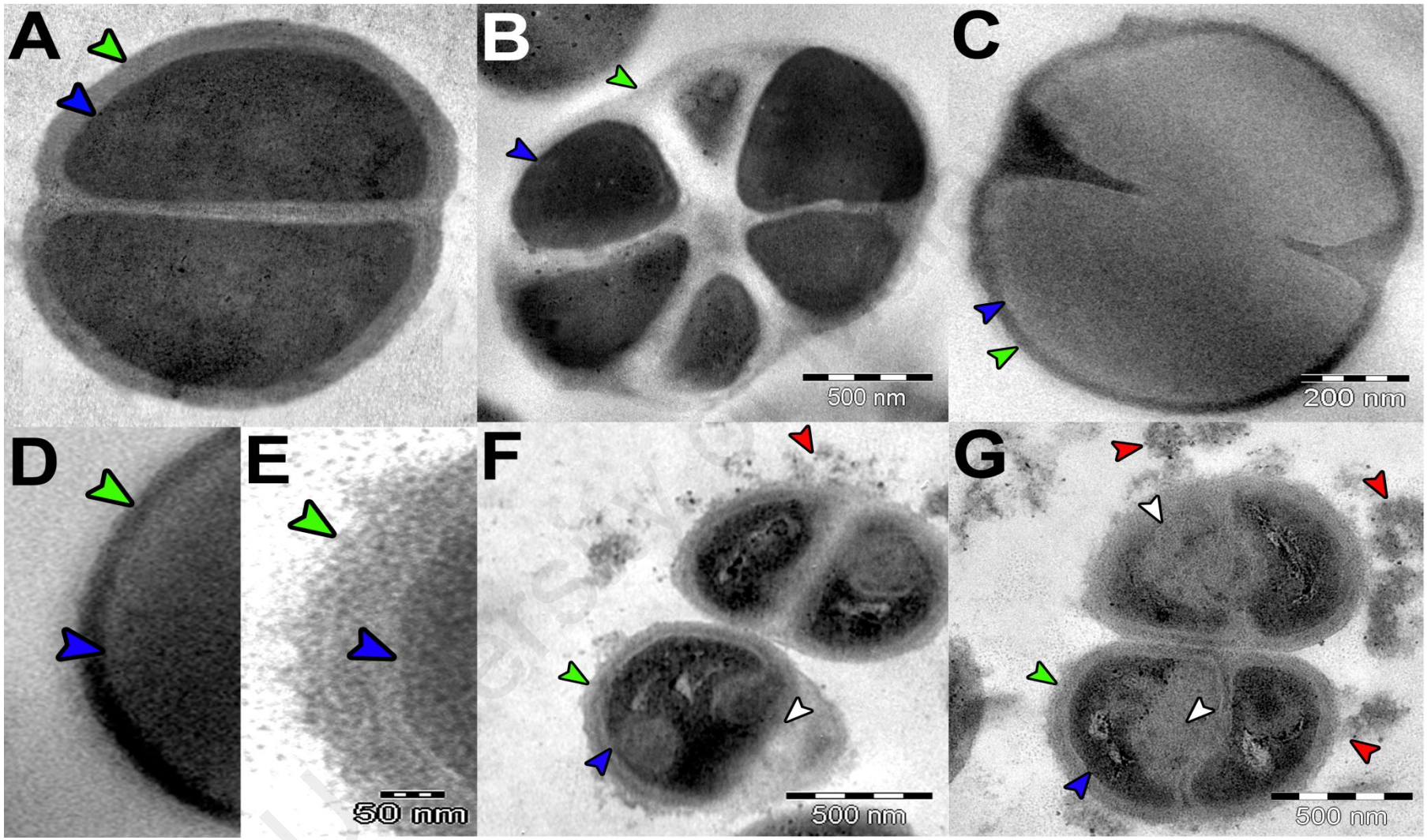

Figure 4.44: Ultrastructural effect of salivaricin B towards *M. luteus* cells. A: Control (untreated), B and C: Salivaricin B treated cells, D: Cell wall of salivaricin B treated cell, E: Cell wall of untreated cell, F and G: Nisin A treated cells. Green arrows indicate cell wall. Blue arrows indicate cell membrane. Red arrows indicate inner cytoplasmic materials oozed out of the cell. White arrows indicate membrane disruption.

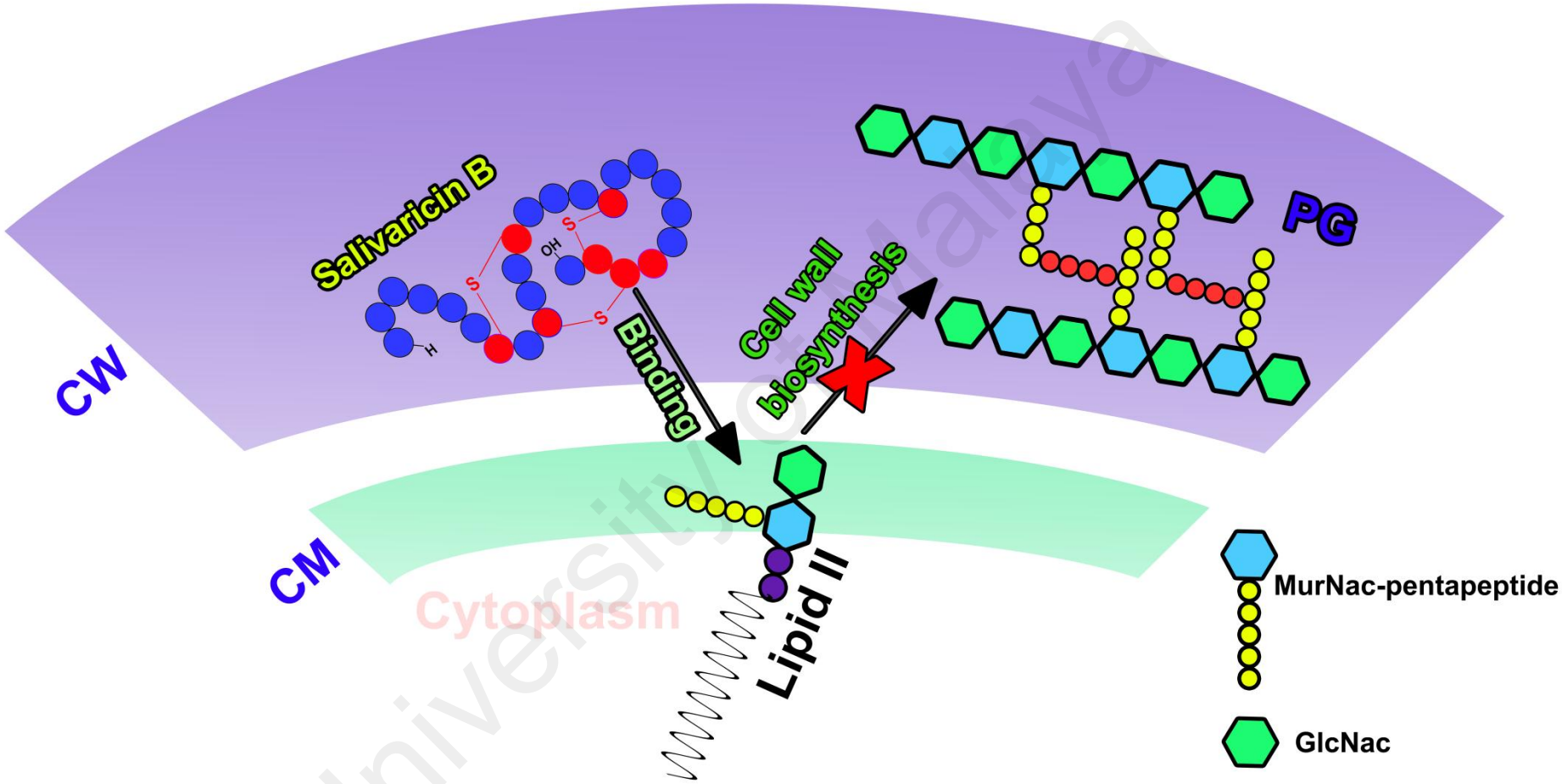

Figure 4.45: Mechanism of action of the lantibiotic salivaricin B. As a conclusion of the study, salivaricin B binds to the cytoplasmic membrane of the targeted bacteria through its lipid II binding motif. This lantibiotic-lipid II binding will result in accumulation of the final soluble cell wall precursor UDP-MurNac-pentapeptide in the bacterial cytoplasm without pore formation or alteration of the membrane integrity. CW: cell wall, CM: cell membrane, PG: peptidoglycan.

CHAPTER 5: DISCUSSION

5.1 Inhibitory activity of lantibiotic-producing *S. salivarius* isolates

S. salivarius is an important lantibiotic-producing bacterial species with a significant impact on human health including the oral cavity and the upper respiratory tract. Developing lantibiotic-producing probiotics is a very current and promising research area and a few *S. salivarius* strains are now established as probiotics. In this present study, lantibiotic-producing *S. salivarius* strains isolated from Malaysian subjects were evaluated and shown to produce different types of BLIS molecules some of which are lantibiotics (sal9 and salG32). A gene encoding a large peptide molecule salMPS (accession number: AGBV01000006) was also detected in some of the strains like GT2 and YU10 isolated during the current study.

Strains K12, NU10 and YU10 produced inhibitory activities when grown on different media including M17 (Difco), BACa, PTNYMES and other media mentioned in the results. On the other hand, strain GT2 failed to produce significant anti-*S. pyogenes* inhibitory activity when grown on media not supplemented with blood but produced significant inhibitory activity against *S. pyogenes* when grown on BACa. This indicated that the production of anti-*S. pyogenes* inhibitory activity by this strain was likely to be dependent on blood components. This characteristic is similar to salivaricin MPS-like peptide which is a large bacteriocin molecule (Wang, 2010). Further analysis showed that strain GT2 harboured the structural genes encoding for salivaricins MPS and MPS variant productions. Other *S. salivarius* strains were also isolated in this study to evaluate the persistence of lantibiotic determinants among *S. salivarius* isolated from Malaysian children in this study. Healthy subjects aged 5-7 years with no serious dental implications were selected for *S. salivarius* isolation. Evaluation of lantibiotic production by these strains using deferred antagonism assay on blood agar suggested

that only a few isolates could be lantibiotic-positive *S. salivarius*. Out of 100 strains isolated from healthy Malaysian children, only 28 showed to produce significant inhibitory activity towards other streptococcal indicator strains.

5.2 Safety assessment of lantibiotic-producing *S. salivarius*

The metabolic profiles, antibiograms and virulence determinants of strains NU10 and YU10 were investigated in this study to assess their potential to be used as probiotics. Both strains K12 and GT2 were also included in this study. All the *S. salivarius* strains tested showed stable metabolic profiles with some variations among the strains as shown in the Results section. Sub-cultured strains over 20 times showed almost identical metabolic profiles when compared to their original culture indicating stable characteristics of the fermentation pathways and phenotypic expression of metabolites of these strains. Moreover, the *β*-glucuronidase activity was negative for strains NU10, YU10, GT2 and K12 and none of the enzymatic reactions or fermentation characteristics of *S. salivarius* isolates were indicative of any deleterious effects for the human host. All *S. salivarius* strains tested in this study did not show any blood hemolytic activity when grown on blood agar media like BaCa. This finding added value to the safe use of *S. salivarius* strains as probiotics in human applications.

The presence of streptococcal virulence genes was first assessed in strains NU10, YU110 and K12 using specific primers of *S. pyogenes* virulence determinants. The PCR screening showed the absence of any of the five streptococcal virulence genes *smez-2*, *scpA*, *speB*, *saga* and *emm* in all *S. salivarius* strains tested. However, all of the five genes were found in *S. pyogenes* ATCC12344 used as a control for this assessment. To extend the safety profile strains, NU10 and YU10 were subjected to whole genome sequencing and both showed to be free of any of the 34 streptococcal virulence genes as mentioned in the results section. This was a strong indication that both strains can be

potential probiotic candidates for safe human consumption. Both genomes of NU10 and YU10 showed to harbour *sbcD* gene since pathogenic streptococcal species lack this gene. This gene's product(s) helps to stabilize the genome effectively by reducing the efficiency of recombination (Bolotin *et al.*, 2004; Burton *et al.*, 2006). *S. salivarius* strains tested in this study showed stable antibiogram after long-term storage or subsequent sub-culturing which indicates stable genomic characteristics. Hence, these strains namely NU10 and YU10 are suitable as probiotics and both showed to be susceptible to conventional antibiotics routinely used to treat upper respiratory tract infections.

5.3 Genomic analysis of lantibiotics loci

Strain NU10 was shown to harbour structural genes encoding salivaricins A and 9 previously but only sal9 could be produced and detected as an active peptide in the present study. Strain YU10 was shown to harbour genes encoding salivaricins A3, G32, streptin and slnA1 lantibiotic-like protein. However, only salG32 was detected and recovered from this strain.

Genome sequencing of strains YU10 and NU10 indicated the presence of megaplasmids designated as pSsal-YU10 and pSsal-NU10 with the size of 148,112 bp and 190,254 bp respectively. Similar megaplasmids were found previously in lantibiotic-producing *S. salivarius* strains. For example, strain K12 was shown to harbour both SalA2 and SalB loci on 190 kb megaplasmid. Plasmid curing treatment of this strain abolished lantibiotics production (Wescombe *et al.*, 2006). Megaplasmids of the same size (190 kb) were also found in *S. salivarius* strains P, G39 and T18A. The same size of megaplasmid was also found in strain NU10 in the current study. However, pSsal-YU10 of size ≈ 148 kb reported in the present study is close in size to the megaplasmids of strains CHR and PAM with both having a size of 150 kb as reported

previously (Wescombe *et al.*, 2006). The pSsal-YU10 megaplasmid was shown in this study to harbour lantibiotic loci of SalA3, SalG32 and streptine. Strain JH reported previously was shown to harbour the same lantibiotic loci like strain YU10 in addition to anti-*S. mutans* lantibiotic salivaricin E reported recently in strain JH (Walker *et al.*, 2016).

In the current study, the full sequencing of SalG32 locus was done for the first time Figure 4.11. This lantibiotic is a homologue of the lantibiotic Streptococcin A-FF22 produced by *S. pyogenes* (Hynes *et al*., 1993) with one difference (absence of lysine in position 2) (Wescombe *et al.*, 2012). In this study, SalG32 locus was found to be distributed among other streptococci such as *S. pyogenes, S. mutans, S. agalactiae, S. dysgalactiae, S. macedonicus* and *S. vestibularis*. Since SalG32 locus is encoded on megaplasmid pSsal-YU10, it is likely that this locus is transferable among different streptococcal species. This study also showed that SalG32 structural gene *slnA* was found in 34% of lantibiotic-positive *S. salivarius* isolates from healthy Malaysian children. Previous study has shown that the *slnA* gene was found in 6 out of 26 *S. salivarius* strains (23%) (Wescombe *et al.*, 2012).

Apparently, lantibiotics loci identified in this study in both strains YU10 and NU10 showed to be megaplasmid borne. The full biosynthesis loci of lantibiotics Sal9, SalA, SalG32 and streptin were fully characterised and analysed in this study. However, only Sal9 and SalG32 were expressed by strains NU10 and YU10 respectively. No SalA activity was detected and this is an indication of a defect in the locus in both NU10 and YU10. It was reported previously that SalA locus although can be found in many streptococci, however, it can be often defective (Hyink *et al.*, 2007; Wescombe *et al.*, 2006). The defect can be either in the structural gene itself, the modification enzyme or the lantibiotic ABC transporter. It was suggested previously that defect in the

modification enzyme can lead to incorrectly processed and cyclized lantibiotic lacking biological activity (Sahl and Bierbaum, 1998; Ross and Vederas, 2011). Mutated lantibiotic transporters also resulted in negative lantibiotic activity and accumulation of the inactive pre-pro peptide in the cytoplasm of the producer cells (Sahl and Bierbaum, 1998). The lantibiotic streptin is originally produced by *S. pyogenes* (Wescombe and Tagg, 2003). However, streptin biosynthesis locus was detected in other streptococcal strains including strains of *S. salivarius* (Wescombe *et al.*, 2006). In the current study, streptin locus was detected in strain YU10. However, no streptin was produced or detected by this strain. Streptin structural gene *srtA* was found only in 6% of lantibiotic-positive *S. salivarius* strains isolated in this study from healthy Malaysian children.

5.4 Development of a lantibiotic production medium

The strains in this study showed some variations in their metabolic profiles. Surprisingly, strain NU10 was negative towards lactose fermentation. When the strain was propagated in growth medium containing lactose as the only carbon source, it showed significantly weaker growth and total absence of any lantibiotic production. A previous study (Barbour *et al.*, 2013) demonstrated that this strain is a producer of salivaricin 9. The maximum yield of lantibiotic activities was recovered when sucrose was used as the carbon source.

The use of commercial media including THB and BHI in aerobic condition resulted in a drop of OD_{600} reading that is apparently attributed to microbial cell lysis. The reason for this lysis in aerobic condition is still unknown, and perhaps the aerobic condition is not ideal for strain K12 and other *S. salivarius* isolates when THB or BHI media are used for propagation. In the current study, a newly developed medium buffered with MES helped to enhance the biomass and bacteriocin production by *S. salivarius* which grew well in an aerobic atmosphere. This finding can solve the

problem of scaling up the culture in large scale bioreactors for probiotic and/or lantibiotic production. Previous study showed that buffering the medium with MES helped to achieve higher biomass levels of *Streptococcus thermophilus* (Somkuti and Gilbreth, 2007). Using organic buffers for bacteriocin production helps to prevent an extreme drop in medium pH due to the production of lactic acid or other substances.

It has been noticed that 60-70% of the bacteriocins recovered in this study were cell-wall associated peptides bound to the producer cells while the rest of the inhibitory peptides were secreted extracellularly into the liquid media. Cell-associated bacteriocins produced by lactic acid bacteria had been reported previously (Mantovani *et al.*, 2002; Tahara and Kanatani, 1997). Hence, this class of bacteriocins can be recovered from producer cells grown in liquid media.

Most lantibiotics appear to be regulated at the transcriptional level in a cell-density-dependent manner in various bacteria (Kleerebezem, 2004). The mode of regulation for lantibiotic production has been shown to involve secreted peptides that act as communication molecules accumulated in the environment during growth. When certain concentrations of these molecules are reached, high level of lantibiotic production is triggered (Kleerebezem, 2004). A previous study demonstrated that the lantibiotic produced by strain NU10 is auto-regulated and the same lantibiotic could induce its production by strain NU10 (Barbour *et al.*, 2013). However, strain NU10 was also shown to encode structural genes for salivaricins A and 9. But it was evident that when an enhanced culture of strain NU10 was analysed using MALDI-TOF MS, salivaricin 9 was the only lantibiotic detected from the purified supernatant. Hence, we can conclude that the presence of structural genes encoding production of salivaricins in *S. salivarius* strains does not necessarily mean that the bioactive molecule is expressed or that the PTNYSMES medium used for the production in aerobic condition did not support the

biosynthesis of that particular peptide. Strain YU10 was shown to produce SalG32 while no salivaricins A, 9 or streptin production was detected. Previous work showed that in contrast to the regulation of sal9, the signal of up-regulation of salivaricin G32 is not the antimicrobial peptide itself but rather some other substances produced by the lantibiotic producer (Wescombe *et al.*, 2012).

Bacteriocins and lantibiotics are not the only unique and useful molecules being produced by the strains described in this study. When sucrose was added to the medium as the only source of carbon, levansucrase enzyme was produced in significant levels. Levan-sucrase (fructosyltransferase) is a unique cell-bound enzyme produced by *S. salivarius* and it plays a major role in the production of levan residues. Levan has been shown to have prebiotic effects and so this production, together with the production of lantibiotics, makes the strain potentially useful for multiple applications. The method described in this study for direct detection of levansucrase from the cell-extract using LC-MS/MS was efficient to detect levansucrase in *S. salivarius* and the full characterization of the gene encoding levansucrase production was elucidated using genome sequencing of the producer strain.

5.5 Developing peptide-membrane interaction model using the intrinsic fluorescence of tryptophan

To understand the mechanism by which small antimicrobial peptides bind and penetrate the membrane vesicles of targeted bacterial strains, a model based on the small cationic peptide cecropin B was developed. The original peptide (cecropin B1) was modified by attaching one tryptophan residue at its C-terminus (cecropin B2). Both peptides were studied to understand the binding mechanism to negatively charged phospholipids of targeted bacterial membrane. Later on, the same model was applied to investigate the binding mechanism of the lantibiotic salivaricin B to the bacterial membrane vesicles since this lantibiotic has one tryptophan residue at position 17. Nisin A was not used in this developed model because it lacks any tryptophan residue.

Boosting bactericidal potency was achieved through end-tagging of antimicrobial peptides with hydrophobic oligopeptides (Schmidtchen *et al.*, 2009). A larger effect was reported for Trp and Phe sequences than for aliphatic ones. The enhancement of bactericidal effects was correlated to a higher degree of bacterial wall rupture. Linking more than one Trp residues (up to five units) to the C-terminal end of some peptides shows substantial peptide adsorption, membrane lysis and bactericidal effect (Strömstedt *et al.*, 2010). It was observed that increasing the peptide helicity correlates with increasing antimicrobial potency (Shalev *et al.*, 2002). The effect of Trp substitutions was demonstrated to increase the peptide's helicity and amphiphilicity thus resulting in higher peptide adsorption, increased peptide-induced liposome leakage and antimicrobial potency (Strömstedt *et al.*, 2010; Schmidtchen *et al.*, 2011).

The results of the current study indicated that cecropin B1 showed a slight enhancement in MIC and MBC values as indicated in Table 4.11 and Table 4.12, respectively. Adding a second Trp to the opposite end of the peptide did not show any

induced enhancement in such activities. This was in line with the fluorescence results which indicated that the Trp residue in cecropin B1 was interacting with the bacterial membrane whereas the second Trp residue in cecropin B2 was not in direct contact with the membrane. The similar blue shift in the fluorescence peak observed for both cecropin peptides pointed to a vertical penetration from the Lys side in which the terminal Trp in cecropin B2 was expected to stay far from the membrane.

It was shown in a previous study that elimination of the peptide positive net charge drastically reduced the bactericidal effect on *P. aeruginosa* (Ringstad *et al.,* 2007). However, short analogues of cecropin B presented in this study with slightly reduced net charge (Table 3.2) retained the bactericidal activity against the proposed pathogen namely *P. aeruginosa*.

A possible mechanism of membrane disruption is thus shown in Figure 4.36 whereby unfolded cationic cecropins tended to create cracks through the negatively charged outer membrane of *P. aeruginosa* by neutralizing the charge over a patch of the outer membrane. The cecropins can directly attach to the cation binding sites on the lipopolysaccharides and depolarize the membrane. After transiting through the outer membrane, cecropins bind to the negatively charged phospholipids where these cationic peptides fold to their amphipathic structure. After insertion into the membrane interface, the cationic cecropins will either aggregate into a micelle-like complex and span across the membrane or flip-flop across the membrane driven by the transmembrane electrical potential gradient (approximately – 140 mV) (Hancock and Chapple, 1999). This caused a huge leakage of the ions and other water-soluble molecules, thereby compromising the cell integrity.

5.6 Elucidating the mechanism of action of the lantibiotic salivaricin B

Generally, lantibiotics exert their inhibitory activity against the targeted bacterial cells by disturbing the integrity of the cytoplasmic membrane through pore formation (Ruhr and Sahl, 1985; Moll *et al.*, 1996; van Kraaij *et al.*, 1998). This mechanism is usually facilitated by using the peptidoglycan precursor lipid II as a docking site (Wiedemann *et al.*, 2001; Wiedemann *et al.*, 2006). However, the mechanisms of action of many lantibiotics belonging to different subclasses are largely unexplored. Salivaricin B, a class AII lantibiotic produced by *S. salivarius,* was shown in this study to exhibit a different mode of action than the typical pore formation. It is usually considered that lantibiotics having a bactericidal mode of action will interfere with the cytoplasmic membrane integrity of susceptible cells. However, lacticin 481 was shown previously to interfere with peptidoglycan biosynthesis by inhibiting transglycosylation without forming pores in the membranes of susceptible bacteria (Knerr *et al.*, 2012).

Like lacticin 481, many other lantibiotics belonging to the same group e.g. nukacin ISK-1, mutacin II and streptococcin SA-FF22 contain a lipid II binding motif (TXS/TXD/EC) present in mersacidin (class B lantibiotic) where X can be any residue. Salivaricin B also has this motif at its ring A which makes lipid II its likely target. Previously it was reported that nukacin ISK-1 has a bacteriostatic mode of action towards *B. subtilis* JCM 1465^{T} cells without causing pore formation or membrane potential dissipation (Asaduzzaman *et al.*, 2009). However, it was found that nukacin ISK-1 could also exhibit a bactericidal mode of action with pore formation ability when tested against *M. luteus* DSM 1790 and *Staphylococcus simulans* 22 (Roy *et al.*, 2014). Mutacin II was shown to possess a bactericidal mode of action by partially depolarizing the transmembrane electrical potential ($\Delta\Psi$) which then recovered shortly after *S. sanguis* Ny101 cells were treated with mutacin II (Chikindas *et al.*, 1995).

It was shown previously that streptococcin SA-FF22 could form relatively unstable, short-lived pores of diameter approximately 0.5-0.6 nm and dissipate the membrane potential with 100mV as a minimum requirement for pore formation (Jack *et al.*, 1994). By using a flow cytometric approach, the current study has shown that salivaricin B was not able to induce pore formation or dissipate the cytoplasmic membrane potential of its targeted cells, exhibiting similar behavior to lacticin 481. This was accomplished with no evidence of short-lived pores or temporary dissipation of the membrane potential. Salivaricin B, mutacin II, streptococcin SA-FF22 and lacticin 481 have one tryptophan residue at positions 17, 20, 18 and 19, respectively. Using spectrofluorometric analysis, it was shown previously that adding artificial phospholipid vesicles to SA-FF22 in aqueous solution shifted the maximum fluorescence of the single tryptophan at position 18 from 352 nm to 337 nm (blue shift) indicating binding of SA-FF22 to the membrane vesicles (Jack *et al.*, 1994). However, no changes in the tryptophan fluorescence spectra of lacticin 481 were observed using the same approach, indicating that the tryptophan residue of lacticin 481 at position 19 played no role in the interaction with cell wall precursor and vesicle membranes (Bottiger *et al.*, 2009). In this study, a new peptide-membrane binding model was developed using synthetic cationic peptides and bacterial vesicles to detect a shift of the maximum fluorescence of the Trp residue. The same peptide-membrane binding model has been used to track the change in the fluorescence of the tryptophan residue of salivaricin B at position 17. The results showed that salivaricin B did not penetrate bacterial membranes, as no typical blue shift in the tryptophan fluorescence was observed, even when different membrane vesicles isolated from *B. cereus* or *E. coli* were used. Salivaricin B was shown in this study to induce intracellular accumulation of the final soluble cell wall precursor UDP-MurNAc-Pentapeptide and ultimately inhibits cell wall biosynthesis Figure 4.45. UDP-MurNAc-pentapeptide accumulation is usually induced by antibiotics inhibiting the late,

membrane-bound steps of cell wall biosynthesis e.g. vancomycin (Reynolds, 1961), bacitracin (Siewert and Strominger, 1967) and ramoplanin (Somner and Reynolds, 1990). Mersacidin and nukacin ISK-1 lantibiotics were also shown to inhibit the same cell wall precursor in *S. simulans* 22 cells (Brötz *et al.,* 1997; Islam *et al.,* 2012).

In this study, salivaricin B-treated *M. luteus* cells showed a significant reduction in cell wall thickness. This phenomenon was also previously observed in nukacin ISK-1-treated *B. cereus* cells and mersacidin-treated *S. simulans* cells (Brotz *et al.,* 1995; Asaduzzaman *et al.,* 2009). Cell wall thinning can be attributed to interference with peptidoglycan biosynthesis resulting in failure to form adequate amounts of the final peptidoglycan chains. Additional and aberrant septum formation was evident in salivaricin B-treated cells and this deregulation and defection of the cell envelopes may lead to a failure to generate daughter cells. The bacterial cell wall is the osmotic barrier to the external environment and is a structural feature of cells, determining their shapes. The loss of shape in salivaricin B treated cells of both *S. pyogenes* and *M. luteus* strongly suggests that these cells lack functional cell walls. This mode of action is apparently irreversible, as salivaricin B activity was shown to be bactericidal against its targeted cells. Only partial lysis of *S. pyogenes* cells was observed after exposure to salivaricin B for 24 hours. Salivaricin B is the first member of the lacticin 481 lantibiotic group shown to cause aberrant septum formation in its targeted bacterial cells without also affecting membrane integrity. Salivaricin B has a functional ABC transporter system (SboFEG) providing immunity and self-protection to the producer cells against the bactericidal action of the lantibiotic. This LanFEG system is very common in lantibiotics, but cross immunity is exceedingly rare among lantibiotic producers (Draper *et al.,* 2015). Although Lipid II is an essential target for many lantibiotics, however, it was suggested previously that the lantibiotic resistance or sensitivity are independent of lipid II levels (Kramer *et al.,* 2004). Moreover, the three-

dimensional structure of a nisin resistance protein (SaNSR) has just been reported recently (Khosa *et al.*, 2016).

It was reported recently that some non-lantibiotic-producing bacteria were shown to possess genes similar to the lantibiotic immunity systems. For example, genes encoding immunity homologues (*spiFEG*) have been found in *Streptococcus infantarius* subsp. *infantarius* BAA-102 with >50% homogeneity to that encoded in nisin U operon. Heterologous expression of these genes in lantibiotic sensitive *strain L. lactis subsp. cremoris* HP confers resistance to nisin U and other members of the nisin family (Draper *et al.*, 2012). Interestingly, the *sboFEG* immunity genes of salivaricin B were found in some *S. pyogenes* strains with 40-60% identity. However, no salivaricin B resistant *S. pyogenes* strains have been reported previously. So far no significant resistance to salivaricin B was reported. Nevertheless, *Solobacterium moorei* CCUG39336 showed an insignificant decrease in sensitivity to *S. salivarius K12* (producer of salivaricins A2 and B) when it was tested in antagonism assay over ten passages (Masdea *et al.*, 2012).

Further work on salivaricin B immunity and resistance may provide vital insights into the emerging evolution of bacterial strains which develop new strategies to avoid elimination due to lantibiotics in the oral cavity.

CHAPTER 6: CONCLUSION

In conclusion, *S. salivarius* strains evaluated in this study showed variations in the type of inhibitory substances produced some of which are lantibiotics sal9 and salG32 produced by strains NU10 and YU10, respectively, while a gene encoding a large bacteriocin molecule salMPS was detected in strains GT2 and YU10. No significant variations in antibiotic susceptibility among the *S. salivarius* isolates were observed after two years of storage (freeze dried stocks) indicating stability of the strains especially in terms of susceptibility towards antibiotics. The metabolic profile studies showed some variations among the tested strains and gave relevant information on the biochemical criteria required by each strain to perform better during fermentation studies. The *in vitro* safety assessment tests showed that the strains are free of virulence genes known to be present in streptococcal pathogens and this finding was supported by genome sequencing of strains NU10 and YU10.

Strains NU10 and YU10 produce sal9 and salG32 lantibiotics, respectively, which are well distinguished from the well characterised *S. salivarius* probiotic strain K12 producing the lantibiotics SalA and SalB. These characteristic differences provide additional options for probiotics use in oral health management with different available lantibiotic molecules.

The developed medium PTNYSMES helped to enhance biomass accumulation of all strains and attempts to recover lantibiotics produced by *S. salivarius* grown in this medium aerobically were successful. A new method for levansucrase detection was also developed and gene encoding levansucrase production was characterised. The ability of *S. salivarius* to produce lantibiotics and levansucrase adds value to this microorganism with dual benefits for probiotic development with prebiotic effects.

The variety of bacteriocins produced by *S. salivarius* isolated from Malaysian subjects makes it interesting to study these molecules, their distribution and prevalence within the Malaysian population. High-throughput genome sequencing of both strains NU10 and YU10 using Illumina's MiSeq genome sequencing confirmed the absence of the streptococcal virulence determinants within both genomes. This finding suggested that some of these strains are promising for development as probiotics as they pass the same initial safety assessments described previously for *S. salivarius* strain K12 (Burton *et al.*, 2006).

In this study, the interaction of synthetic antimicrobial peptides with bacterial membrane vesicles has been investigated using fluorescence measurements to detect the change in the fluorescence signal of the intrinsic Trp residue in the peptide chain. The results indicated perpendicular penetration of the peptides into the membrane from the Lys side. A degree of flexibility inside the membrane was observed in the form of two fluorescence decay components for the Trp residue in which the cecropin molecule can adapt in two different rotamers that represent two phases of the membrane.

This advanced peptide-membrane binding model was applied on the lantibiotic salivaricin B to elucidate the mechanism of interaction by measuring the fluorescence of the tryptophan residue at position 17. As a result, salivaricin B interacted with the bacterial membrane vesicles but failed to penetrate the membranes.

The killing action of salivaricin B required micro-molar concentrations of the lantibiotic whereas the prototype lantibiotic nisin A was shown to be potent at nano-molar levels. Unlike nisin A, salivaricin B did not induce pore formation or dissipate the membrane potential in susceptible cells. On the other hand, salivaricin B interfered with cell wall biosynthesis, as shown by the accumulation of the final soluble cell wall precursor UDP-MurNAc-pentapeptide which forms the backbone of the bacterial

peptidoglycan. Transmission electron microscopy of salivaricin B-treated cells also showed a reduction in cell wall thickness together with signs of aberrant septum formation in the absence of visible changes to cytoplasmic membrane integrity.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

Research articles:

- **Barbour A**, Philip K (2014) Variable Characteristics of Bacteriocin-Producing *Streptococcus salivarius* Strains Isolated from Malaysian Subjects. ***PLoS ONE,*** 9(6): e100541. doi: 10.1371/journal.pone.0100541.

- Abou-Zied, O. K., **Barbour, A.**, Al-Sharji, N. A., & Philip, K. (2015). Elucidating the mechanism of peptide interaction with membranes using the intrinsic fluorescence of tryptophan: Perpendicular penetration of cecropin b-like peptides into *Pseudomonas aeruginosa*. ***RSC Advances***, 5(19), 14214-14220.

- **Barbour, A.,** Tagg, J., Abou-Zied, O. K., & Philip, K. (2016). New insights into the mode of action of the lantibiotic salivaricin B. ***Scientific Reports***, 6, 31749; doi: 10.1038/srep31749 (2016).

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- Philip, K., & **Barbour, A.** (2015). Salivaricin 9 lantibiotic and method to produce thereof. Google Patents, WO2015187000 A1.

Seminar:

- Venue: Faculty of Science, ISB, Unit of Genetics and Molecular Biology, Seminar Room B6, 10 March 2016. Semester 2, Session 2015/2016. Title: Characterisation and Mechanism of Action of Lantibiotics Produced by *Streptococcus saliva*rius.

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Variable Characteristics of Bacteriocin-Producing *Streptococcus salivarius* Strains Isolated from Malaysian Subjects

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Abstract

Background: Salivaricins are bacteriocins produced by *Streptococcus salivarius*, some strains of which can have significant probiotic effects. *S. salivarius* strains were isolated from Malaysian subjects showing variable antimicrobial activity, metabolic profile, antibiotic susceptibility and lantibiotic production.

Methodology/Principal Findings: In this study we report new *S. salivarius* strains isolated from Malaysian subjects with potential as probiotics. Safety assessment of these strains included their antibiotic susceptibility and metabolic profiles. Genome sequencing using Illumina's MiSeq system was performed for both strains NU10 and YU10 and demonstrating the absence of any known streptococcal virulence determinants indicating that these strains are safe for subsequent use as probiotics. Strain NU10 was found to harbour genes encoding salivaricins A and 9 while strain YU10 was shown to harbour genes encoding salivaricins A3, G32, streptin and slnA1 lantibiotic-like protein. Strain GT2 was shown to harbour genes encoding a large non-lantibiotic bacteriocin (salivaricin-MPS). A new medium for maximum biomass production buffered with 2-(N-morpholino)ethanesulfonic acid (MES) was developed and showed better biomass accumulation compared with other commercial media. Furthermore, we extracted and purified salivaricin 9 (by strain NU10) and salivaricin G32 (by strain YU10) from *S. salivarius* cells grown aerobically in this medium. In addition to bacteriocin production, *S. salivarius* strains produced levan-sucrase which was detected by a specific ESI-LC-MS/MS method which indicates additional health benefits from the developed strains.

Conclusion: The current study established the bacteriocin, levan-sucrase production and basic safety features of *S. salivarius* strains isolated from healthy Malaysian subjects demonstrating their potential for use as probiotics. A new bacteriocin-production medium was developed with potential scale up application for pharmaceuticals and probiotics from *S. salivarius* generating different lantibiotics. This is relevant for the clinical management of oral cavity and upper respiratory tract in the human population.

Citation: Barbour A, Philip K (2014) Variable Characteristics of Bacteriocin-Producing *Streptococcus salivarius* Strains Isolated from Malaysian Subjects. PLoS ONE 9(6): e100541. doi:10.1371/journal.pone.0100541

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Introduction

Bacteriocin or bacteriocin-like inhibitory substances (BLIS) are peptide molecules produced by Gram-positive bacteria and some genera of Gram negative bacteria [1–3]. Lactic acid bacteria are generally considered to be non-pathogenic (with some exceptions such as *Streptococcus mutans* which causes dental caries) and can produce different kinds of bacteriocins such as nisin produced by *Lactococcus lactis* [4–7], plantaricins produced by *Lactobacillus plantarum* [8–10], mutacins produced by *Streptococcus mutans* [11–15] and salivaricins produced by *Streptococcus salivarius* [16–20]. *S. salivarius* is a species of lactic acid bacteria colonizing the human oral cavity [21].

Some strains of *S. salivarius* such as strains K12 and M18 are now being used as probiotics worldwide due to their capability to produce different kinds of bacteriocins called lantibiotics [18,22,23]. Lantibiotics are heat stable ribosomally synthesized small molecules produced by some strains of gram positive bacteria with therapeutic potential in treating infectious diseases [24–29].

To compete better in the oral ecosystem, *S. salivarius* produce different kinds of lantibiotics such as salivaricin A, salivaricin B, salivaricin 9 and salivaricin G32 [16–18,20]. It has been noticed that bacteriocin or BLIS molecules are not the only useful metabolites produced by *S. salivarius*. Levan-sucrase is one of the important molecules secreted by *S. salivarius* [30]. Levan-sucrase or fructosyltransferase (FTF) attack the fructose moiety of sucrose and polymerize it into fructans which possess levan structure. Levan is a homo-polysaccharide, non-mutagenic, non-toxic, soluble dietary fiber with significant prebiotic effects through stimulating the growth and activity of selected probiotic bacteria in the colon which can improve the host's health [31]. Levan may also

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Elucidating the mechanism of peptide interaction with membranes using the intrinsic fluorescence of tryptophan: perpendicular penetration of cecropin B-like peptides into *Pseudomonas aeruginosa*

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The importance of small molecular weight antimicrobial peptides as novel therapeutic agents stems from their ability to act against bacteria, viruses, and fungi. As part of the innate immune system, they are also capable of killing cancerous cells. Herein, we study the interaction between a synthetic cecropin B peptide and a target *Pseudomonas aeruginosa* (PA) membrane using steady-state and time-resolved fluorescence measurements in order to elucidate the mechanism of membrane rupture. The importance of synthetic cecropin B as a therapeutic peptide stems from its effect against a wide range of bacteria which is indistinguishable from that of naturally occuring cecropins. Fluorescence of cecropin B results from the sole tryptophan residue in the peptide. In order to understand the mechansim of peptide–membrane binding, we modified the original peptide (cecropin B1: KWKVFKKIEKMGRNIRNGIV) by attaching a terminal tryptophan residue (cecropin B2: KWKVFKKIEKMGRNIRNGIVW). Both peptides show a large inhibition effect against a wide range of bacteria, compared to naturally occurring peptides. The fluorescence results show an enhancement in the peak intensity of cecropin B1 upon mixing with the membrane, accompanied by a blue shift. For cecropin B2, a blue shift was observed upon mixing with the PA membrane, but no enhancement in intensity was observed. The results indicate perpendicular penetration of cecropins B1 and B2 from the Lys side where the Trp residue of cecropin B1 is immersed in the PA membrane. Partial quenching of the Trp fluorescence by acrylamide was observed and the values of the Stern–Volmer constants (K_{sv}) indicate that the Trp molecule penetrates into the membrane, but resides close to the interface region. Two fluorescence lifetimes were measured for the cecropin B1–PA complex which are for two rotamers of Trp. The results point to a degree of flexibility of the local environment around the Trp molecule. A mechanism of membrane disruption is proposed in which the cecropin peptide creates cracks through the negatively charged outer membrane of PA.

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1 Introduction

Antimicrobial peptides (AMPs) are oligopeptides consisting of amino acid residues and can be found in both prokaryotes and eukaryotes.[1,2] To date, more than 5000 AMPs have been discovered and characterized.[3] Cecropins are lytic antimicrobial peptides originally isolated from the haemolymph of the *Hylophora cecropia*.[4] Cecropins were first isolated in 1980 and usually are of 35 to 37 residues length.[5] Cecropin B was found to be not only effective against a range of gram positive and gram negative bacteria but also cytotoxic to a range of mammalian cancer and non-cancer cell-lines.[6] Attempts to generate recombinant and chemically synthesized cecropin B were successful and the results indicate that naturally produced and synthetic cecropins are indistinguishable in terms of their antibacterial activity.[7] Cecropin B and its analogs showed to penetrate bacterial cell membranes and this permeabilization depends on the liposome composition in the targeted cells.[8] The structure of cecropin A has been studied previously by NMR spectroscopy and shown to consist of an amphiapathic α-helical N-terminus (which plays the main role in the antibacterial activity of cecropins), a glycine–proline bend and a hydrophobic C-terminal α-helix.[9] Unlike other amphiapathic α-helical peptides such as maganin[10] and dermaseptin,[11] cecropins do not lyse erythrocytes. The mode of action of cecropin is membrane permeabilization *via* peptide–lipid interaction rather than receptor-mediated recognition.[12]

In the present study, we investigate a model of peptide–membrane interaction using synthetic cecropin B-like peptides against the outer membrane of *Pseudomonas aeruginosa*. The

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SCIENTIFIC REPORTS

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New insights into the mode of action of the lantibiotic salivaricin B

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Salivaricin B is a 25 amino acid polycyclic peptide belonging to the type AII lantibiotics and first shown to be produced by *Streptococcus salivarius*. In this study we describe the bactericidal mode of action of salivaricin B against susceptible Gram-positive bacteria. The killing action of salivaricin B required micro-molar concentrations of lantibiotic whereas the prototype lantibiotic nisin A was shown to be potent at nano-molar levels. Unlike nisin A, salivaricin B did not induce pore formation or dissipate the membrane potential in susceptible cells. This was established by measuring the fluorescence of the tryptophan residue at position 17 when salivaricin B interacted with bacterial membrane vesicles. The absence of a fluorescence blue shift indicates a failure of salivaricin B to penetrate the membranes. On the other hand, salivaricin B interfered with cell wall biosynthesis, as shown by the accumulation of the final soluble cell wall precursor UDP-MurNAc-pentapeptide which is the backbone of the bacterial peptidoglycan. Transmission electron microscopy of salivaricin B-treated cells showed a reduction in cell wall thickness together with signs of aberrant septum formation in the absence of visible changes to cytoplasmic membrane integrity.

Lantibiotics are ribosomally-synthesized antimicrobial peptides containing intramolecular ring structures introduced through the thioether-containing lanthionine (Lan) and/or methyllanthionine (MeLan) residues formed by post-translation modification[1]. Although most of the currently-described lantibiotics are produced by Gram-positive bacteria[2] certain isolates of *Streptomyces* have also been shown to produce lantibiotics or lantibiotic-like peptides[3,4]. Lantibiotics are widely-considered to assist the survival of the host bacteria in their favoured ecosystem by suppressing the growth of competitor bacteria in that particular ecological niche.

The most well-known lantibiotic is nisin, which was first described in 1928[5] and then subsequently widely used in the dairy industry as an effective and safe preservative[6]. Lantibiotics from gram positive bacteria are classified into two major groups based on their modes of action and structural variations[7]. Nisin, epidermin and Pep5 are members of the type A lantibiotic group and they act mainly by forming pores in the cytoplasmic membrane of the targeted bacterial cells[8]. On the other hand, type B lantibiotics such as mersacidin form complexes with their membrane bound substrates and inhibit peptidoglycan synthesis[9–13]. While type AI lantibiotics (the nisin group) are elongated and flexible, type AII (the lacticin 481 group) display an unbridged N-terminal extremity and a globular C-terminal part. Type AIII lantibiotics consists of lactosin S and the two-component system lantibiotics[14–16].

Salivaricin B is a type AII lantibiotic produced by *Streptococcus salivarius* strain K12 and having a ring topology similar to that of the *Lactococcus lactis* lantibiotic, lacticin 481[16–18] (Fig. 1).

S. salivarius is a commonly-occurring member of the human oral microbiota, typically colonizing the mouth and upper respiratory tract within a few hours of birth. Some *S. salivarius* are equipped to compete with predominant bacterial pathogens involved in upper respiratory tract infections due to their production of various lantibiotics, which include salivaricin A, salivaricin B, salivaricin G32 and salivaricin 9[18–24]. Salivaricin B is particularly potent, with a broad inhibitory spectrum that includes all 9 standard indicator strains used in the production (P-) typing method that was developed specifically for the categorization of bacteriocin-producing streptococci[18,25].

One important characteristic of the members of the lacticin 481 group is that they contain a mersacidin-like lipid II binding motif and in this regard salivaricin B is no exception[16,18,26]. Although salivaricin B and lacticin 481 are classified as class AII lantibiotics they also contain an important membrane binding motif found in class B lantibiotics, which makes it interesting to study the mechanism of action of these lantibiotics and to determine

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APPENDIX

APPENDIX A

PUTATIVE MEGAPLASMID SEQUENCE OF *Streptococcus salivarius* strain NU10

Contains:
Salivaricin A locus
Salivaricin 9 locus
Putative non-ribosomal peptide synthetase system
Putative ParA-like plasmid partitioning (segregation) system
Putative DNA primase (DNA replication)
Putative TraE-like plasmid transfer system
Putative bacteriophage (possibly inactive)

```
LOCUS       Nu10-2015_hybrid2_c11       190254 bp    DNA     linear   UNK
DEFINITION  Contig Nu10-2015_hybrid2_c11 from Streptococcus salivarius
            Nu10-Hyb2X_All
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FEATURES             Location/Qualifiers
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                     /db_xref="taxon: 1304"
                     /genome_md5=""
                     /project="hengnck_1304"
                     /genome_id="1304.166"
                     /organism="Streptococcus salivarius Nu10-Hyb2X_All"
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                     YLRTSSGTLEKFYKTGDIGRIGHDSLLYYKSRKDRQVKIRGFRVELSEIETQVMKSDG
                     VSKCVVDFNKDNLSRSLSLVYEGSILSKDLREYMIKTLPPYMIPNEIKHVQELKLNIN
                     GKLEQELSYIEEIKEEHLIGKDSSNNTKIENIIFEAIKETLQIKYVNKDVDFYELGID
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                     PIQKWFFLTQHENKSLNHFNQTFLIKLNSDIENDDILRGLQEIIDVYPIFNTCFSIKN
                     NKWYQTMKDTSKYFIETYNVENYNEFEKLLAALQGSLDIHEKLYNFCLINFNGRNYLF
                     FVIHHLIIDGVSWRVFLDSFSRRLMSIEKNGEKIIAPNFSEWVRYIEDYKVSEDVSEY
                     WNQFELYPQKNNISFSTVDHEVIEFSSRETERFKEIVNESYFADMESCLLSMVSNAFY
                     KNFVSQKFLVKIEGHGRPWKAEQFNDSLGWFTSVYPFQSTTNDNLKDSIIEIHNRLGS
                     VPNKGFDFQLENDLNFDSDFSFNFMGEFSSNSYKNFEICSMFRKDDFDLNLFSTDLVS
                     FVPIIVDGKLQLRVSYARNLVNETCIKHTLESFINSMNNFIKGSHSRYLPATSLQSSL
                     LLKDVSEVDTGAYVIQWSGYFKELNFSKFQSSINKLIRNTDTLRSVFEFSGTNAVQLI
                     MKPEEFFANSYIKVLDWSVYSKEESELKLETYLNDNRSKGFDLSNGPLFRVTVIKLGT
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                     IWALVLSFTFGREDILLGVSLSGRSTFPINILNSIGMFVTTLPCRIKNIESYTNINTL
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                subunit"
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                HEKNHFLDARHYLTSVRGLSDETINFFGRQGVLSQALLKERNDSTEPVVVFKYLDHRG
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APPENDIX B

PUTATIVE MEGAPLASMID SEQUENCE OF *Streptococcus salivarius* strain YU10

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ATCCS"
/product="**Salivaricin G32 peptide**"

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ATCCS"
/product="**Salivaricin G32 peptide**"

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HLITVKGLGYKLVI"
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/product="Paratox"

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                     /product="Choline binding protein D"
                     /product="C-terminal portion of putative salivaricin MPS"

LOCUS       Yu10-Hybrid1_c34        68594 bp    DNA     linear   UNK
DEFINITION  Contig Yu10-Hybrid1_c34 from Streptococcus salivarius Yu10-Hybrid1
ACCESSION   unknown
FEATURES             Location/Qualifiers
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APPENDIX C:

Ethical approval used for *S. salivarius* isolation

UNIVERSITY OF MALAYA
The Leader in Research & Innovation

MEDICAL ETHICS COMMITTEE
FACULTY OF DENTISTRY
ADDRESS: 50603, KUALA LUMPUR, MALAYSIA
TELEPHONE: 03-79676461 FAXIMILE: 03-79676456

NAME OF ETHICS COMMITTEE/IRB: Medical Ethics Committee, Faculty of Dentistry **ADDRESS:** Faculty of Dentistry, University of Malaya, 50603, Kuala Lumpur	**ETHICS COMMITTEE/IRB REFERENCE NUMBER:**
PROTOCOL NO: **TITLE:** Isolation and Identification of Streptococcus salivarius in the Oral Cavity of Children	OF BS1401/0017(L)
PRINCIPAL INVESTIGATOR: Prof Madya Dr Koshy Philip / Prof Dr. Dasan Swaminathan **TELEPHONE:** 03-79675839	

The following item [√] have been received and reviewed in connection with the above study to be conducted by the above investigator.

[√] Investigator's Checklist — Ver date: 17 February 2014
[ ] Approval Form for Presentation at Department — Ver date: 14 March 2014
[√] Application Form
[√] Study Protocol
[√] Brief CV of Main Investigator
Patient Information Sheet (PIS):
[√] BM version
[√] English version
[ ] Others: _____
Consent Form:
[√] BM version
[√] English version
[ ] Others: _____

and have been

[√] Approved
[ ] Conditionally approved (identify item and specify modification below or in accompanying letter)
[ ] Rejected (identify item and specify reasons below or in accompanying letter)

Investigator are required to:

1) follow instructions, guidelines and requirements of the Medical Ethics Committee.
2) report any protocol deviations/violations to Medical Ethics Committee.
3) comply with International Conference on Harmonization – Guidelines for Good Clinical Practice (ICH-GCP) and Declaration of Helsinki
4) note that Medical Ethics Committee may audit the approved study.
5) ethics approval by the Medical Ethics Committee, Faculty of Dentistry is only valid for 2 years from the date of approval. Please update your project status (on-going/ completed) by submitting study report/ study closure report form (UM-DMEC-SR01).

Date of approval: 14 March 2014

c.c Dean
Faculty of Dentistry

Dean
Faculty of Science

Secretary
Medical Ethics Committee
Faculty of Dentistry

PROF. DR. NOOR HAYATY ABU KASIM
Chairperson
Medical Ethics Committee