ISOLATION, PURIFICATION AND ANTIMICROBIAL ACTION OF PEPTIDES PRODUCED BY *STREPTOCOCCUS SALIVARIUS* ISOLATED FROM MALAYSIAN SUBJECTS

ABDELAHHAD BARBOUR

DISSERTATION SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

INSTITUTE OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2013

UNIVERSITI MALAYA

ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: **ABDELAHHAD BARBOUR** I/C/Passport No: **N005819343**

Regisration/Matric No.: **SGR110003**

Name of Degree: **MASTER OF SCIENCE**

Title of Project Paper/Research Report/Dissertation/Thesis ("this Work"):

"ISOLATION, PURIFICATION AND ANTIMICROBIAL ACTION OF PEPTIDES PRODUCED BY *STREPTOCOCCUS SALIVARIUS* ISOLATED FROM MALAYSIAN SUBJECTS"

Field of Study: **MICROBIOLOGY**

I do solemnly and sincerely declare that:

(1) I am the sole author/writer of this Work,
(2) This Work is original,
(3) Any use of any work in which copyright exists was done by way of fair dealing and for permitted purposes and any excerpt or extract from, or reference to or reproduction of any copyright work has been disclosed expressly and sufficiently and the title of the Work and its authorship have been acknowledged in this Work,
(4) I do not have any actual knowledge nor do I ought reasonably to know that the making of this work constitutes an infringement of any copyright work,
(5) I hereby assign all and every rights in the copyright to this Work to the University of Malaya ("UM"), who henceforth shall be owner of the copyright in this Work and that any reproduction or use in any form or by any means whatsoever is prohibited without the written consent of UM having been first had and obtained,
(6) I am fully aware that if in the course of making this Work I have infringed any copyright whether intentionally or otherwise, I may be subject to legal action or any other action as may be determined by UM.

(Candidate Signature) Date:

Subscribed and solemnly declared before,

Witness's Signature Date:

Name: **ASSOC. PROF. DR KOSHY PHILIP**

Designation

Witness's Signature Date:

Name: **PROFESSOR DR SEKARAN A/L MUNIANDY**

Designation

Abstract

Streptococcus salivarius is known to produce different bacteriocin-like inhibitory substances (BLIS). In this study six *S. salivarius* strains were isolated from different Malaysian subjects. The isolates were identified using morphological and biochemical characterization and 16S rDNA gene sequencing. Levansucrase enzyme was characterized from sucrose-enriched culture of strain *S. salivarius* YU10.

BLIS production was investigated using simultaneous and deferred antagonism tests that showed YU10 and NU10 as the best BLIS-producing strains among the Malaysian isolates in this study. Strain HJEFF was the only *S. salivarius* isolate which produced a more limited quantity of BLIS in liquid medium. However, all other strains failed to produce any BLIS activity in liquid media. The distribution of *salA*, *sboB* and *sivA* genes encoding the production of salivaricin A, B and 9 was investigated in this study using BLIS producing strains NU10 and YU10. Strains NU10 and K12 (commercial probiotic) were the only strains that harboured the *sboB* structural gene. Both strains (NU10 and YU10) but not K12 harbored the *sivA* structural gene. Isolation of BLIS was done using acidic methanol extraction of the producer cells and freeze thaw extraction. BLIS-YU10 proved bacteriostatic while BLIS-NU10 was bactericidal. When added to different growth phases of sensitive bacteria, BLIS-NU10 reduced its growth significantly. BLIS-NU10 was purified from its crude form using different steps of initial purification namely ammonium sulphate precipitation, gel filtration, XAD-2 and solid phase extraction chromatography.

BLIS-NU10 showed to be auto-regulated whereby it enhanced the production of the same molecule when added to NU10 culture. The induction ability was used to develop BLIS-production in liquid medium. BLIS-NU10 showed to be of cationic nature and it was

purified using a cation exchange column. The cation exchange chromatography was performed using Fast Protein Liquid Chromatography system. Tris-ticine SDS page of the active fractions showed that BLIS-NU10 had a molecular weight of approximately 3,000 Da. The pure fraction was subjected to MALDI-TOF MS analysis. The only known salivaricin detected in BLIS-NU10 was salivaricin 9 (2560 Da). Other peptides where also detected in BLIS-NU10 with molecular weights of approximately 2,000 Da.

BLIS-NU10 showed to have permeability activity towards selected target cells and showed to induce pore formation in the cytoplasmic membrane of targeted cells. The stability of BLIS-NU10 was also investigated in this study. BLIS-NU10 exhibited thermo-stability when exposed to 100^{o}C for 30 minutes and retained biological activity when subjected to different pH values ranging from 2 to10. When treated with proteinase K or peptidase, BLIS-NU10 lost the antimicrobial activity. One major difficulty with strain NU10 was its erratic BLIS production. However, although strain NU10 harbors three different genes encoding known lantibiotics, analysis of pure BLIS-NU10 showed the presence of only lantibiotic salivaricin 9 in addition to other proline-rich peptides. The reason for the absence of salivaricin A and B in the purified BLIS-NU10 is still unknown and worthy of further investigation. Due to its uniqueness, strain NU10 can be further studied using whole genome sequencing approach to determine if this strain can be a potential probiotic.

Abstrak

Streptococcus salivarius dikenali untuk menghasilkan bahan-bahan yang melarang bakteriosin seperti berbeza (BLIS). Dalam kajian ini enam jenis *S. salivarius* telah diasingkan daripada mata pelajaran yang berbeza di Malaysia. Pencilan telah dikenal pasti menggunakan pencirian morfologi dan biokimia dan 16S rDNA urutan gen. Enzim Levansucrase dicirikan dari budaya yang kaya dengan sukrosa ketegangan *S. salivarius* YU10. Pengeluaran BLIS telah disiasat menggunakan ujian permusuhan serentak dan tertunda yang menunjukkan YU10 dan NU10 sebagai yang terbaik jenis BLIS yang menghasilkan antara pencilan Malaysia dalam kajian ini. Terikan HJEFF adalah satu-satunya *S. salivarius* mengasingkan yang menghasilkan kuantiti yang lebih terhad BLIS dalam medium cecair. Walau bagaimanapun, semua jenis lain gagal mengemukakan apa-apa aktiviti BLIS dalam media cecair. Pengagihan *salA, sboB* dan *sivA* gen pengekodan pengeluaran salivaricin A, B dan 9 telah disiasat dalam kajian ini menggunakan BLIS menghasilkan tekanan NU10 dan YU10. Terikan NU10 dan K12 (probiotik komersial) adalah satu jenis yang melabuhkan gen struktur *sboB*. Kedua-dua jenis (NU10 dan YU10) tetapi tidak K12 menaruh gen *sivA* struktur. Pengasingan BLIS telah dilakukan dengan menggunakan pengeluaran methanol berasid daripada sel sel pengeluar dan pengeluaran cair beku. BLIS-YU10 membuktikan bacteriostatic manakala BLIS-NU10 adalah bakteria. Apabila dimasukkan ke dalam fasa pertumbuhan yang berbeza bakteria sensitif, BLIS-NU10 dikurangkan pertumbuhan ketara. BLIS-NU10 telah disucikan dari bentuk mentah dengan menggunakan langkah-langkah yang berbeza pembersihan awal iaitu hujan ammonium sulfat, penapisan gel, XAD-2 dan pepejal fasa kromatografi pengekstrakan. BLIS-NU10 menunjukkan untuk menjadi auto-terkawal di mana ia meningkatkan

pengeluaran molekul yang sama apabila ditambah kepada NU10 budaya. Keupayaan induksi telah digunakan untuk membangunkan BLIS pengeluaran dalam medium cecair. BLIS-NU10 menunjukkan kepada bersifat kationik dan ia telah disucikan menggunakan ruang pertukaran kation. Pertukaran kromatografi kation dilakukan menggunakan Protein Sistem Kromatografi cecair segera. Tris-ticine SDS halaman pecahan aktif menunjukkan bahawa BLIS-NU10 mempunyai berat molekul kira-kira 3,000 Da. Pecahan tulen adalah tertakluk kepada MALDI-TOF analisis MS. Salivaricin hanya diketahui dikesan dalam BLIS-NU10 adalah salivaricin 9 (2560 Da). Peptida lain di mana juga dikesan dalam BLIS-NU10 dengan berat molekul kira-kira 2,000 Da. BLIS-NU10 menunjukkan mempunyai aktiviti kebolehtelapan terhadap sel-sel sasaran terpilih dan menunjukkan kepada mendorong pembentukan liang dalam membran cytoplasmic sel sel yang disasarkan. Kestabilan BLIS-NU10 juga diselidiki dalam kajian ini. BLIS-NU10 dipamerkan termo-kestabilan apabila terdedah kepada 100°C selama 30 minit dan mengekalkan aktiviti biologi apabila tertakluk kepada nilai pH yang berbeza antara 2 tersebut adalah 10. Apabila dirawat dengan proteinase K atau peptidase, BLIS-NU10 hilang aktiviti antimikrobial. Salah satu masalah utama dengan tekanan NU10 adalah pengeluaran BLIS tidak menentu itu. Walau bagaimanapun, walaupun ketegangan NU10 pelabuhan tiga gen yang berbeza pengekodan lantibiotics dikenali, analisis tulen BLIS-NU10 menunjukkan kehadiran hanya lantibiotic salivaricin 9 di samping lain lain peptida proline yang kaya. Sebab ketiadaan salivaricin A dan B dalam suci BLIS-NU10 masih tidak diketahui dan memerlukan siasatan lanjut. Disebabkan keunikannya, ketegangan NU10 boleh lagi dikaji dengan menggunakan pendekatan keseluruhan genom penjujukan untuk menentukan sama ada tekanan ini boleh menjadi potensi probiotik.

Acknowledgments

I would like to express my deepest appreciation to my Supervisor Associate Professor Dr. Koshy Philip for his support, comments and remarks throughout my Master's project. It was a great opportunity to work with him and be under his supervision. He was very supportive especially when I was depressed and worried about my family in my beloved country Syria during the war. I also wish to thank my co-supervisor Professor Dr Sekaran Muniandy for his input on the research project.

I wish to acknowledge the facilities at University of Malaya and the High Impact Research – Ministry of Higher Education Grant UM.C/625/1/HIR/MOHE/SC/08 under the Principal Investigator Associate Professor Dr Koshy Philip.

I also wish to acknowledge the facilities provided at the Malaysia Genome Institute, Bangi, Malaysia and the Proteomics Center at the University of Victoria, Canada to analyze some of my samples.

Furthermore I must acknowledge and thank Professor Dr. John Tagg for providing the probiotic strain K12 as a control for the study.

Special thanks go to my parents and family for being there for me, praying for me and believing in me. Last but not least thank you Alissar for all what you did to make things easier. You are my angel forever.

"For with God nothing shall be impossible"

Luke 1:37

Table of contents

List of Figures

List of Tables

List of Abbreviations

µ	Micro
ABC	ATP-binding cassette
AU	Activity unit
BACa	Blood agar for antagonism test
BLAST	Basic local alignment search tool
BLIS	Bacteriocin-like inhibitory substances
bp	Base pair
CAB	Columbia agar base
$CaCO_3$	Calcium carbonate
CFU	Colony forming unit
CO_2	Carbon dioxide
Da	Dalton
DEAE	diethylaminoethyl
EDTA	Ethylene diamine tetraacetic acid
FPLC	Fast protein liquid chromatography
g	Gram(s)
h	hour
kDa	Kilodalton
LAB	Lactic acid bacteria
Lan	lanthionine
LC	Liquid chromatography
MALDI	matrix-assisted laser desorption ionization

MeLan	methyllanthionine
ml	milliliter
MS	Mass spectrometry
MSA	Mitis Salivarius Agar
NaCl	Sodium chloride
$^{\circ}$C	Degree Celsius
PAGE	Poly acrylamide gel electrophoresis
PCR	Polymerase chain reaction
SDS	Sodium dodecyl sulphate
SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscopy
THB	Todd Hewitt Broth
TOF	Time of flight
TSB	Trypticase Soy Broth
UV	Ultra violet
V	volt
v/v	Volume per volume
w	Weight(s)
YE	Yeast extract

Chapter One

Introduction

Different genera of lactic acid bacteria (LAB) can produce different kinds of antimicrobial peptides and bacteriocins such as plantaricin from *Lactobacillus plantarum* (Tiwari and Srivastava, 2008), enterococcin from *Enterococcus faecium* (Klocke *et al.*, 2005), leucocin from *Leuconostoc carnosum* (Wan *et al.*, 2012), pediocin from *Pediococcus acidilactici* (Papagianni and Anastasiadou, 2009) and several others. The scientific interest in bacteriocins has increased significantly recently due to their antimicrobial activity towards Gram-positive pathogens (Abee *et al.*, 1995; Lee *et al.*, 2011; Wilson-Stanford and Smith, 2011). Bacteriocins produced by many oral microorganisms have been reported previously (Hyink *et al.*, 2007; Nicolas *et al.*, 2011; Wescombe *et al.*, 2012; Wescombe *et al.*, 2006; Wescombe *et al.*, 2011). Most of the bacteriocins produced by human oral streptococci are understood to be controlled by quorum sensing, whereby it can only produce the bioactive bacteriocin when grown on solid or semi-solid medium supplemented with agar or agarose (Hyink *et al.*, 2007; Nicolas *et al.*, 2011; Ross *et al.*, 1993). Other lantibiotics such as nisin could be produced in significant quantity when the producer is grown in liquid medium (Flores and Alegre, 2001; Gonzalez-Toledo *et al.*, 2010; Lv *et al.*, 2005). In recent years methods to enhance and optimize bacteriocin production have been developed (Cheigh *et al.*, 2005; Liu *et al.*, 2010; Pongtharangkul and Demirci, 2006) due to the potential importance of bacteriocin-producing strains in replacement therapy (Cotter *et al.*, 2005). Many strains produce bacteriocins that have already been used as probiotics. *Streptococcus salivarius* K12 is an oral probiotic producing two kinds of antimicrobial peptides referred as salivaricin A2 and salivaricin B (Hyink *et al.*, 2007). Both bacteriocins can be produced by freeze thaw extraction method after the producer is grown on solid medium. Pore forming mechanism is a common mode of action of lantibiotics (Garcera *et al.*, 1993; van Heusden *et al.*, 2002). The permeabilization of the cytoplasmic membrane of the targeted

cells by lantibiotics has been studied in the past to investigate whether the bioactive lantibiotic can penetrate the cell membrane of the potential pathogens (Chun and Hancock, 2000; Zendo *et al.*, 2010).

In this study a number of *S. salivarius* strains were isolated from the oral cavity of Malaysian subjects. Some of the identified strains showed to produce BLIS molecules with antimicrobial activity against selected oral pathogens. New techniques to produce BLIS-NU10 in liquid medium were developed for the first time using *Streptococcus salivarius* strain NU10 isolated from a Malaysian subject. The purification method used to recover BLIS-NU10 was planned in this study through the use of XAD-16 chromatography followed by cation exchange chromatography. MALDI-TOF MS analysis showed the presence of salivaricin 9 lantibiotic in addition to proline-rich peptides. The current research work also included an investigation on the mechanism of action of the pure BLIS-NU10 using SYTOX green and Real-Time PCR system.

Scanning electron microscopy was used to detect the morphological changes of the targeted indicator microorganisms that are sensitive on exposure to BLIS-NU10. Investigating the mechanism of action of lantibiotics produced by oral streptococci was carried out with the intent of furthering knowledge in future development of new antimicrobials and probiotics that might enhance the health of the human oral anatomy and the upper respiratory tract.

Main objectives of the study:

1- Isolation, identification and characterization of BLIS producing *S. salivarius* strains and their antimicrobial activity using simultaneous and deferred antagonism assays.

2- Enhanced production, purification and characterization of the antimicrobial peptide using different methods.

3- Investigation of *salA*, *sboB* and *sivA* structural genes distribution among Malaysian isolates.

4- Elucidation of the mechanism of action of BLIS-NU10.

Chapter Two

Literature Review

2.1 Lactic acid bacteria

Joseph Lister obtained the first pure culture of lactic acid bacteria which was designated as *Bacterium lactis* in 1873. In 1890 the first starter culture to be used for cheese production was introduced. Lactic acid bacteria (LAB) were classified according to Orla-Jensen who divided the LAB according to morphology into rods (*Lactobacillus* and *Corynebacterium*) and cocci (all other genera) and this classification system was only at the genus level and this first monograph was the first basis in LAB classification (Orla-Jensen, 1919). LAB are Gram-positive, oxidase and catalase negative, non-sporulating, non-motile bacteria which includes many genera namely: *Lactobacillus, Enterococcus, Pediococcus, Lactococcus, Oenococcus, Leuconostoc, Streptococcus* and others. All these LAB are acid tolerant and can produce lactic acid as a major end product. Current taxonomic outline of lactic acid bacteria is stated in table 2.1. Next differentiation of LAB genera was introduced according to the mode of glucose fermentation under standard conditions (e.g. non limited supply with: glucose, amino acids, nucleic acid precursors, vitamins and oxygen limitation) under these fermentation characteristics LAB can be divided into two groups:

I. Homofermentative bacteria.

II. Heterofermentative bacteria.

Based on a biochemical perspective, LAB comprise homofermenters (which basically yield lactic acid) and heterofermenters (which produce different fermentation products e.g. ethanol, acetic acid, carbone dioxide and formic acid) (Kleerebezem and Hugenholtz, 2003). LAB use sugars as primary energy sources to grow on substrates (de Vos, 1996). Phosphotransferase system (PTS) and ATP-

binding cassette (ABC) both are basic systems which contribute in the uptake of carbohydrates in LAB. In *Lactococcus lactis* lactose is hydrolyzed after been transported by PTS resulting in transformation of the galactose-6p moiety by the tagatose pathway, while in *Streptococcus thermophilis* only glucose moiety of lactose is fermented while the galactose moiety is released into the medium (Vaughan *et al.*, 2001). LAB can also produce bacteriocins to compete in their environments. Most of these bacteriocins are small peptides with antimicrobial properties toward bacteria of the same taxonomically related species (Castellano *et al.*, 2008). After the advent of genome sequencing genes encoding bacteriocin production in LAB were well understood and studied. Clustered genes associated with bacteriocin-coding sequences were well studied in 7 different *Lactobacillus* genomes (Makarova and Koonin, 2007). Bacteriocins play a role in cell signaling. Bacteriocin producing LAB also confer self-protection by possessing immunity mechanisms (Cotter *et al.*, 2005). LAB have plenty of applications which contribute to human health like using those species designated as generally recognized as safe (GRAS) in the probiotics industry (Burton *et al.*, 2006).

Table 2.1: Current taxonomic outline of lactic acid bacteria (König *et al.*, 2009)

Phylum	Class	Order	Family	Genus
Firmicutes	Bacilli	Lactobacillales	**Lactobacillaceae**	*Lactobacillus*
				Paralactobacillus
				Pediococcus
			Aerococcaceae	*Aerococcus*
				Abiotrophia
				Dolosicoccus
				Eremococcus
				Facklamia
				Globicatella
				Ignavigranum
			Carnobacteriaceae	*Carnobacterium*
				Agitococcus
				Alkalibacterium
				Allofustis
				Alloiococcus
				Desemzia
				Dolosigranulum
				Granulicatella
				Isobaculum
				Lactosphaera
				Marinilactibacillus
				Trichococcus
			Enterococcaceae	*Enterococcus*
				Atopobacter
				Melissococcus
				Tetragenococcus
				Vagococcus
			Leuconostocaceae	*Leuconostoc*
				Oenococcus
				Weissella
			Streptococcaceae	*Streptococcus*
				Lactococcus

2.2 *Streptococcus salivarius* and oral cavity health

Streptococcus salivarius is a member of lactic acid bacteria group or family which form an important part of the normal flora of the human oral cavity and upper respiratory tract (Kang *et al.*, 2006; Sherman *et al.*, 1943). Among all the dominant streptococci of the oral cavity *S. salivarius* is numerically the most significant colonist, especially on the tongue (McCarthy *et al.*, 1965). It is also inhabits the oral epithelial surfaces and is one of the first microorganisms to be established in the mouths of infants (Carlsson *et al.*, 1970). It has been reported that some strains of *S. salivarius* can produce many bacteriocins, most of which are lantibiotics (Birri *et al.*, 2012; Hyink *et al.*, 2007; Ross *et al.*, 1993; Wescombe *et al.*, 2006). These bacteriocins or bacteriocin-like inhibitory substances (BLIS) have been reported to inhibit some oral pathogens such as *Corynebacterium diphtheriae* (Bill and Washington, 1975), *Streptococcus pneumonia* (Johanson *et al.*, 1970) and *Streptococcus pyogenes* (Sanders and Sanders, 1982). *S. salivarius* usually colonize the tongue dorsum and can be found in great numbers there (Aas *et al.*, 2005). Because of the bacteriocin molecules that some of *S. salivarius* can produce, the oral cavity will remain healthy and protected. The protection comes from the reduction in oral pathogens numbers. *S. salivarius* strain K12, discovered by Professor John Tagg from Otago University, is the first oral probiotic which can produce more than one kind of bacteriocin- the lantibiotics salivaricin A2 and salivaricin B (Hyink *et al.*, 2007). This protective mouth bacterium can prevent sore throats which can lead to rheumatic fever especially in children. BLIS produced by *S. salivarius* had been described previously (Bill and Washington, 1975; Tagg *et al.*, 1983). BLIS are extracellular peptides or protein molecules released by some bacteria which can kill some closely related bacteria. BLIS-producer bacteria use these substances

to compete better in their environment by reducing the numbers of other related bacteria, some of which may cause some diseases in the host.

2.3 Bacteriocins:

Reports of antimicrobial molecules produced by either Gram-positive or Gram-negative bacteria have increased gradually through the last decade. Usually these antimicrobials are called bacteriocins, specially those of protein nature. Proteinaceous antimicrobial bacteriocins which have the physiological capability of interfering with the growth of other bacteria have taken researchers attention especially after the significant drug-resistant reports due to over use of conventional antibiotics. The first bacteriocins to be investigated were the colicins produced by different members of the *Enterobacteriaceae* family (Jack *et al.*, 1995).

2.3.1 Bacteriocins from gram negative bacteria

A variety of bacteriocins can be produced by Gram-negative bacteria e.g. klebsin produced by *Klebsiella pneumoniae*, marcescin produced by *Serratia marcescens*, alveicin produced by *Hafina alvei* and colicin produced by *E. coli*. On the basis of their size the bacteriocins of Gram-negative bacteria can be divided in three groups:

- colicin-like bacteriocins (25-80 kDa)
- microcins (<10 kDa)
- phage tail-like bacteriocins (multimeric peptide assemblies)

2.3.1.1 Colicin-like bacteriocins

Colicins are large plasmid-encoded ribosomally-synthesized bacteriocins produced by many strains of *E. coli*. Colicin synthesis repression is controlled by an inducible DNA repair system called the SOS response which helps many bacteria to survive under circumstances of exposure to DNA damaging agents (Michel, 2005). Colicins have three killing domains which form the functional modules which catalyze deleterious changes in the targeted cell. The first step of colicin activation is the binding of central domain (located within the central region of the colicin molecule) to the specific receptor (Brunden *et al.*, 1984). Translocation of the colicin N-terminus across the outer membrane of the targeted cell is the second step of colicin activation. This N-terminal domain differs among colicins depending on which import system is used (Pilsl and Braun, 1995). The third step in the colicin killing mechanism is formation of pores in the cytoplasmic membrane of the target cell and this step is carried out by the C-terminal domain. The killing activity of some colicins can be caused by nuclease activity which cut up the nucleic material DNA of the target cell (Martinez *et al.*, 1983).

2.3.1.2 Microcins

Members of this group of bacteriocins from Gram-negative bacteria are low molecular weight peptides which have a similarity to bacteriocins produced by Gram-positive bacteria (Jack and Jung, 2000; Moreno *et al.*, 2002). Microcins are non-SOS-inducible peptides which inhibit transcription by binding to the DNA-dependent RNA polymerase RNAP (the central enzyme of bacterial gene expression) secondary channel. Binding of microcin

within this channel blocks nucleotide substrates from entering the enzyme active site (Yuzenkova *et al.*, 2002).

2.3.1.3 Phage tail-like bacteriocins

Phage tail-like bacteriocins are rod-like molecules resembling a bacteriophage tail. Members of this group are nuclease and protease-resistant rod-like bacteriocins. Cytoplasmic membrane depolarization is the mechanism of action of phage tail-like bacteriocins toward sensitive cells (Strauch *et al.*, 2003). Pyocins (bacteriocin produced by *Pseudomonas aeruginosa*) are of two types; R2 related to P2 phage and Pyocin F2, similar to lamda phage (Nakayama *et al.*, 2000).

2.3.2 Bacteriocins from gram positive bacteria

Bacteriocins are antimicrobial proteins or peptides produced by many genera of bacteria. Many microbiologists are now focusing their interest on bacteriocins which can be produced by gram positive bacteria, especially LAB, because the number of bacteriocins discovered from this group is increasing exponentially every year. LAB is a sub group of gram positive bacteria and can produce four classes of bacteriocins:

Class I: Lanthionine-containing bacteriocins

Class II: non- modified bacteriocins

Class III: large bacteriocins

Class IV: cyclic peptides

2.3.2.1 Class I: Lanthionine containing bacteriocins (Lantibiotics)

Lantibiotics are heat-stable, posttranslationally modified, ribosomally synthesized small peptides (Al-Mahrous and Upton, 2011; Islam *et al.*, 2012; Perin *et al.*, 2012; Zhao, 2011). Unlike other bacteriocins, this group contains some unusual residues e.g., (Lan) Lanthionine and (MeLan) β-methellanthionine (Al-Mahrous and Upton, 2011; Bierbaum and Sahl, 1993, 2009; Cotter *et al.*, 2005). Prior to cleavage of the leader peptide posttranslational modification happens to the propeptide region of the precursor molecule. The modifications are the result of the dehydration of serine (Ser) and threonine (Thr) residues to 2,3-dehydoalanine (Dha) and (Z)-2,3-dehydrobutyrine (Dhb) while neighboring cysteines (Cys) link covalently to Dha and Dhb resulting in formation of (Lan) Lanthionine and (MeLan) β-methellanthionine bridges which give lantibiotics their distinctive ring structure (Willey and van der Donk, 2007). Then the leader peptide of 23-59 amino acids is proteolytically removed to give the lantibiotic in its bioactive form (Xie and van der Donk, 2004).

Many factors play roles in lantibiotic activity, while Lan and MeLan (positively charged amino acids) interact with the negatively charged phospholipid head group in the cytoplasmic membrane of targeted microorganism, other amino acids like cystine, didhydrobutyrine and didhydroalanine increase the bio-activity of the peptide (Morris *et al.*, 1984).

Lantibiotics can be subdivided into three subclasses (type A, type B and type C) depending on their antimicrobial activity and the mechanisms by which the peptide gains maturity.

2.3.2.1.1 Type A lantibiotics

These lantibiotics are screw-shaped and amphipathic molecules with molecular masses varying from 2164 to 3488 Da, with 2 to 7 net positive charges. Nisin produced by *Lactococcus lactis* is an example of Type A lantibiotics. Genes encoding nisin production are located in a 70 kb conjugative transposon (Rodriguez and Dodd, 1996). Nisin has long been used in food preservation (Delves-Broughton *et al.*, 1996; Pajohi *et al.*, 2011; Udompijitkul *et al.*, 2012). Nisin production is affected by posttranslational modification of a propeptide molecule (Bierbaum and Sahl, 1993; Sahl *et al.*, 1995; Xie and van der Donk, 2004) before a subtilisin-like protease (encoded by NisP) removes the leader peptide to give the nisin bioactive form. The same gene has been found in other lantibiotics (Schnell *et al.*, 1992; Siezen *et al.*, 1995; van der Meer *et al.*, 1993; Ye *et al.*, 1995). The first stage of the nisin killing activity is an electrostatic interaction between the positively charged C-terminal residues of nisin and the negatively charged membrane phospholipid of the targeted bacteria. After nisin insertion membrane pores are formed and this leads to cell death (Breukink *et al.*, 1997). Type A lantibiotics have bacteriocidal activity against gram positive bacteria, but not against gram negative bacteria unless the integrity of the outer membrane is first disrupted (Blackburn, 1989). *S. salivarius* can also produce type A lantibiotics which are called salivaricins. Hyink *et al*. (2007) reported that *S. salivarius* strain K12 can produce two different kinds of bacteriocins (salivaricins A & B) both having strong inhibitory activity against *Streptococcus pyogenes* (oral pathogen causes rheumatic fever). However, *S. salivarius* strain 5M6C has been reported to produce a trypsin resistant lantibiotic (salivaricin D) which is effective against *Bacillus subtilis*, *Clostridium bifermentans* and *Clostridium butyricum* (Birri *et al.*, 2012). Salivaricin 9 (lantibiotic

produced by probiotic *S. salivarius* M18 can inhibit all *Streptococcus* pyogenes strains tested so far) has been described by Wescombe *et al*. (2011). Usually the genes encoding salivaricin production could be located on a megaplasmid (for example, the genes encoding salivaricin A & B (Wescombe *et al*., 2006), or the gene locus can be a DNA chromosomal segment like salivaricin D (Birri *et al.,* 2012). However, the genetic locus of salivaricin 9 can be located on either the chromosome or megaplasmid. The production of salivaricin is also dependent on the strain itself, with some strains producing the BLIS in liquid media others failing. Strain 5M6C produces salivaricin D when it is grown in MRS broth, but strain K12 does not because no detectable activity can be recovered from this strain in liquid media. Other ways of obtaining salivaricin from this strain are from cultures on solid media (freeze thaw of agar cultures) or methanol extraction of the cells (Wescombe *et al.,* 2006). Salivaricin 9 produced by strain M18 can be detected by freeze thaw extraction of the agar cultures or in liquid media if an inducer of the bacteriocin is added to the liquid culture. Some bacteriocins are cell-associated, like SA-FF22 from *Streptococcus pyogenes*, and mutacin K8 produced by *Streptococcus mutans* (Robson *et al.,* 2007).

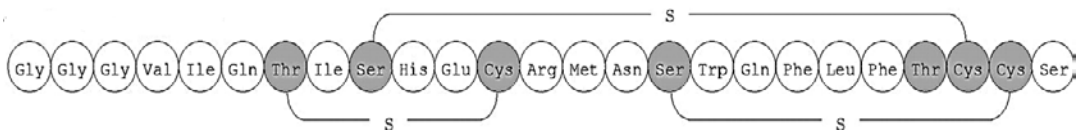


Figure 2.1: Predicted dehydration/bonding pattern of salivaricin B (Hyink *et al.*, 2007).

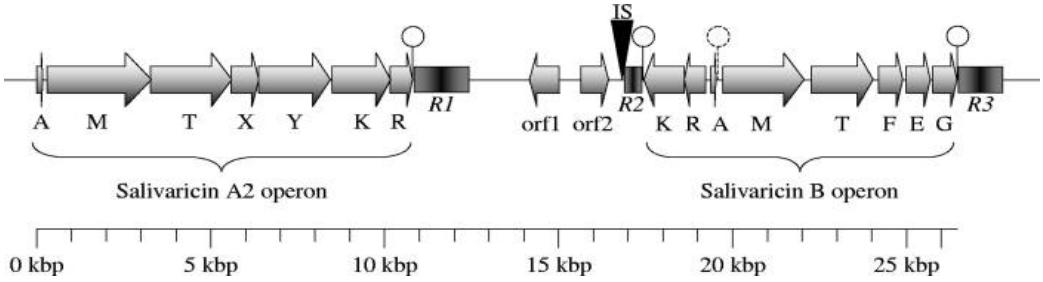


Figure 2.2: Arrangement of the two gene clusters encoding salivaricins A and B in *S. salivarius* K12 (Hyink *et al.*, 2007).Predicted transcriptional terminators for both loci are indicated by stem-loop symbols, with the dashed stem-loop showing a possible weak terminator. Two putative genes are located between the two lantibiotic loci. The three repeat regions (R1, R2, and R3) identified are also indicated, as is the position of a small insertion sequence (IS; black triangle) identified upstream of the salivaricin B locus in some strains.

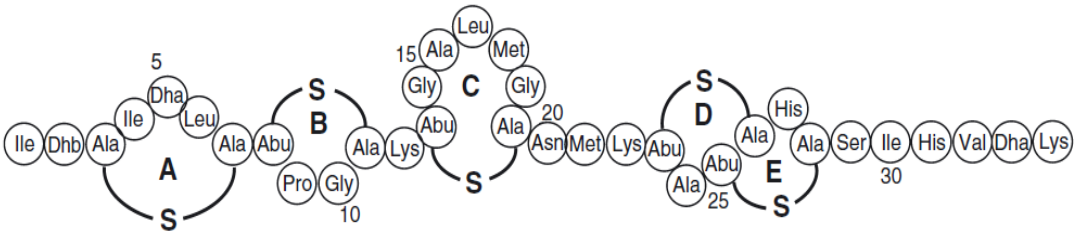


Figure 2.3: Nisin, an example of type A bacteriocins

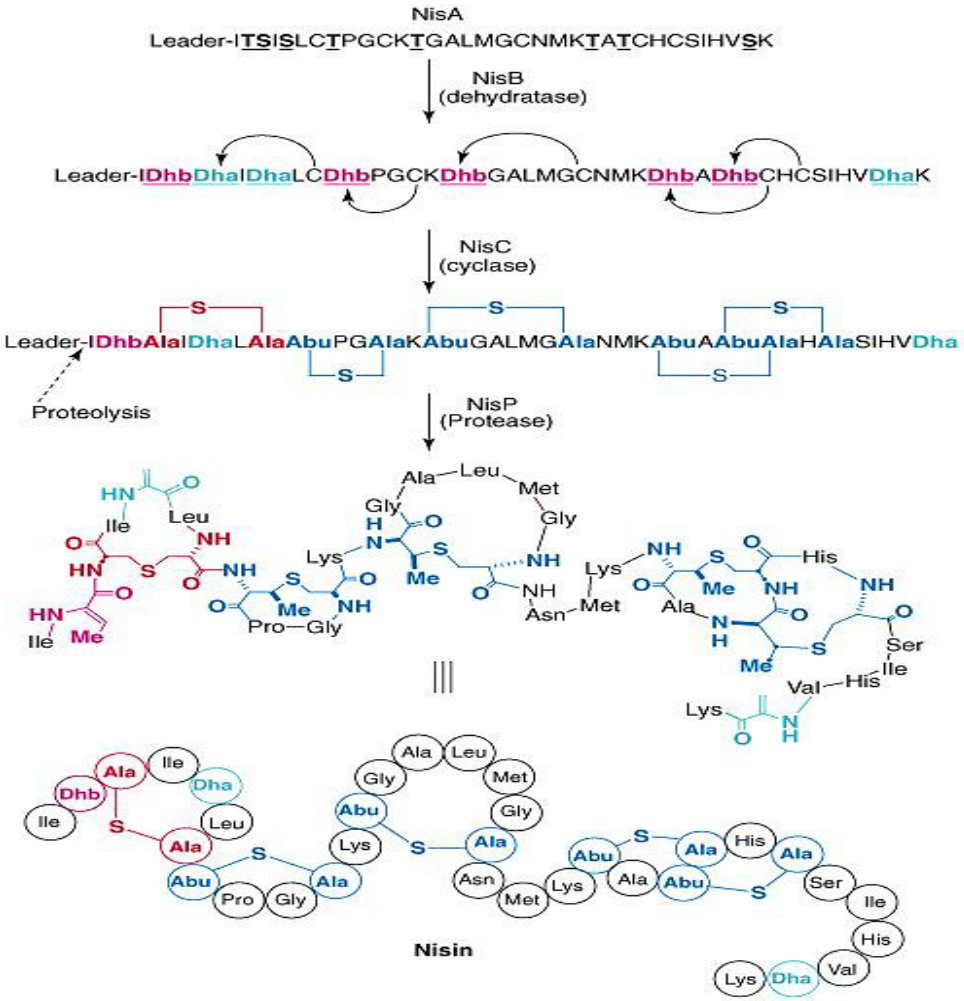


Figure 2.4: Post translation modifications during nisin biosynthesis (Xie and van der Donk, 2004)

2.3.2.1.2 Type B lantibiotics

There are some structural modifications between this subclass and type A lantibiotics. The molecular mass of known type B lantibiotics is 1959 to 2041 Da with zero or negative net charge (Altena *et al.*, 2000). Mersacidin produced by *Bacillus spp* is an example of type B lantibiotics which contains three ring-form structures of MeLan and a compact size of 20 amino acids (Herzner *et al.*, 2011). The mechanism of action of type B lantibiotics is not cytoplasmic membrane pore formation, but inhibition of cell wall synthesis by targeting the cell wall precursor lipid II (Brotz *et al.*, 1995; Sass *et al.*, 2008). Merasacidin is active against methicillin-resistant *Staphylococcus aureus* (MRSA) and it can be used as a potential alternative to vancomycin.

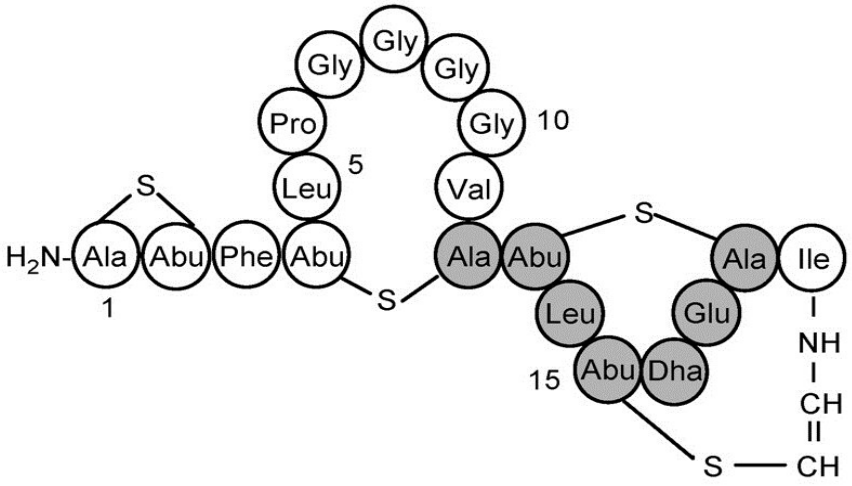


Figure 2.5: Merasacidin, an example of type B lantibiotics

2.3.2.1.3 Type C lantibiotics:

Type C lantibiotics basically consist of two peptides working synergistically in a sequential pathway with pore forming activity of the cytoplasmic membrane of the targeted cells. Lacticin 3147 is an example of this subclass of lantibiotics which consists of two peptides: Ltnα, which has a similar structure to type B lantibiotics and Ltnβ which is similar to type A lantibiotics in adopting an elongated linear conformation (Martin *et al.*, 2004). The activity starts when Ltnα binds to lipid II, followed by interaction of Ltnβ with the Ltnα-lipid II complex, which results in more peptide insertion and pore formation (Morgan *et al.*, 2005).

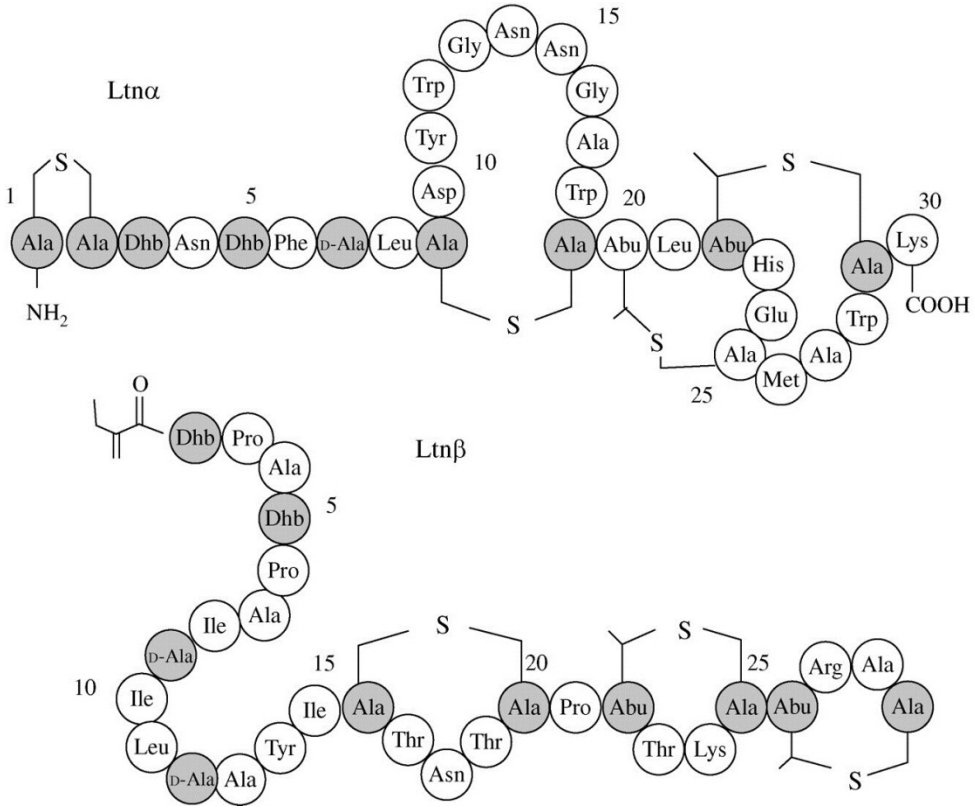


Figure 2.6: Lacticin 3147, an example of type C lantibiotics

Table 2.2: Lantibiotics produced by lactic acid bacteria

Lantibiotic	Producing LAB	Reference
Nisin A	*Lactococcus lactis*	(Gross and Morell, 1971)
Nisin Z	*Lactococcus lactis*	(Mulders *et al.*, 1991)
Nisin F	*Lactococcus lactis*	(de Kwaadsteniet *et al.*, 2008)
Nisin U	*Lactococcus lactis*	(Wirawan *et al.*, 2006)
Nisin U2	*Lactococcus lactis*	(Wirawan *et al.*, 2006)
Nisin Q	*Lactococcus lactis*	(Zendo *et al.*, 2003)
Mutacin B-NY266	*Streptococcus mutans*	(Mota-Meira *et al.*, 1997)
Mutacin 1140	*Streptococcus mutans*	(Hillman *et al.*, 1998)
Mutacin I	*Streptococcus mutans*	(Tsang *et al.*, 2005)
Mutacin K8	*Streptococcus mutans*	(Robson *et al.*, 2007)
Mutacin II	*Streptococcus mutans*	(Chikindas *et al.*, 1995)
Streptin	*Streptococcus pyogenes*	(Wescombe and Tagg, 2003)
Macedocin	*Streptococcus macedonicus*	(Georgalaki *et al.*, 2002)
Lacticin 481	*Lactococcus lactis*	(Piard *et al.*, 1993)
Streptococcin A-FF22	*Streptococcus pyogenes*	(Tagg and Wannamaker, 1978)
Salivaricin A1	*Streptococcus salivarius*	(Wescombe *et al.*, 2006)
Salivaricin A2	*Streptococcus salivarius*	(Wescombe *et al.*, 2006)
Salivaricin A3	*Streptococcus salivarius*	(Wescombe *et al.*, 2006)
Salivaricin A4	*Streptococcus salivarius*	(Wescombe *et al.*, 2006)
Salivaricin A5	*Streptococcus salivarius*	(Wescombe *et al.*, 2006)
Salivaricin A	*Streptococcus salivarius*	(Ross *et al.*, 1993)
Salivaricin B	*Streptococcus salivarius*	(Hyink *et al.*, 2007)
Salivaricin 9	*Streptococcus salivarius*	(Wescombe *et al.*, 2011)
Plantaricin C	*Lactobacillus plantarum*	(Turner *et al.*, 1999)
Plantaricin W	*Lactobacillus plantarum*	(Holo *et al.*, 2001)
Lacticin 481	*Lactococcus lactis*	(Piard *et al.*, 1993)
Lacticin 3147	*Lactococcus lactis*	(McAuliffe *et al.*, 1998)
Lacticin J46	*Lactococcus lactis*	(Huot *et al.*, 1996)
SmbB	*Streptococcus mutans*	(Yonezawa and Kuramitsu, 2005)
BhtA	*Streptococcus rattus*	(Hyink *et al.*, 2005)
Lactocin S	*Lactobacillus sake*	(Mortvedt *et al.*, 1991)
Bovicin HJ50	*Streptococcus bovis*	(Xiao *et al.*, 2004)
Bovicin HC5	*Streptococcus bovis*	(Mantovani *et al.*, 2002)
Cytolysin	*Enterococcus faecalis*	(Gilmore *et al.*, 1996)

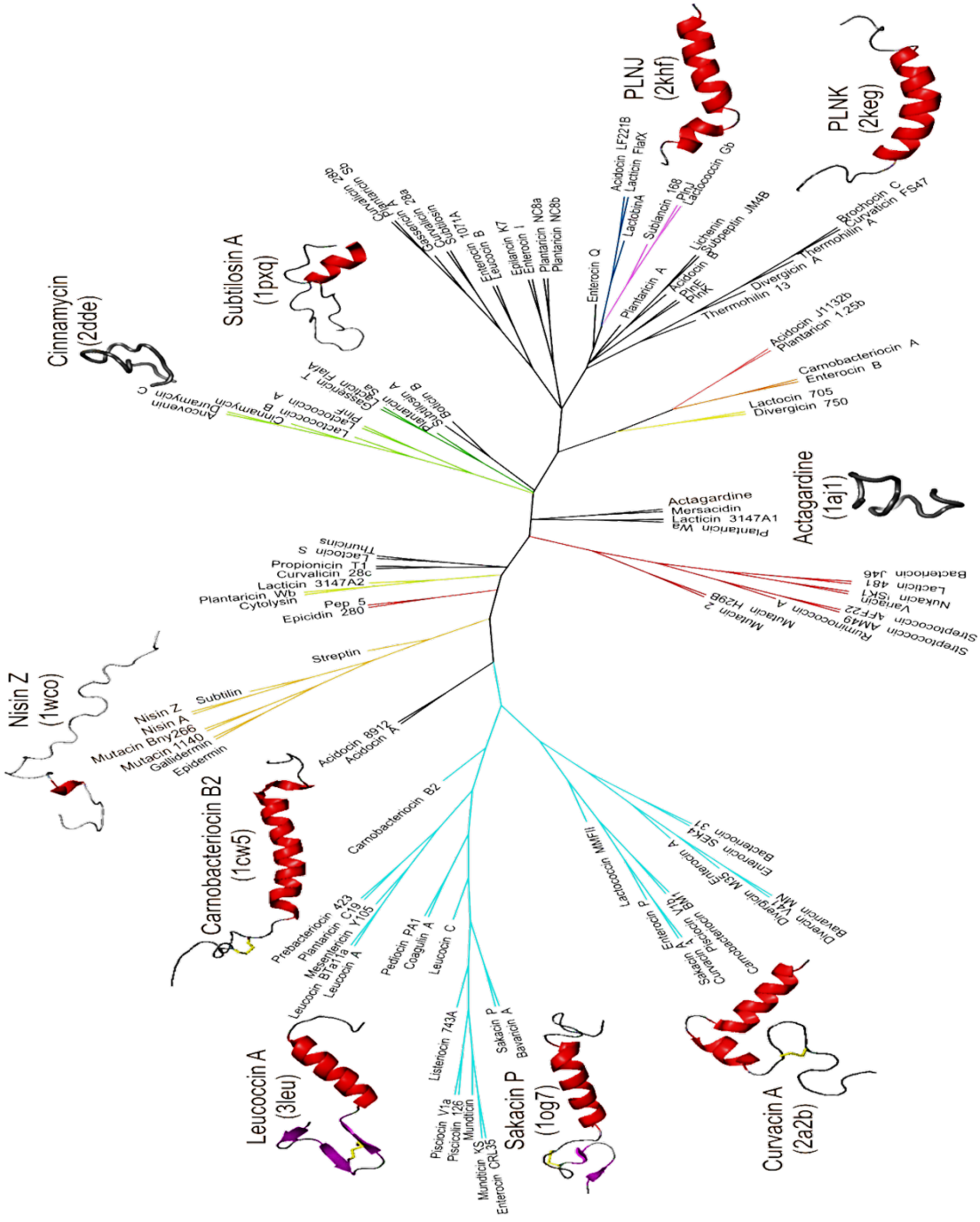


Figure 2.7: Some of bacteriocins types produced by different microorganisms http://bactibase.pfba-lab-tun.org. (Hammami *et al.*, 2010).

2.3.2.2 Class II: Non- modified bacteriocins

This group of bacteriocins can be divided into three subclasses:

Subclass IIa: Pediocin-like bacteriocins

Subclass IIb: Two peptide bacteriocins

Subclass IIc: Other peptide bacteriocins

2.3.2.2.1 Subclass IIa

The pediocin-like antimicrobial peptides are typical single functional peptides of the class II bacteriocins and usually include two cysteine residues that form a disulfide bridge (Eijsink *et al.*, 1998). Class IIa bacteriocins can be produced by many LAB, namely *Pediococcus, Enterococcus, Carnobacterium* and *Lactobacillus*. Pediocin-like peptides contain a YGNGV amino acid sequence in their N-terminus. Many studies suggested that the C-terminal of pediocin-like peptides play a significant role in their inhibitory activity spectrum (Fimland *et al.*, 1996). The mode of action of pediocin-like peptides is similar to type A lantibiotics and includes binding, insertion and pore formation in the cytoplasmic membrane of the targeted bacterial cell. The hinge motifs between the N-terminal and the C-terminal regions play a role in pediocin-like peptide penetration into the targeted membrane (Fimland *et al.*, 2005).

2.3.2.2.2 Subclass IIb

The complementary activity of two peptides is the basic characteristic of this kind of bacteriocin (Nissen-Meyer *et al.*, 1992). Sometimes the individual peptide cannot be active alone but clear synergistic activity is observed upon combination of the two peptides (Anderssen *et al.*, 1998).

2.3.2.2.3 Subclass IIc

This group of bacteriocins does not contain any of lantibiotics or subclass IIa and IIb bacteriocins but represents bacteriocins derived from a variety of LAB. These bacteriocins are non-cyclic and non-lantibiotic small peptides. The activity of this group of bacteriocin is due to either single or multiple bioactive peptides.

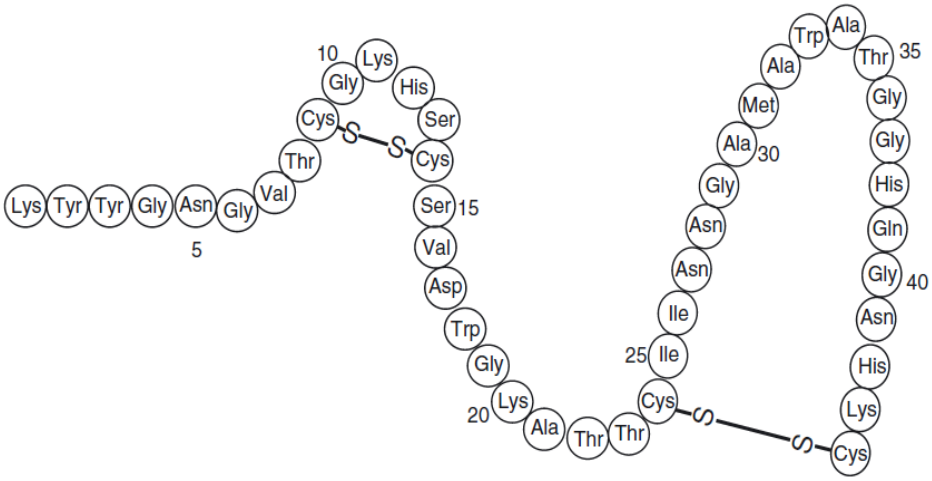


Figure 2.8: Pediocin example of Class II (unmodified bacteriocins)

2.3.2.3 Class III large bacteriocins

Members of this group are large >10 kDa, heat labile bacteriocins produced mostly by streptococcal bacteria. Zoocin produced by *Streptococcus equi. subsp zooepidemicus* is an example of this kind of bacteriocin. Zoocin is 27.8 kDa, antimicrobial peptide with similarity to lysostaphin (Simmonds *et al.*, 1997). The mechanism of action of zoocin can be summarized by binding to the putative receptor-recognition region of the targeted cell by the C-terminal domain of zoocin, while the N-terminal domain is involved in the peptidase activity of the molecule which targets the interpeptide crossbridge, which leads to cell wall hydrolysis. Dysgalacticin (21.5 kDa anionic protein produced by *Streptococcus dysgalactiae subsp. equisimilis*) is also a member of this group of bacteriocins and it differs from zoocin in the mode of action in that dysgalacticin kills its target without inducing lysis. Dysgalacticin inhibits sugar uptake into the sensitive cell which leads to loss of intracellular potassium ions and disruption of membrane integrity (Heng *et al.*, 2006; Swe *et al.*, 2009).

2.3.2.4 Class IV: Cyclic peptides

This group comprises the post translationally modified cyclic peptides whose first and last amino acids are covalently joined forming a head-to-tail linkage (Maqueda *et al.*, 2004). Enterocin AS-48 produced by *Enterococcus faecalis subsp. liquefaciens* is a member of this group. In enterocin AS-48 there is a peptide linkage between C-terminal Try-70 and N-terminal Met-1with no thioether residues in the structure. This bacteriocin is heat and pH stable (Maqueda *et al.*, 2004). Enterocin can be used in food preservation due to its effectiveness at inhibiting *Bacillus coagulans* and enhancing anti-spore activity when

combined with high temperature thermal treatment (Lucas *et al.*, 2006). Uberolysin produced by *Streptococcus uberis* is a 7048 Da cyclic peptide which induces lysis of the metabolically susceptible targeted cell. Uberolysin is post-translationally and covalently modified to form a head-to-tail monocycle (Wirawan *et al.*, 2007).

Chapter Three

Methodology

3.1 *Streptococcus salivarius* isolates and indicator strains:

The *Streptococcus salivarius* strains used in this study were strains HJEFF originally isolated and maintained in the culture collection of Koshy Philip (Fermentation Laboratory, Microbiology Division, Institute of Biological Sciences, University of Malaya), NU10, GT2, YU10, IND9 (isolated from healthy Malaysian subjects), SAM3, 7YE (isolated in the Dental Clinic of University of Malaya from Malaysian subjects with dental problems) and K12™ (producer of salivaricin A2 and salivaricin B) provided by John Tagg (University of Otago, New Zealand). *S. salivarius* strain ATCC13419 (BLIS-negative strain) was used as a reference strain. The indicator test strains (Table 3.1) were from the culture collection maintained in the Fermentation Laboratory, Microbiology Division, Institute of Biological Sciences, University of Malaya and American Type Culture Collection (ATCC).

Table 3.1: Indicators strains

Microorganisms	**Strains**
Actinomyces naeslundii	TG2
Bacillus cereus	ATCC14579
Corynebacterium spp	GH17
Gemella sanguinis	TGH12
Haemophilus parainfluenzae	TONEJ11
Lactococcus lactis	ATCC11454
Micrococcus luteus	ATCC10240
Staphylococcus aureus	RF122
Streptococcus equisimilis	ATCC12388
Streptococcus gordonii	ST2
Streptococcus mutans	GEJ11
Streptococcus pyogenes	ATCC12344
Streptococcus pyogenes	ATCC12384

3.2 Bacterial growth media and cultures

Difco Mitis Salivarius Agar (MSA) was used to obtain pure colonies of *S. salivarius* since it is a selective medium used for the isolation of *S. salivarius*, *Streptococcus mitis* and enterococci. Todd Hewitt Broth (THB) was used to propagate all *S. salivarius* isolates and indicator strains. Difco Columbia Agar Base (CAB) was used for antimicrobial activity assays (well diffusion and spot-on-lawn). Difco Columbia Agar Base supplemented with 0.1% (w/v) calcium carbonate and 5% (v/v) whole human blood (BACa) was used in simultaneous and deferred antagonism assays. Difco Tryptic Soy Broth supplemented with 1% (w/v) yeast extract, 0.1% (w/v) calcium carbonate (TSYECa) was also used in deferred antagonism tests. Difco M17 Broth supplemented with 1% sucrose and 0.1% calcium carbonate M17SUCa was used to produce BLIS activity in strains K12, YU10 and NU10.

3.3 Sterilization and storage conditions

All the liquid media used in this study were steam autoclaved at 121^{o}C for 15 minutes and cooled to 30^{o}C prior to use. Agar media was also prepared in the same way as the liquid media but they were cooled to 50^{o}C in water bath prior to pouring into petri dishes (20 ml each plate). After the agar had set, the plates were inverted and incubated overnight at 37^{o}C to ensure its contamination free condition before bacterial inoculation. Both liquid and agar media were stored at 4^{o}C for subsequent use.

3.3.1 Ultra violet (UV) sterilization

The unopened agar plate and surface was sterilized by exposing to UV light source (270 nm) for 25 minutes in a covered hood. Tips, pipettes, inoculation loops and all items used for bacterial sub-culture and inoculation were sterilized the same way prior to use.

3.3.2 Chloroform sterilization

The agar surface was inverted over a Whatman filter paper no 1 (soaked with chloroform) for 30 minutes before the agar surface was aired for another 30 minutes to evaporate any residue of chloroform left over. This sterilization technique was used specially for the deferred antagonism assay.

3.4 Isolation and identification of *Streptococcus salivarius*

Streptococcus salivarius strains were isolated from Malaysian subjects using sterilized cotton swabs, either from the dorsal surface of the tongue or from saliva. Each subject who agreed to participate in this study was required to sign a consent form for isolation of *S. salivarius* from their oral cavity. All protocols used were based on Good Laboratory Practices and isolation from subjects conducted with the necessary approval of the University Ethics Committee and complied with the principles of the Helsinki Declaration. The cotton swabs were kept in 0.85% NaCl before streaking on MSA plates within 1 hour of isolation. The plates were incubated anaerobically for 18 h at 37^{o}C and dome shaped blue colonies were selected and subsequently sub-cultured on fresh MSA (commonly used

for the detection of oral streptococci in human and animals) (Takada *et al.*, 2006). Glycerol stocks of the pure cultures were kept at -20^{o}C. Some of the *S. salivarius* isolates have rough shaped colony morphology which can adhere to the agar medium like strain GT2, while the others appeared smooth. Gram staining, catalase and oxidase tests were applied before using 50 CHL and 20 Strep API kits (Biomerieux) to identify the biochemical characteristics of the isolates and the final bacteria identification was done using 16S rDNA gene sequencing. DNA was extracted using i-genomic mini kit from iNtRON Biotechnology, Korea, with some modifications. The bacterial pellets were washed thrice with NaCl 85% to reduce the amount of proteins attached to the cell wall and then incubated 10 hours with lysozyme (50 mg ml^{-1}) at 37^{o}C during the lysis step. Universal primers 27f (AGAGTTTGATCATGGCTCAG) and 1492r (TACGGCTACCTTGTTACGACTT) were used to amplify the 16S rDNA gene. The 16S rDNA gene sequence of the samples was compared to the human oral microbiome Database BLAST tool.

For further investigation of the morphology of the isolates, transmission electron microscopy TEM was applied to visualize and determine the morphology and size of *S. salivarius* cells and how they replicated to form the typical chaining arrangements.

Levansucrase enzyme was detected in the cell-free supernatant of strain YU10 as follows; M17 medium supplemented with 20% sucrose was used to produce the enzyme from strain YU10 after 18 hours of incubation aerobically at 37^{o}C. The sample was subjected to LC-MS/MS analysis (Thermo Fisher Scientific, UVic Genome BC PROTIOMICS CENTRE) and the raw data were analysed with Proteome Discoverer 1.3.0.339 software suite (Thermo Scientific).

3.5 Simultaneous antagonism test

A sterilized toothpick was used to pick colonies of *S. salivarius* grown on MSA and stabbed into a fresh lawn of *Corynebacterium spp* GH17 or *Micrococcus luteus* ATCC10240 seeded on blood agar supplemented with 0.1% $CaCO_3$. This assay was done in duplicate plates which were then incubated aerobically at 37^{o}C for 18 hours. A clear zone of inhibition surrounding the site where the producer was stabbed was recorded as positive bacteriocin-like activity. *S. salivarius* K12™ (BLIS Technologies) was used as a positive control in this study (Figures 4.13 and 4.14)

3.6 Deferred antagonism test

The deferred antagonism test described previously (Tagg and Bannister, 1979) was used with minimum modification. The producer strains were streaked across Tryptic Soy agar plates supplemented with 1% yeast extract and 0.1% calcium carbonate (TSYECa) or BACa plates as a 1 cm wide strip using a sterilized cotton swab. Then the plates were incubated anaerobically at 37^{o}C for 20 hours. Then the visible growth of the producer strain was removed by using a glass slide and then the agar surface was sterilized by exposing to chloroform vapors by inverting the plate over a filter paper soaked with chloroform for 30 min to kill any living but invisible bacterial cells. The plates were aired for 30 min to remove any chloroform residue. Two colonies of the indicator strains already grown on CAB (Columbia agar base) were suspended in 3 ml of 0.85% NaCl. Then a cotton swab was immersed in this suspension and streaked across the agar plate at a right angles to the

producer streak. The plates were again incubated aerobically for 18 hours at 37^{o}C. Zones of growth inhibition indicated the presence of inhibitory substances produced by the producer strain. Results were recorded as follows: + inhibition zone < 0.75 cm, ++ inhibition zone = 0.75 - 1 cm, +++ inhibition zone > 1 cm, - no inhibition.

3.7 Distribution of *salA*, *sboB* and *sivA* structural genes in *S. salivarius* isolates

Primers to detect *salA*, *sboB* and *sivA* (Table 4) were synthesized according to Wescombe *et al.*, 2006 and Wescombe *et al.*, 2011). The PCR reactions included 30 cycles that included denaturation at 95^{o}C for 30 s, annealing at 55 (*salA* and *sivA*) or 44^{o}C (*sboB*) for 30 s and finally extension at 65^{o}C for 30 s. The final PCR mixture volume was maintained at 50 µl for each single reaction and consisted of 41.5 µl of ultrapure DNA-free water, 5 µl of 10X buffer (i-genomic, Korea), 1 µl of dNTP (i-genomic, Korea), 1 µl of each forward and reverse primer (10 pmol ml^{-1}) and 0.5 µl of i-Taq (5 U ml^{-1}) (i-genomic, Korea).

Table 3.2: Primers used to detect the genes encoding peptide (salivaricin A, B and 9)

Lantibiotic	Primer name	Primer sequence	Reference
SalivaricinA	salAUS	GTAGAAAATATTTACTACATACT	(Ross *et al.*, 1993)
	salADS	GTTAAAGTATTCGTAAAACTGATG	
SalivaricinB	salBF	GTGAATTCTCTTCAAGAATTGACTCTT	(Hyink *et al.*, 2007)
	salBR	AAAATATTCATACCGCTCTTCC	
Salivaricin9	sivF	AAAAAGGCGCTTCTATATCCATGA	(Wescombe *et al.*, 2011)
	sivR	ATCTTTACCTCAAACTTTTAAGTCCATT	

3.8 Evaluation of inhibitory activity

3.8.1 Production of inhibitory activity in liquid media

All *S. salivarius* strains failed to express detectable amount of inhibitory substances in liquid media, except strain HJEFF which yielded a very small quantity of inhibitory activity while growing in Mueller Hinton broth (MHB). Growth experiments were performed in a 2-litre fermenter (BRAUN BIOTECH INTERNATIONAL) using 2 litre of MHB or THB. *S. salivarius* [4.8 x 10^4colony forming units (CFU) ml^{-1}] was inoculated and the culture was incubated at 37^{o}C for 41 h. Cell viability was determined by the plate dilution method using MSA. After enumerating the CFU every hour to determine the growth patterns of *S. salivarius* early stationary phase the cells were collected for BLIS extraction. The cells were sedimented by centrifugation 11,000 x g for 5 min followed by filtration of the supernatant using a Millipore filter (0.22 µm pore size). Then ammonium sulphate was added to attain 50% saturation. After stirring and centrifugation (15,000 x g for 20 min at 4^{o}C), the precipitate was collected and then dissolved in a minimal volume of phosphate buffer saline (PBS) made up to pH 7.0. The crude bacteriocin preparation was desalted by using 3,000 Da cut-off dialysis centrifuge tube and then tested for inhibitory activity.

3.8.1.1 Binding of BLIS produced by strain HJEFF to DEAE anion exchanger column

Desalted crude peptide produced by strain HJEFF was manually injected onto a DEAE anion exchanger column equilibrated in Buffer A (20 mM Tris, pH 8.0) at a flow rate of 1ml min^{-1}.

Table 3.3: Column and buffers used in anion exchange study.

Column*	Buffer A	Buffer B
DEAE FF (1 ml)	20 mM Tris, pH 8.0	20 mM Tris, pH 8.0 + 1 M NaCl

*HiTrap DEAE FF diethylaminoethyl Sepharose, fast flow (weak anion exchanger).

The column was then washed with 10 ml of buffer A and the effluent was designated as the "unbound" fraction. Fractions containing the inhibitory activity started to elute from the column following application of buffer B. Each fraction was tested for inhibitory activity by the spot-on-lawn method using *Corynebacterium spp* as the indicator strain.

3.8.2 Acidic methanol extraction

This method was as described by Wescombe *et al*. (2006) as a standard procedure for recovery of Sal-A like peptides, with little modification. One liter of THB base supplemented with 0.1% (w/v) calcium carbonate was inoculated with *S. salivarius*. The fermentation process was done by using a BRAUN BIOTECH INTERNATIONAL fermenter at 32^{O}C for 18 h. The initial preparation of the BLIS was obtained by extracting the cells obtained from the fermentation process mentioned above with 200 ml of 95% methanol (adjusted to approximately pH 2 by adding 25 mmol l^{-1} of HCl) at 4^{o}C for 20 h. After centrifugation (15000 x g), the supernatant was subjected to rotary evaporation to

remove the methanol. The extract was then lyophilized and re-dissolved in 1 ml of PBS buffer (pH 7). This preparation was used in antimicrobial assay (well-diffusion) to check for inhibitory activity.

3.8.3 Freeze thaw extraction

It was noticed that when the producer strain, *S. salivarius* NU10, is grown in the presence of a small quantity of its own bacteriocin product large quantities of bacteriocin are produced. A newly modified medium was used in this production experiment that included M17 broth supplemented with 1% sucrose, 0.1% calcium carbonate, 0.1 fetal calf serum (Sigma) and 0.7% agarose. After the media was autoclaved and cooled down to 50^{o}C, 0.05 g mL^{-1} of crude NU10 peptide (4 AU/ml) and 1% of an 18-hour old *S. salivarius* NU10 culture grown in THB were added to the production medium prior to pouring it into petri dishes (20 ml per dish). After the agarose was set, the plates were incubated anaerobically at 37^{o}C for 22 hour before they were transferred to -80^{o}C freezer and kept there for 10 hours. Then the plates were taken out of the freezer and thawed at room temperature where all the plates' contents (liquid and agarose) were transferred to sterilized gauze to separate the agarose debris. The liquid was collected in sterilized Scott bottle. After the cells were removed by centrifugation at 12000 x g for 20 min at 4 ºC, the supernatant was collected and stored at 4ºC for further purification.

3.9 Purification of BLIS produced by *Streptococcus salivarius* NU10

After isolation of the bacteriocin by freeze thaw extraction, solid ammonium sulphate was added to the crude peptide to achieve 65% saturation before the solution was stirred overnight at 4^{o}C to dissolve the ammonium sulphate. The peptide pellet was collected after centrifugation at 18000 x g for 25 minutes at 4^{o}C and the peptide was dissolved in a minimal volume of 25 mM phosphate buffer pH 5.8. This preparation was desalted on SEPHADEX G 25 column (sigma) (Column volume 100 ml and diameter 1.5 cm). The column was washed with twice the column volume of 25 mM phosphate buffer pH 5.8 before the sample of 5ml was loaded at a flow rate of 3ml min^{-1}. Protein concentration of the fractions was measured using Nanodrop 2000 at 280 nm (Figure 4.22). All the fractions were tested for bacteriocin activity by loading 50µl of each fraction into pre-cut wells on CAB freshly seeded with *Micrococcus luteus* ATCC® 10240. Fractions showing antimicrobial activity were pooled and lyophilized for storage purpose. The lyophilized fraction was dissolved in distilled water and transferred to a shaker flask with 20 g of Amberlite® XAD-2 resin added. The mixture was incubated at 4^{o}C for 2 hours on 150 rpm orbital shaker. The matrix was packed into glass chromatography column and washed with 200 ml of distilled water, 200 ml 70% methanol and 200 ml 95% methanol at pH 2. Fractions were collected at a flow rate of 5 ml/min. The methanol was evaporated and the pellet was dissolved in ultrapure water and filtered by 0.2 nm millipore membrane before injecting into Sep-Pack C18 classic cartridge (Waters Model). The cartridge was washed with 10 ml of 95% methanol at pH 2 followed by 10 ml ultrapure water before the sample was loaded. To elute the activity, 2 ml of different concentrations of methanol were used as

follows: 50%, 60%, 70%, 80% and 95% adjusted to pH 2. The fractions were subjected to speed vacuum to evaporate the methanol before antimicrobial activity was tested.

3.10 BLIS-NU10 induction assay

BLIS-NU10 production was shown to be auto regulated in this study whereby a small amount of the active peptide could induce its production in a large scale production system. One colony of an 18-hour old culture of *S. salivarius* NU10 grown anaerobically on BACa agar was used to inoculate 10 ml of M17YESUCa broth and incubated again under the same conditions before the cells were collected by centrifugation and washed 3 times in saline buffer to reduce the amount of BLIS-NU10 attached to the cells. Two tubes containing 0.9 ml of M17YESUCa were inoculated with 0.1 ml of the cell suspension. 0.05 ml of BLIS-NU10 preparation (titre 4 AU/ml) was added to one of the tubes marked as "induced" and the other marked as "control". Both tubes were incubated anaerobically for 18 h before 0.05 ml BLIS-NU10 preparation (titre 4 AU/ml) was added to the control tube. 50µl of each sample (induced and control) was used to test for antimicrobial activity by well diffusion assay. The positive control was recorded as a clear zone of inhibition representing the induced sample and the inhibition representing the control sample appeared nil or faint (Table 4.9).

3.11 Production of BLIS-NU10 in liquid medium

Usually bacteriocins produced by *S. salivarius* can be isolated from cultures grown on solid medium using the freeze thaw method described previously (Hyink *et al.*, 2007). Strain NU10 is also able to produce the bioactive peptide in solid phase medium and showed extremely weak expression of BLIS-NU10 in the liquid media. To overcome this limitation, a new technique was used in this study to enhance the production of the BLIS-NU10 in liquid media. Strain NU10 was grown on MSA plate for 18 h under anaerobic condition at 37^{o}C before one colony (dome-shape) was used to inoculate 20 ml of M17YESUCa broth and incubated under the same conditions mentioned above on an orbital shaker at 150 rpm. The broth culture was centrifuged at 6000 x g for 5 min at 4^{o}C before the cells were collected and washed twice in 0.85% NaCl sterile solution and then resuspended in 20 ml of the same solution. The cell suspension was used to inoculate 50 ml of fresh M17YESUCa broth at 37^{o}C in anaerobic condition for 20 h. The resulting culture was fed with fresh 50 ml of the same medium and 0.05 g/ml of pure BLIS-NU10 was added to the broth medium as an inducer before the 100 ml culture was incubated for another 18 h. 80 ml of the final culture was used to inoculate 1.5 litres of M17YESUCa broth. The pH was adjusted to 6.5 using concentrated HCl. CFU and AU was measured for colony forming units and arbitrary units per ml during production (Figure 4.31).

3.12 Cation exchange chromatography

Induced culture of *S. salivarius* NU10 was centrifuged at 8000 x g to pellet the cells. The cell free supernatant (CFS) was filtered through 0.2 µm sterilized cellulose membrane (Millipore). The filtered crude bacteriocin (800 ml) was passed through 100 ml XAD-16 particles (Sigma) packed in a glass column. The column was washed with 400 ml of distilled water followed by 200 ml of 70% methanol before the active fraction was eluted using 200 ml of 95% methanol (adjusted to pH 2) at a flow rate of 15 ml/min. The methanol was removed using a rotary evaporator and the resulting peptide was lyophilized and stored at -20^{o}C. The lyophilized peptide pellets were dissolved in 20 ml of 20 mM sodium phosphate pH 5.8 (binding buffer) Then the sample was injected into fast protein liquid chromatography (FPLC) system (ÄKTA Purifier™, Malaysia Genome Institute) using SP FF 5ml strong cation exchanger column (GE Healthcare) equilibrated with the same buffer at a flow rate of 1 ml/min. Then the column was washed with 10X column volume of the binding buffer before BLIS-NU10 was eluted using linear gradient of 0 to 1M NaCl in 20 mM sodium phosphate buffer at pH 5.8 (Figure 4.32). All bound and unbound fractions were tested for inhibitory activity using *Micrococcus luteus* GAB13 as the indicator microorganism.

Table 3.4: Column and buffers used in cation exchange study.

Column*	Buffer A	Buffer B
SP FF (5 ml)	20 mM sodium phosphate PH 5.8	20 mM sodium phosphate PH 5.8 + 1 M NaCl

*HiTrap SP FF is prepacked with SP Sepharose Fast Flow and is a strong cation exchanger.

3.13 Tris-Tricine SDS PAGE

The active fractions eluted from the FPLC system were subjected to 16.5% sodium dodecyl sulphate (SDS) as described previously (Schagger, 2006) and Tris-Tricine running buffer system. Mini-Protean Tetra Cell (Bio Rad) was used according to the manufacturer's instructions. Dual Xtra protein marker (Bio Rad) was used to estimate the molecular weight of the pure BLIS-NU10. The gel was run at 125 V for 45 min and stained using SimplyBlue™ SafeStain (Life Technologies-Invitrogen). After de-staining, the gel was visualized and the molecular weight was estimated (Figure 4.34).

3.14 Activity assay of the purified BLIS-NU10 peptide

The samples were subjected to two-fold serial dilutions and 20µl samples were spotted onto the surface of CAB freshly seeded with *Micrococcus luteus* ATCC® 10240. After incubation at 37^{o}C for 18 hours, zones of inhibition were observed and the arbitrary units of the inhibitor peptide per ml were measured. The arbitrary unit (AU) is the reciprocal of the highest dilution of the pure peptide that showed clear inhibition of *Micrococcus luteus* growth (Mantovani and Russell, 2008) as shown in Figure 4.25.

3.15 Characterization of BLIS-NU10 peptides

The pure FPLC fraction was subjected to nano liquid chromatography electrospray ionization mass spectrometry LC/MS-ESI analysis. The sample was injected into a Symmetry C18 pre-column (5 µm, 20 mm x 180 µm) attached to BEH C18 reversed phase analytical column (1.7 µm, 20 cm x 75 µm) using nano ACQUITY@ UPLC system at the Malaysia Genome Institute located in Bangi, Malaysia. Elution was carried out with solvent A (0.1 formic acid in water) and solvent B (0.1 formic acid in acetonitrile) with a gradient of 4-45% of solvent B in solvent A at a flow rate of 3 µl /min for 70 min. The eluted peptide molecules were subjected directly to positive electrospray ionization. Q-TOF premier system and Protein Lynx Global SERVER V 2.4 software (Malaysia Genome Institute) were used to search for peptide spectra. Data directed analysis (DDA) was used for de novo amino acids sequencing. For further analysis pure BLIS-NU10 was analyzed by matrix-assisted laser desorption ionization - time of flight (MALDI-TOF) mass spectrometry (MS) at the Medical Biotechnology Laboratory, Faculty of Medicine, University of Malaya (Figure 4.35 and 4.36).

3.16 Mode of action bacteriostatic, bactericidal and bacteriolytic

One ml of purified peptide was added to 10 ml of log-phase culture of the indicator strain (*Micrococcus luteus* ATCC) grown in THB. The optical density was measured at 620 nm at various times to check if there is any bacterial growth in the bacteriocin-containing culture. One ml of distilled water was added to the sensitive culture as a negative control. If the indicator bacteria succeeded in growing after sometime, it means the mode of action of the inhibitor is bacteriostatic but if it fails then it is bactericidal as shown in figure 4.27. Growth kinetics of the indicator bacteria was recorded and analyzed by Microsoft Excel software. The bactericidal effect of pure BLIS-NU10 was studied at different phases of growth as shown in figure 4.28 where salivaricin was added to freshly inoculated culture after 10, 15, 30, 60, 120 and 210 minutes at 37^{o}C and the bacterial growth was recorded by measuring OD_{600}.

3.17 Membrane permeabilization assay

Indicator bacteria *Micrococcus luteus* ATCC 10240, *Streptococcus equisimilis* ATCC12388 and *Corynebacterium spp* GH17 were subcultured on CAB (Columbia agar base). One colony was used to inoculate 1 ml of THB (Todd Hewitt broth) and incubated at 32^{o}C aerobically for 18 hours at 150 rpm. 1 ml of test strain culture was used to inoculate 10 ml of THB and incubated with the same conditions mentioned above. The culture was grown to an OD^{600nm} value of 0.6 (exponential growing phase). Then the bacterial CFU was enumerated using Miles and Misra method (surface viable count) (Heritage *et al.*, 1996). At

OD^{600nm} = 0.6 the CFU count was 3.4 X 10^5. The bacterial culture was centrifuged at 2,000 x g for 15 minutes, after which the supernatant was discarded. Bacterial pellets were washed with 10mm sodium phosphate buffer at pH 7.2 and centrifuged at 2,000 x g for 15 minutes. This washing step was performed twice. Pellet was suspended with 10 ml of 10 mM sodium phosphate sterile buffer pH 7.2. The bacteria was diluted again to reach OD_{600} = 0.6 in sodium phosphate buffer. Final volume was made up to 10 ml. 5 μl^{-1} of SYTOX stock solution was added to 5 ml of bacterial preparation mentioned above. 10 µl of peptide solution to be tested at different concentrations was added to 90 µl of the (bacteria cells + SYTOX) preparation in 96-Well Real Time PCR Plate. Positive control (acidic methanol extract from *S.salivarius* K12 and 70% ethanol) was also added to the same bacterial preparation. For negative control bacterial cells without addition of any peptide or positive control was used. Each experiment was performed in triplicate. The wells were sealed by using optical cap strips and the plate was placed as soon as possible in the Real-Time PCR. The real-time PCR (Applied Biosystems StepOnePlus™Real-Time PCR Systems) was programmed with SYBR Green filter selected and 60 cycles of 1 min duration at 37^{o}C read at the end of each cycle. Raw data was exported into Excel. Results were expressed as fluorescence units (FU). Each sample was tested in triplicate. Mean values and standard deviation were calculated using Microsoft Excel.

3.18 Scanning electron microscopy (SEM)

SEM was used to visualize the mechanism of antimicrobial action of BLIS NU10. Indicator bacteria *Micrococcus luteus* ATCC 10240 was grown in THB for 10 hours till it reached the exponential phase with CFU count of 1.2×10^6. The cells were then centrifuged at 8000 x g for 5 minutes and washed with 20 mM HEPES buffer pH 7 (washing step was repeated twice). Cells were centrifuged again at 9000 x g for 2 minutes, supernatant was discarded and bacterial pellet was resuspended in 1 ml of purified BLIS NU10 containing 8 AU/ml. The samples were fixed with 8% glutaraldehyde in 1:1 (v/v) Sorensen's phosphate buffer for one hour. Samples were then washed with Sorensen's phosphate buffer and water mixture 1:1 (v/v) before the samples were fixed with 4% OsO_4 mixed with 1:1 (v/v) H_2O and then left overnight. After washing with deionized water for 15 minutes, the samples were dehydrated in increasing concentrations of ethanol as follows: 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100%. Dehydration with ethanol-acetone mixture was applied before the samples were finally washed with pure acetone as the last step of preparation before critical point drying (CPD). After the samples were prepared and coated with gold particles the mechanism of antimicrobial action was visualized using JEOL JSM-7001F Scanning Electron Microscope. Control sample (without BLIS NU10 treatment) and treated sample were then compared for morphological changes.

3.19 Stability tests on BLIS-NU10

Heat stability of pure salivaricin was studied by treating the peptide at different temperatures for one hour following which the sample was centrifuged and the supernatant was tested for inhibitory activity. The stability of the peptide at different pH values was also evaluated and tested similarly. For enzymatic effects on salivaricin stability, two wells of 6mm diameter each separated by a distance of 4 mm were prepared in a CAB plate. One well was filled with 50µl of the pure salivaricin while the other was filled with 50µl of the enzyme (1 mg/ml). The plate was incubated at 50^{o}C for 2 hours and then at 37^{o}C overnight before the indicator strain was seeded onto the agar surface using a cotton swab. Then the plate was re-incubated at 37^{o}C for 18 hours and the zones of inhibition that appeared indicated the stability of salivaricin, while absence of inhibition zones indicated the denaturation of the antimicrobial peptide by the applied enzymes namely proteinase K, peptidase, lyticase and catalase The stability of BLIS-NU10 to the chemicals namely EDTA, SDS, urea, NaCl and β-merchaptoethanol was examined by adding 1% of the chemicals to the bacteriocin followed by incubation for two hours at RT before the samples were centrifuged and the supernatant was tested as described above. If the chemical was a solvent e.g. β-mercaptoethanol, the solvent was evaporated before testing for antimicrobial activity (Table 4.11).

Chapter Four

Results

4.1 *Streptococcus salivarius* isolation and identification

All the strains were isolated from the oral cavities of Malaysian subjects. Some of them were isolated from the dorsal surface of the tongue while the others from the saliva. It appeared that all isolates were negative for both catalase and oxidase tests with gram positive characteristics when viewed under the light microscope after gram staining Figure (4.1-4.4).

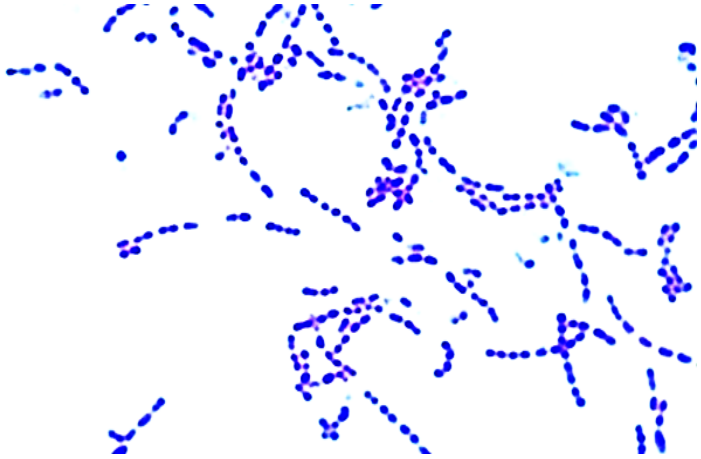


Figure 4.1: Gram staining of *Streptococcus salivarius* NU10

Figure 4.2: Gram staining of *Streptococcus salivarius* YU10

Figure 4.3: Gram staining of *Streptococcus salivarius* K12

Figure 4.4: Gram staining of *Streptococcus salivarius* GT2

Table 4.1 summarizes the basic characteristics of the isolates with results from the simultaneous antagonism assay to evaluate the ability of these strains for producing

antimicrobial agents. Strains NU10 and YU10 were most significant for inhibitory activity as shown in figure 4.13 where a large halo zone can be observed surrounding the spot where the producer was stabbed. Strain GT2 also showed some activity, but it appeared as a smaller halo zone. Further study showed that the inhibitory activity of this strain can be extracted using the acidic methanol extraction method of the GT2 cells. All the isolated colonies appear blue dome-shaped when grown on MSA plate. Some of the strains appeared to have rough colony morphology like strain GT2 which adhered strongly to the surface of the agar. This was challenging, especially when the deffered antagonism test was applied because the macroscopic growth (visible to the naked eye) of the producer strain needed to be scraped off the agar surface before indicator bacteria were applied by swabbing. Other colonies of the strains in the study appeared smooth. All the isolates appeared to have a dome shape on MSA and this puffy gum shape is due to the production of levan as levansucrase enzyme was also detected from the methanol extract of the producer's cells. Some modification on the manufacturer's protocol was applied to isolate the total DNA by adding 100 mg/ml of lysozyme with overnight incubation at 37^{o}C increased the yield of DNA recovered significantly. The thick cell wall of *S. salivarius* makes it difficult to lyse in the presence of lysozyme for a short period of incubation (30 min). But the cell lysis was enhanced significantly with overnight incubation in lysozyme.

The 16s rDNA gene sequences of the isolates were assembled using DNA baser software. After that, the data was blasted on the human oral microbiome database www.homd.org. All strains scored ≥99% on the database. 16S rDNA gene identification finally confirmed the identity of the isolates.

Table 4.1: The basic characteristics of *Streptococcus salivarius* isolates

S. salivarius isolate	Gram Stain	Shape	Catalase Test	Oxidase Test	Isolation source	Antagonism activity*	
						Micrococcus luteus	*Corynebacterium Spp*
NU10	Positive	Cocci	-	-	Tongue	+	+
YU10	Positive	Cocci	-	-	Tongue	+	+
7YE	Positive	Cocci	-	-	Saliva	-	-
GT2	Positive	Cocci	-	-	Tongue	±	-
HJEFF	Positive	Cocci	-	-	Tongue	-	-
IND9	Positive	Cocci	-	-	Saliva	-	-
SAM3	Positive	Cocci	-	-	Saliva	-	-

* Simultaneous antagonism test on BACa plates.

16s rDNA gene sequencing identification scores according to BLAST tool used in human oral microbiome database are listed in Table 4.3. The NCBI data base was used to confirm the identity of the isolates. All the isolates showed to be *S. salivarius*. Finally the 16s rDNA gene sequences of all the isolates were deposited in NCBI gene bank.

API tests were used to determine the metabolic profile of the strains as shown in Table 4.2. Strains NU10 and YU10 differ from strain K12. While K12 was able to metabolize D-galactose, both strains NU10 and YU10 were negative to this biochemical test. Both strains K12 and YU10 were able to metabolize D-tagatose while strain NU10 showed otherwise. Inulin metabolism was positive for both strains K12 and NU10 while this test was negative when strain YU10 was used. However, strain YU10 metabolized both D-sorbitol and GENtiobios, but both strains K12 and NU10 did not metabolize these substrates. Strain NU10 was the only strain that showed positive reaction when incubated with AMYgdaline. The entire biochemical test for all the strains showed a similar result when API 20 Strep was used as they are listed in Table 4.2. The main reason for conducting the API biochemical tests was to identify the metabolic profile of strains NU10 and YU10 as they showed the best production pattern and it was useful to compare their metabolic pattern with that of strain K12 the commercial probiotic which can produce both salivaricin A2 and salivaricin B.

Table 4.2: Metabolic profile of *S. salivarius* isolates using API 20 Strep and API 50 CH.

API 20 Strep	K12	NU10	YU10
Acetoin production	+	+	+
β-Glucosidase	+	+	+
Pyrrolidonyl arylamidase			
6-Bromo-2-naphthyl _-D-galactopyranoside	-	-	-
Naphthol AS-BI_-D-glucuronate	-	-	-
D- galactosidase	-	-	-
Alkaline phosphatase	+	+	+
Leucine arylamidase	+	+	+
Arginine dihydrolase	-	-	-
Ribose	-	-	-
L-Arabinose	-	-	-
Mannitol	-	-	-
Sorbitol	-	-	-
D-Lactose	+	-	+
Trehalose	+	+	+
Inulin	+	+	-
Raffinose	+	+	+
Starch (2)	-	-	-
Glycogen	-	-	-
API 50 CH			
D-Galactose	+	-	-
D-Glucose	+	+	+
D-Fructose	+	+	+
D-Mannose	+	+	+
N-Acetylglucosamine	+	+	+
Arbutine	+	+	-
Salicin	+	+	+
D-Cellobiose	+	+	+
D-Maltose	+	+	+
D-Lactose	+	-	+
D-Saccharose	+	+	+
D-Trehalose	+	+	+
Inulin	+	+	-
D-Raffinose	+	+	+
D-Tagatose	+	-	+
AMYgdaline	-	+	-
D-Sorbitol	-	-	+
GENtiobios	-	-	+

Several of the *S. salivarius* isolates were observed by transmission electron microscopy (TEM) to determine the size of the bacteria and details of the bacterial cell morphology. TEM pictures showed that the chain-forming bacteria are surrounded by capsular material.

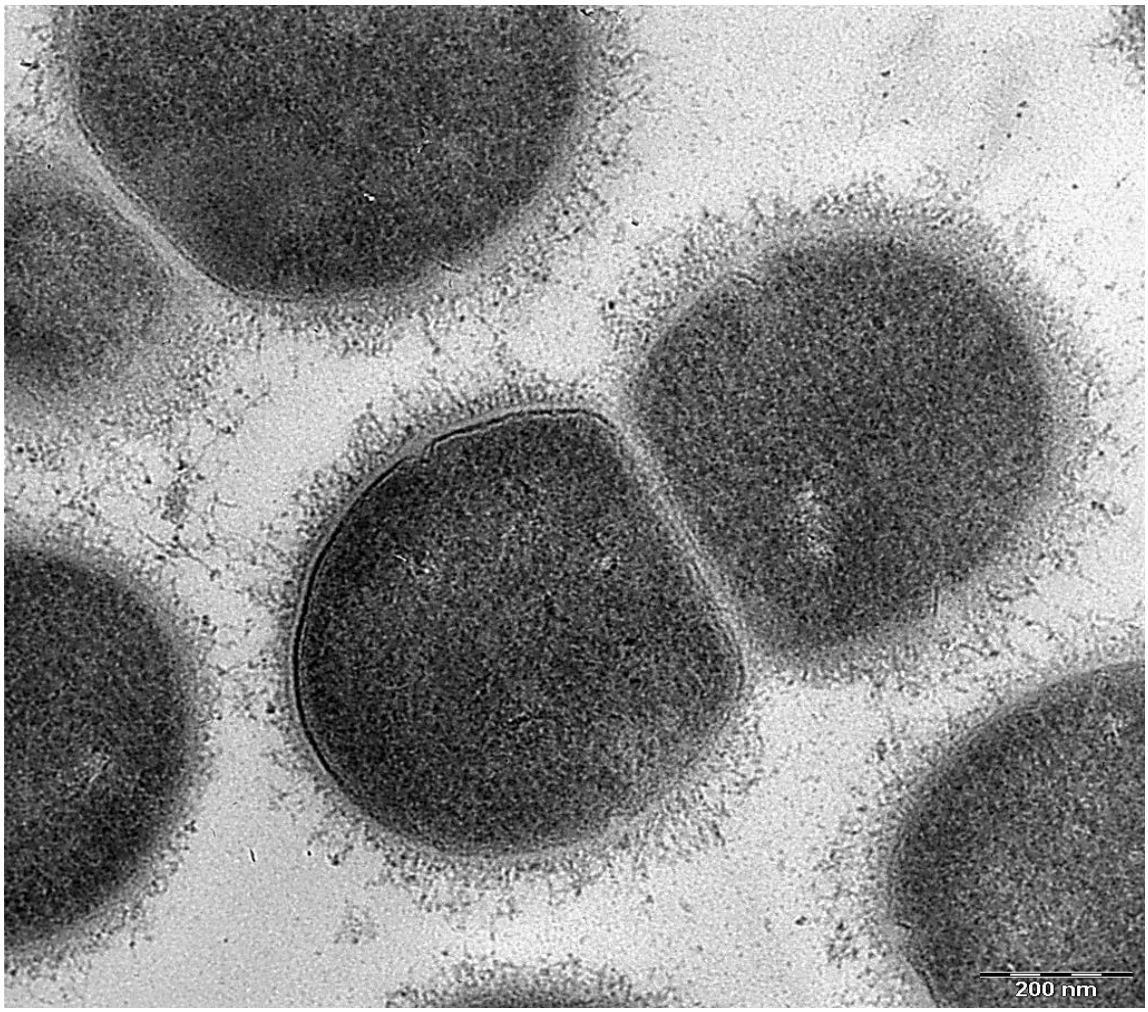


Figure 4.5: TEM section of *Streptococcus salivarius* NU10.

The width of each *S. salivarius* cell was recorded as ≤ 400 nm for bacterial cells in the log phase of growth.

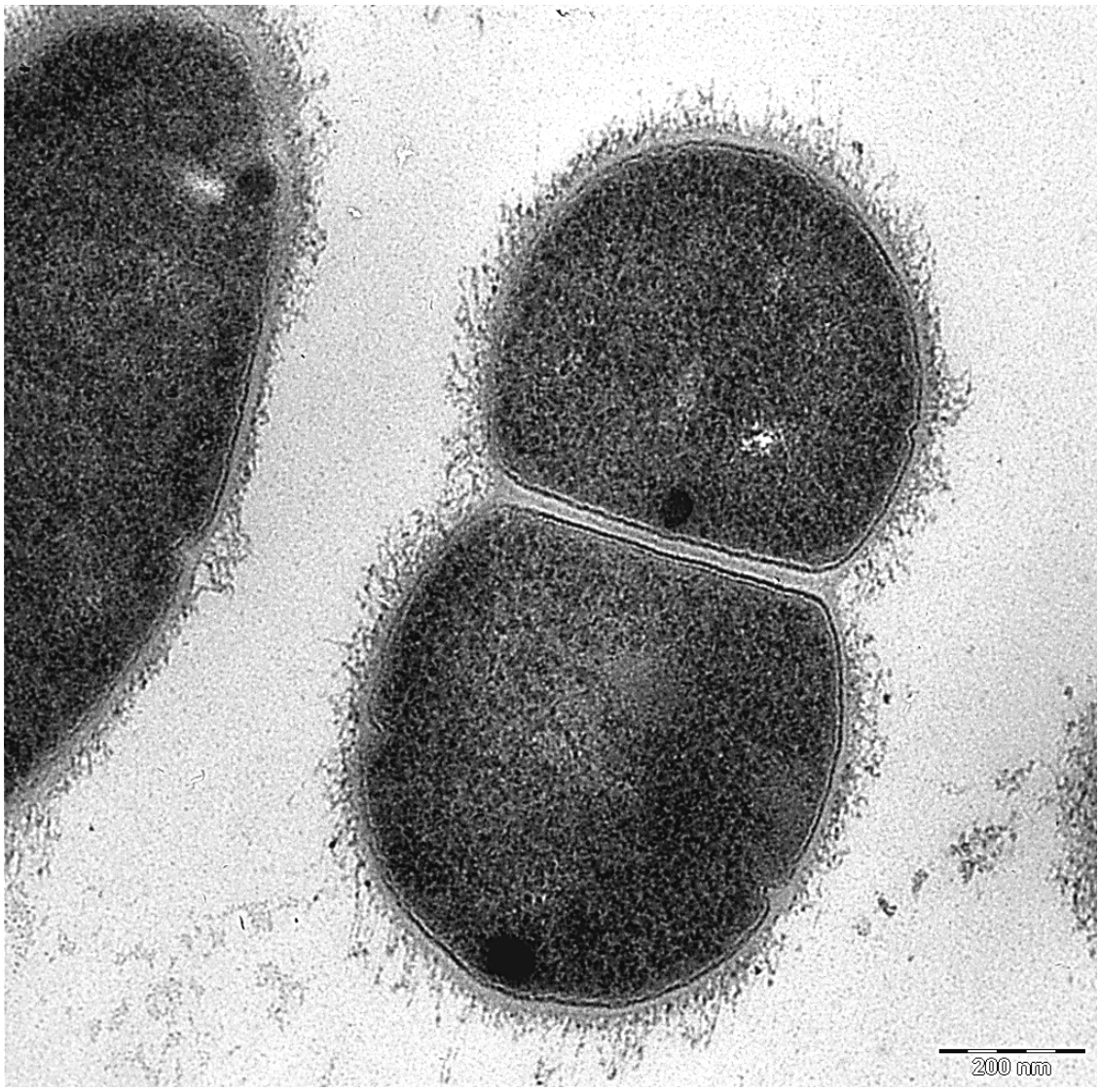


Figure 4.6: TEM section of *Streptococcus salivarius* YU10.

No flagella was detected in all isolates of *S. salivarius* since this bacteria is not motile.

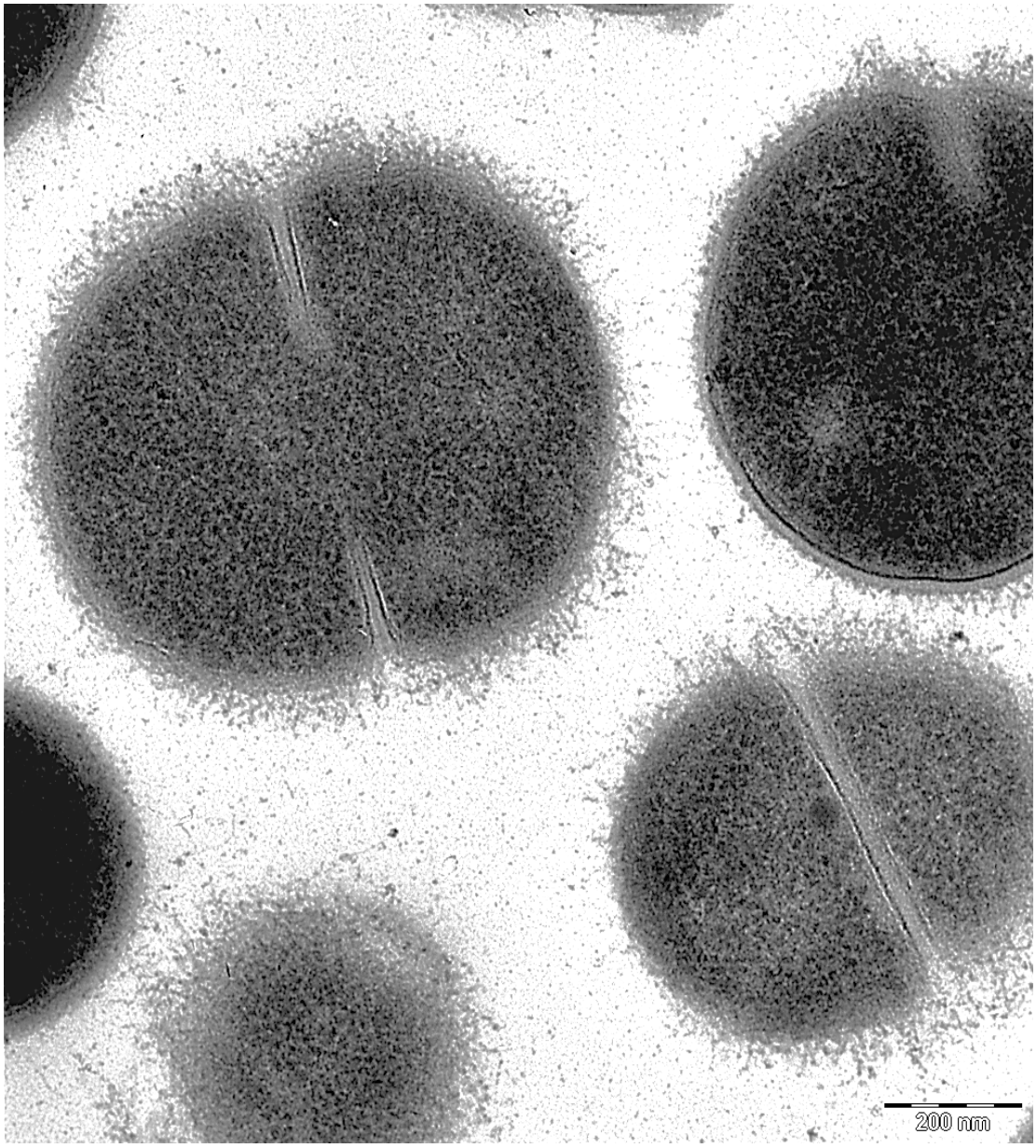


Figure 4.7: TEM section of *Streptococcus salivarius* HJEFF

Sometimes *S. salivarius* can be shown as single cocci depending on the plane of the section.

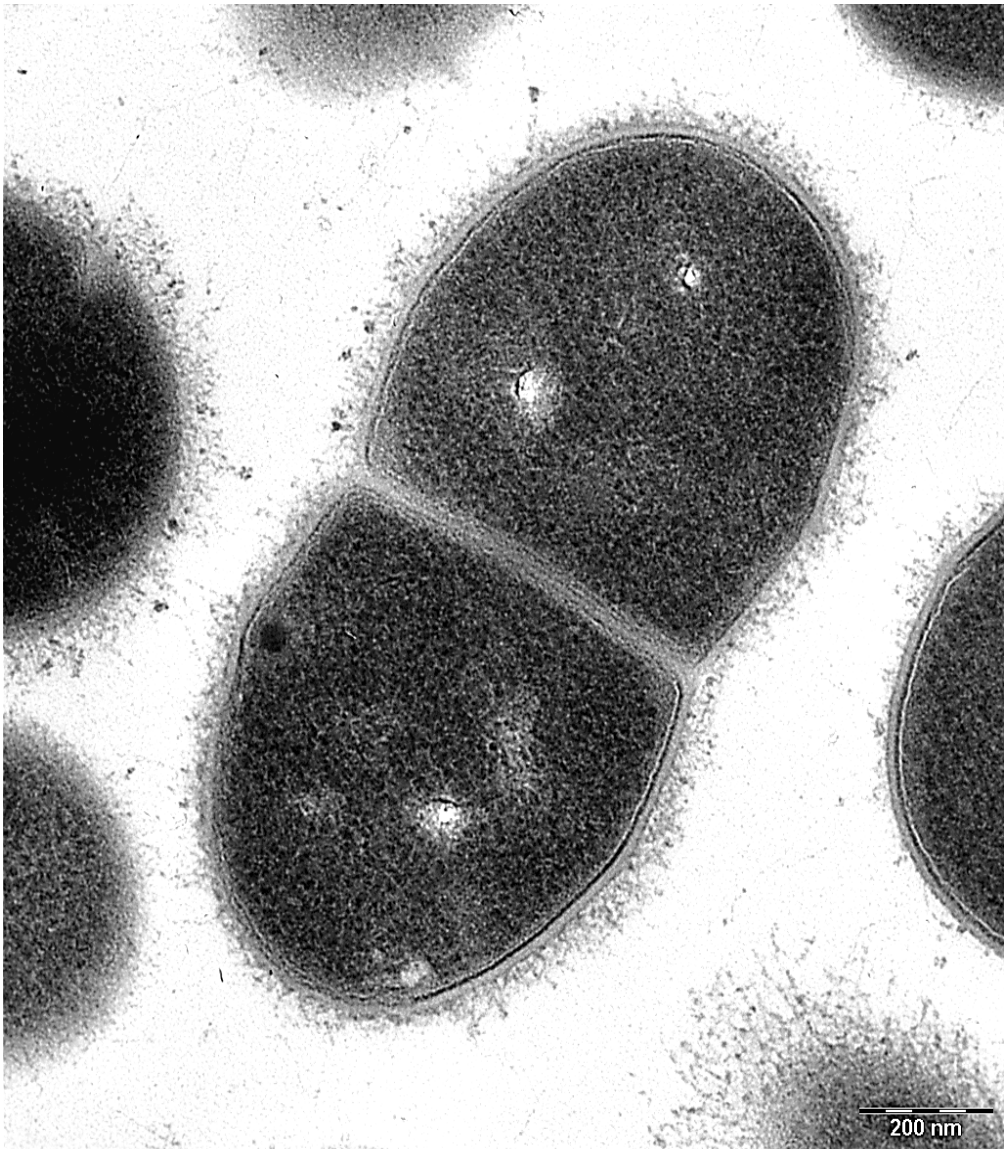


Figure 4.8: TEM section of *Streptococcus salivarius* 7YE

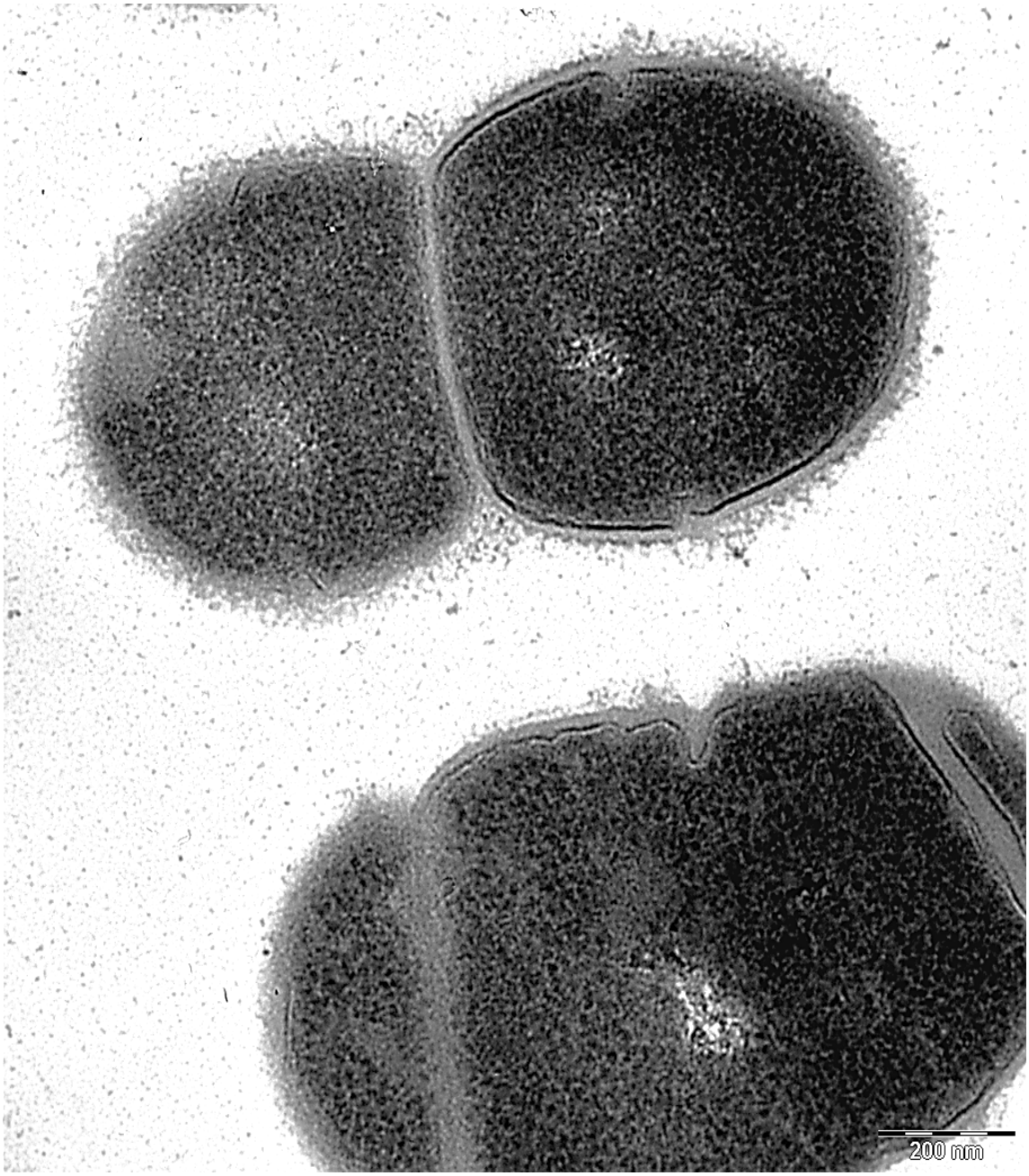


Figure 4.9: TEM section of *Streptococcus salivarius* GT2

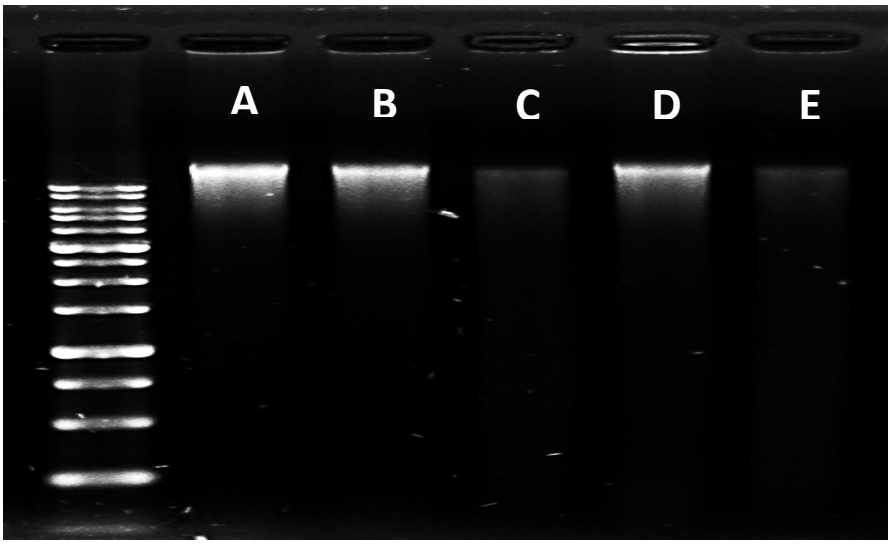


Figure 4.10: The genome DNA extracted from *Streptococcus salivarius* isolates. A, B and D: DNA extracted from *S. salivarius* strains K12, NU10 and YU10 respectively after cells lysis for overnight in presence of 100 mg/ml of lysozyme. C and E: DNA extracted from *S. salivarius* strains NU10 and YU10 respectively after incubation with lysozyme was for only 30 min. incubating the cells with lysozyme for overnight enhance the yield of DNA recovered significantly.

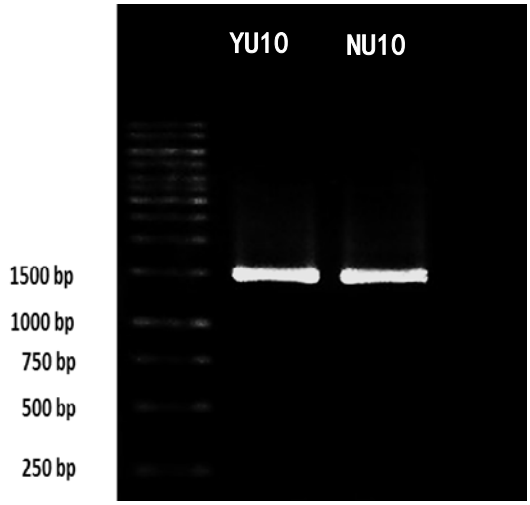


Figure 4.11: Gel electrophoresis of 16S rDNA gene sequencing of *Streptococcus salivarius* strains YU10 and NU10 isolated from Malaysian subjects. The PCR product showed a single band of amplified gene. The PCR product was purified and sequenced before the results were blasted on www.homd.org.

Table 4.3: 16s rDNA gene sequencing identification

Strain	Significant alignment	Score	E value	Identities
NU10	*Streptococcus salivarius*	2109	0.0	99.9
YU10	*Streptococcus salivarius*	2118	0.0	99.9
7YE	*Streptococcus salivarius*	2198	0.0	99.9
GT2	*Streptococcus salivarius*	2176	0.0	99.9
HJEFF	*Streptococcus salivarius*	2210	0.0	99.9
IND9	*Streptococcus salivarius*	2111	0.0	99.9
SAM3	*Streptococcus salivarius*	2204	0.0	99.9

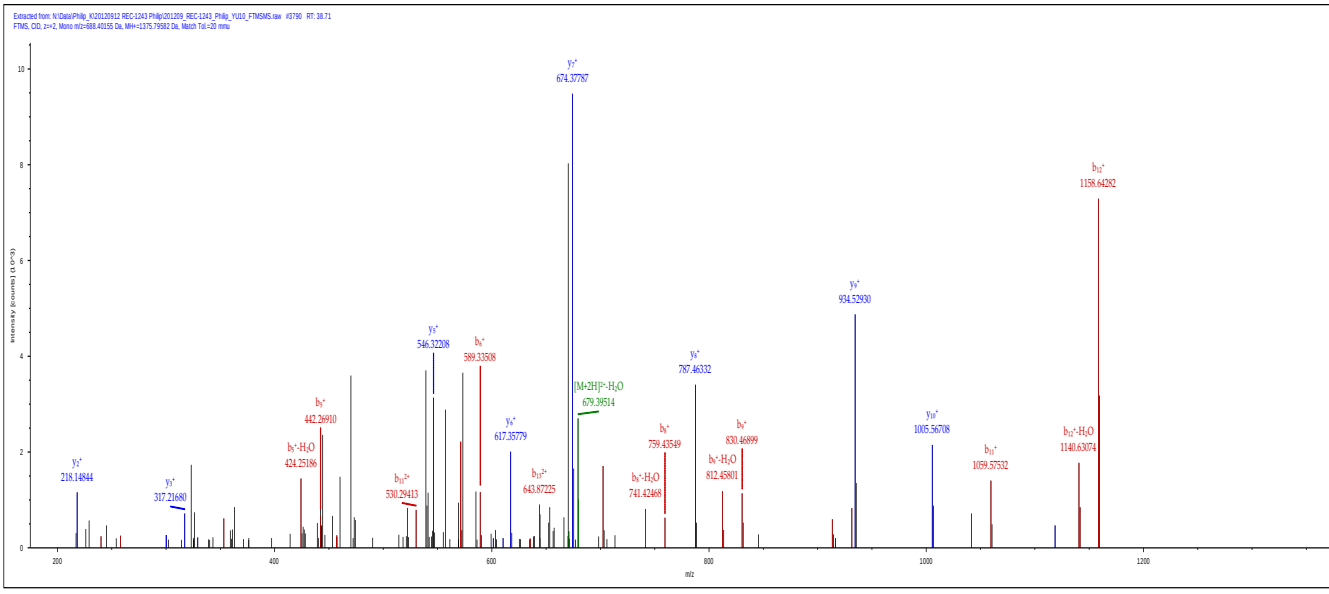


Figure 4.12: MS/MS spectrum evidence for VGTLAFLGATQVKA (Levansucrase enzyme) produced by *Streptococcus salivarius* YU1-0

Levansucrase was detected in the acidic methanol extract of the YU10 cells. The bacteria were grown into M17SUCa medium and the extract showed to have antimicrobial activity against selected indicators. The detection of cell-bound levansucrase enzyme at a significant quantity indicates that this enzyme is a major product of strain YU10 when sucrose was used as the only sugar source in the production medium since the detection method was developed to detect the most intensive peak. On solid medium levansucrase usually polymerizes fructose moiety of sucrose into fructans which possess either inulin or levan and this will resulted in a dome-shape colony morphology of the *S. salivarius* when it is grown on MSA (sucrose-enriched selective medium).

4.2 Simultaneous antagonism test

Simultaneous antagonism test was used initially to screen for bacteriocin-producing *S. salivarius* strains. Some of the *S. salivarius* stabbed into BACa medium were able to produce antimicrobial activity (Figures 4.13 and 4.14). In this assay a sensitive bacteria like *Micrococcus luteus* was used against which K12, YU10 and NU10 produced the largest inhibition zones. HJEFF and GT2 also produced inhibition zones but the activity was faint indicating perhaps inhibition due to acidic metabolites.

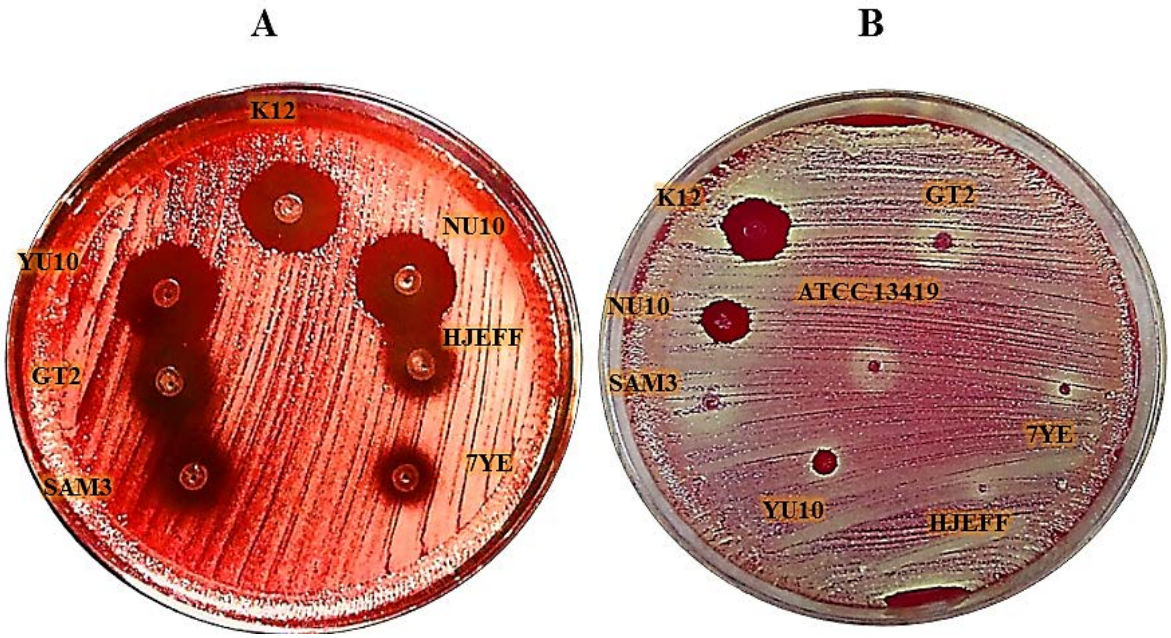


Figure 4.13: Simultaneous antagonism test of *Streptococcus salivarius* isolates. A, the test was done using *Micrococcus luteus* as an indicator strain. B, the test was done using *Corynebacterium spp* as indicator strain. The *S. salivarius* strains were stabbed into a fresh lawn of indicator strain seeded on BACa agar. Strains K12, NU10 and YU10 were the best producers.

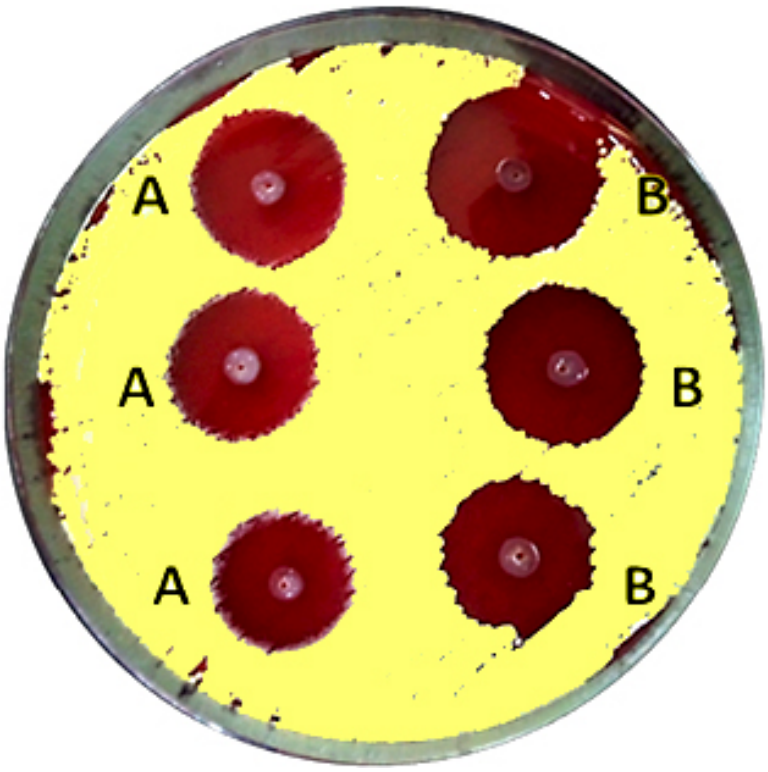


Figure 4.14: Simultaneous antagonism test for strains NU10 and K12. A: NU10 was stabbed as producer, B: K12 was stabbed as producer. Strain NU10 showed similar activity when compared with strain K12. The production of the bacteriocins was on BACa. The edges of the inhibition zones caused by antimicrobial activity produced by strain K12 are relatively sharp compared with that of strain NU10 and this may be due to the production of multiple bacteriocins by strain K12. *Micrococcus luteus* ATCC 10240 was used as indicator.

4.3 Deferred antagonism test

For the deferred antagonism assay, two different kinds of production media were used. Of these, TSYECa medium seemed to be good especially for strains HJEFF, 7YE, GT2 and SAM3. However, it must be noted that the production appeared inconsistent and inhibition was not shown always with the strains from the unhealthy subjects from the Dental Clinic. But the strains from the healthy Malaysian subjects showed relatively stable production of inhibitory activity. Although the inhibitory activity was not so strong, some strains like HJEFF, 7YE and GT2 could also inhibit the growth of *Bacillus cereus*. *Haemophilus parainfluenzae* was also inhibited by HJEFF and 7YE. After supplying the media with 0.4% of calcium carbonate, the inhibitory activity was reduced. BACa medium is a routinely used medium for this test and it gave a better bacteriocin production especially with strains YU10, NU10 and K12.

One of the reasons for absence of inhibitory activity against some of the indicator bacteria when using BACa in deferred antagonism assays is that the inhibitory activity seen when using TSYECa might be due to lactic acid produced by the *S. salivarius. S. salivarius* strain HJEFF in later experiments produced minimum inhibition of *Bacillus cereus* in liquid media and the inhibitor was recovered by ammonium sulphate precipitation which proved the presence of an inhibitor(s) of protein or peptide constitution produced by HJEFF strain. Strains GT2 and K12 were the only producers that caused inhibition of *Streptococcus mutans* in deferred antagonism tests on BACa.

Figure 4.15: Deferred antagonism test on BACa agar plates. (Left) K12 was used as producer strain. (Right) NU10 was used as a producer strain. Indicator bacteria from top to bottom: *Micrococcus luteus* ATCC 10240, *Streptococcus gordonii* ST2, *Streptococcus pyogenes* ATCC 12344, *Corynebacterium spp* GHG17, *Streptococcus equisimilis* ATCC 12388 and *Lactococcus lactis* ATCC 11454.

The inhibition characteristic of strain NU10 isolated from a Malaysian subject is similar to strain K12. At other times when the deferred antagonism test was repeated, the zones of inhibition were more or less. The reason for this fluctuation in production is still unknown. However, when *Streptococcus pyogenes* ATCC 12344 was diluted to OD_{600} of 0.2 before using it as indicator in this test, a significant inhibition zone was observed.

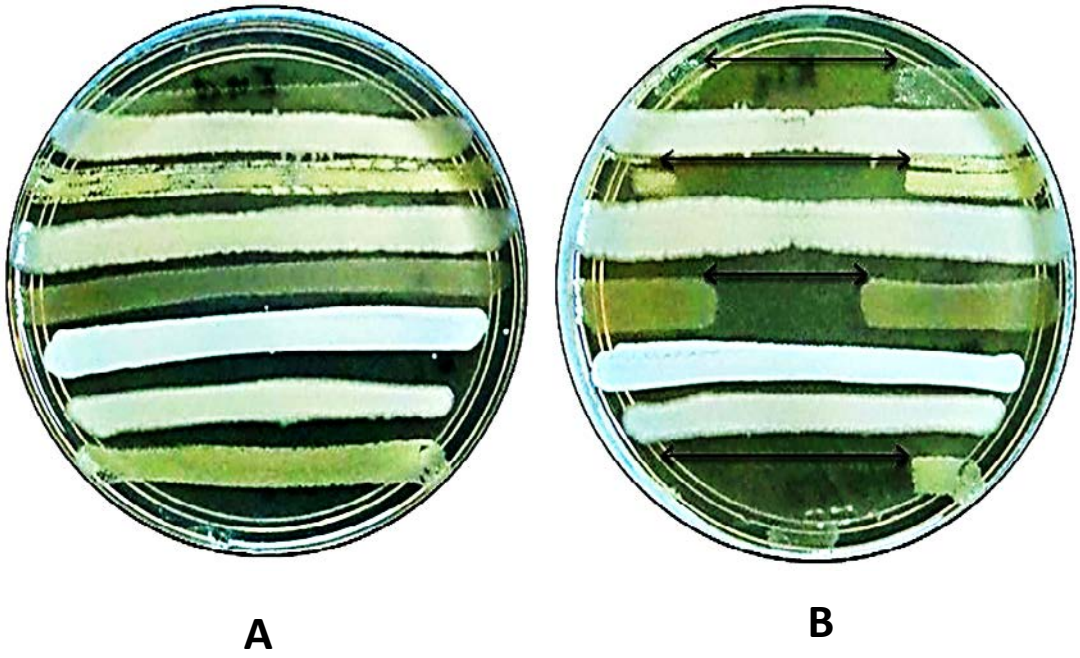


Figure 4.16: Deferred antagonism test on TSYECa agar plates. A: BLIS-negative reference strain ATCC13419. B: producer strain K12. Indicator bacteria from top to bottom: *Lactococcus lactis, Bacillus cereus, Corynebacterium spp, Haemophilus parainfluenza, Streptococcus mutans, Staphylococcus aureus, Gemella sanguinis, Micrococcus luteus.* Strain ATCC13419 could not inhibit any of the indicator strains while K12 secreted BLIS which inhibited four of the indicator strains used. Table 4.4 summarizes the production pattern of all *S. salivarius* isolates using BACa and TSYECa plates in deferred antagonism test.

Table 4.4: Deferred antagonism test to evaluate BLIS production *by Streptococcus salivarius* isolates using two different production media.

Indicator strain	Deferred antagonism test using two different type of production media (TSYECa and BACa)															
	HJEFF		7YE		SAM3		GT2		NU10		YU10		IND9		K12™	
	TSY ECa	**BACa**	**TSY ECa**	**BACa**	**TSY ECa**	**BACa**	**TSY ECa**	**BACa**	**TSY ECa**	**BACa**	**TSY ECa**	**BACa**	**TSY ECa**	**BACa**	**TSY ECa**	**BACa**
Bacillus cereus	++	-	++	-	-	-	+	-	-	-	+	-	-	-	-	-
Haemophilu sparainfluenza	++	-	++	-	-	-	+	-	-	-	-	-	-	-	-	-
Lactococcus lactis	-	-	-	-	-	-	-	-	-	+	-	++	-	-	+++	+++
Corynebacterium spp	+++	+	+++	+	++	+	+++	++	+++	+++	+++	+++	-	-	+++	+++
Streptococcus mutans	-	-	-	-	-	-	++	++	-	-	-	-	-	-	+++	+++
Staphylococcus aureus	-	-	-	-	-	-	+	-	-	-	-	-	-	-	++	++
Streptococcus equisimilis		-		-		-	-	-	+++	+++	+++	+++	-	-	+++	+++
Actinomyces naeslundii	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Micrococcus luteus	+	+	+	+	+	+		+	+++	+++	+++	+++	-	-	+++	+++
Streptococcus gordonii	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Streptococcus pyogenes	-	-	-	-	-	-	-	-	+	+	+	+	-	-	++	+++

+ inhibition zone < 0.75 cm, ++ inhibition zone = 0.75 - 1 cm, +++ inhibition zone > 1 cm, - no inhibition

4.4 Production of BLIS activity in liquid media

All the isolates failed to produce antimicrobial activity while growing in liquid medium. Strain HJEFF was able to grow in Muller Hinton broth and the concentrated cell free supernatant showed some inhibitory activity. The production of this activity was not stable where only small quantity of inhibitory activity could be recovered. When the same experiment was repeated no inhibitory activity was recovered.

Table 4.5: production of BLIS activity using different production methods

S.salivarius strain	Production of BLIS in liquid media	Production of BLIS by freeze thaw extraction	Production of BLIS by methanol extraction of producer cells
NU10	-	+	+
YU10	-	+	+
7YE	-	-	+
GT2	-	+	+
HJEFF	+$^{\alpha}$	-	+
IND9	-	-	-
SAM3	-	-	+
K12™	-	+	+
ATCC13419	-	-	-

α Very small quantity of inhibitory activity could be recovered from the concentrated supernatant of this strain

The growth curve of strain HJEFF was studied and the BLIS activity was recovered from the late log phase and early stationary phase. Because no activity could be detected directly

from the cell free supernatant, ammonium sulphate was added to the supernatant at a saturation of 50% to concentrate the activity.

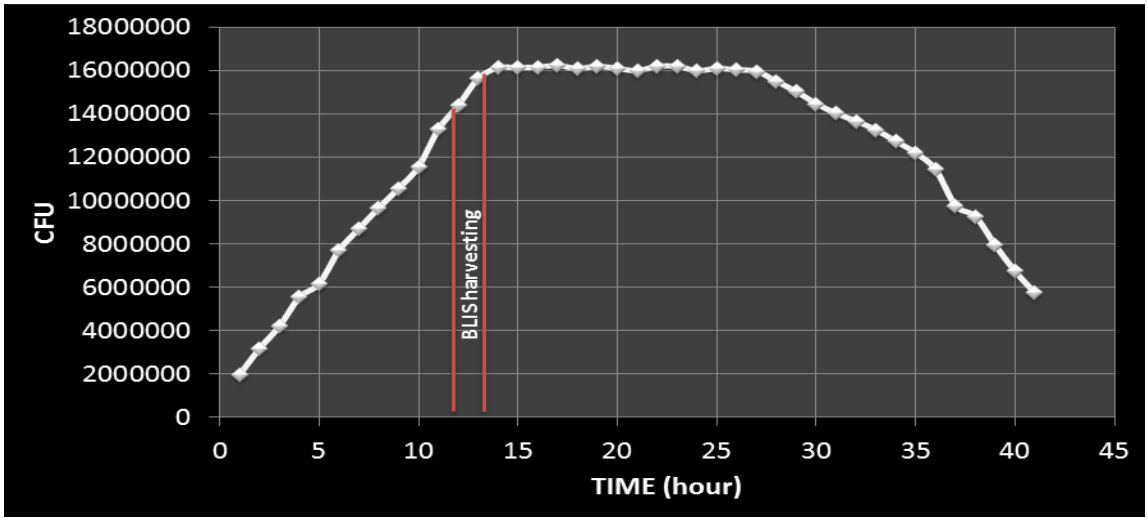


Figure 4.17: Growth curve of strain HJEFF that produce minimum inhibitory activity in liquid medium.

The bacteria reached the stationary phase after 14 hours of incubation in Mueller Hinton broth. The antimicrobial activity test from the supernatant did not detect any inhibitory activity during the production. The ammonium sulphate precipitation of cell free supernatant harvested from the late log phase and early stationary phase revealed inhibitory activity especially against *Bacillus cereus*. When the production experiment was repeated using other media like M17 or THB no activity could be recovered neither from the supernatant nor from the ammonium sulphate precipitate. In this case, maybe MH broth which has less nutrients comparing with M17 and THB played a role in making the bacterial environment more stressful. The inhibitory activity in the ammonium sulphate precipitate was purified using DEAE anion exchanger. The inhibitory agent was able to

bind to the column and eluted out using 1M NaCl. No activity could be recovered from the unbound fractions while three fractions eluted once 1M NaCl was applied showed antimicrobial activity against *Corynebacterium* spp.

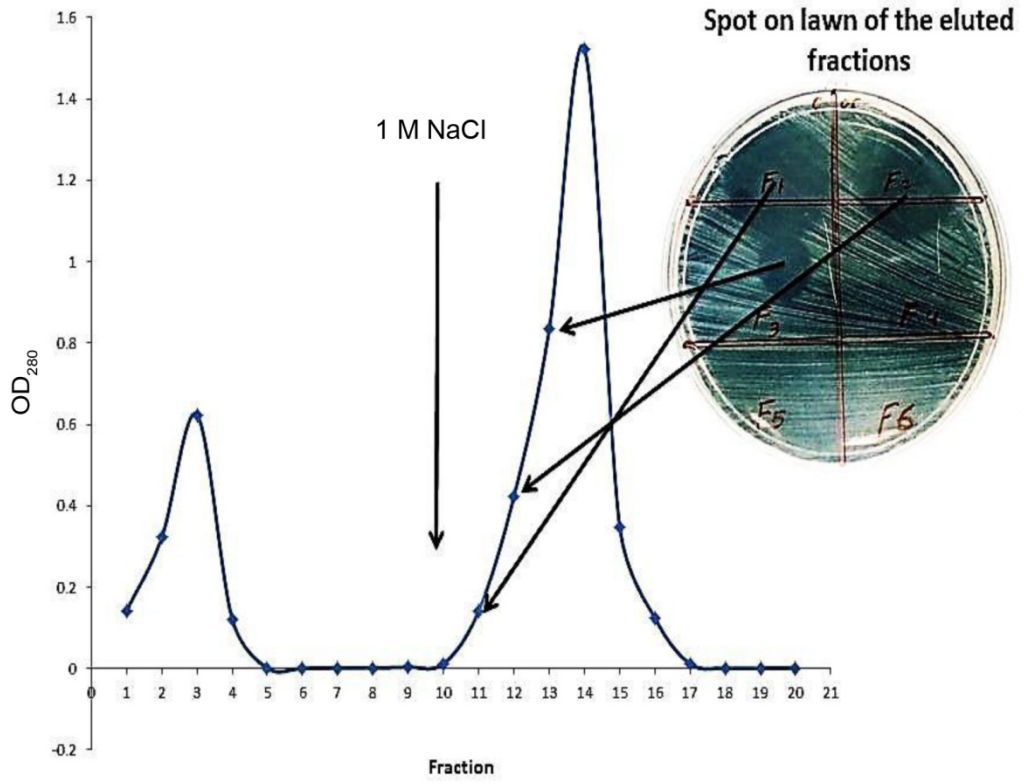


Figure 4.18: Binding of inhibitory activity produced by strain HJEFF in liquid medium to the anion exchanger DEAE column. The activity was eluted using 1M NaCl and the fractions were tested against *Corynebacterium spp.* Fractions 1-6 bounded to the DEAE column and eluted after washing the column with high salt buffer. The protein was detected at wavelength of 280 nm.

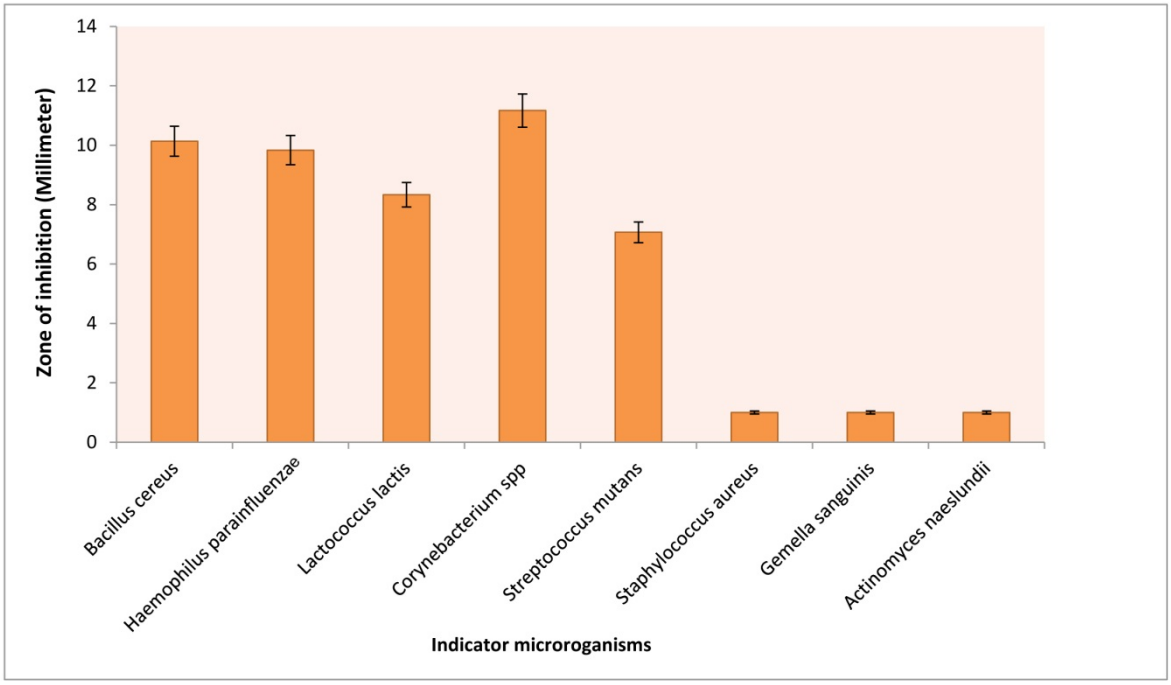


Figure 4.19: well diffusion assay of the BLIS activity extracted from cell-free supernatant of *S.salivarius* HJEFF grown in liquid medium after the ammonium sulphate precipitation. 50µl of the crude inhibitory activity dissolved in distilled water was loaded in each well previously cut in CAB plate. After incubation at 37^{o}C for 18 hours clear zones of inhibition surrounding the well was measured. Each test was done in triplicate and the standard deviation was calculated using Microsoft Office Excel.

4.5 Isolation of antimicrobial peptides using acidic methanol extraction

This method has been use for long time to isolate lantibiotics from lactic acid bacteria especially in the medical industry were this method can give relatively pure peptide with strong activity. In this project the cell-associated peptide was eluted from the cells surfaces using 95% methanol pH 2. To enhance the quantity recovered, the producer bacterial strain was grown in 2 liters bioreactor and the production media was supplemented with 0.1% calcium carbonate which worked as a buffer to reduce the changing in pH condition of the culture. Since the peptide was recovered from the cells surfaces it was very important to

reduce the acidity of the culture by supplying it with calcium carbonate. If the media got more and more acidic, the peptide will start to be eluted out from the bacterial surface into the supernatant (desorption), but when the media condition during bacterial growth was maintained between pH of 4-6, the extra cellular metabolite (in this case antimicrobial peptide) will adsorbed to the surface of the producer strain (adsorption). This method was ideal in peptide recovering and the peptides isolated using this method was reasonably pure peptide comparing with that recovered by freeze thaw method. From the figure 4.20 we can see that the most sensitive indicator bacteria for peptide preparations using acidic methanol extraction are *Corynebacterium spp* and *Micrococcus luteus.* This support the results achieved from deferred antagonism test, where these strains were the most sensitive for the producers and this is evidence that the same peptide secreted into agar and cause strong inhibition for both sensitive indicators during deferred antagonism assay is the same produced and isolated with acidic methanol extraction method. Peptide extracted by this method achieved 64 $AU.ml^{-1}$ against *Corynebacterium spp* and 128 $AU.ml^{-1}$ against *Micrococcus luteus* when *S. salivarius* NU10 was used as producer strain while *S. salivarius* YU10 gave a titer of 32 $AU.ml^{-1}$ and 64 $AU.ml^{-1}$ against *Corynebacterium spp* and *Micrococcus luteus* respectively.

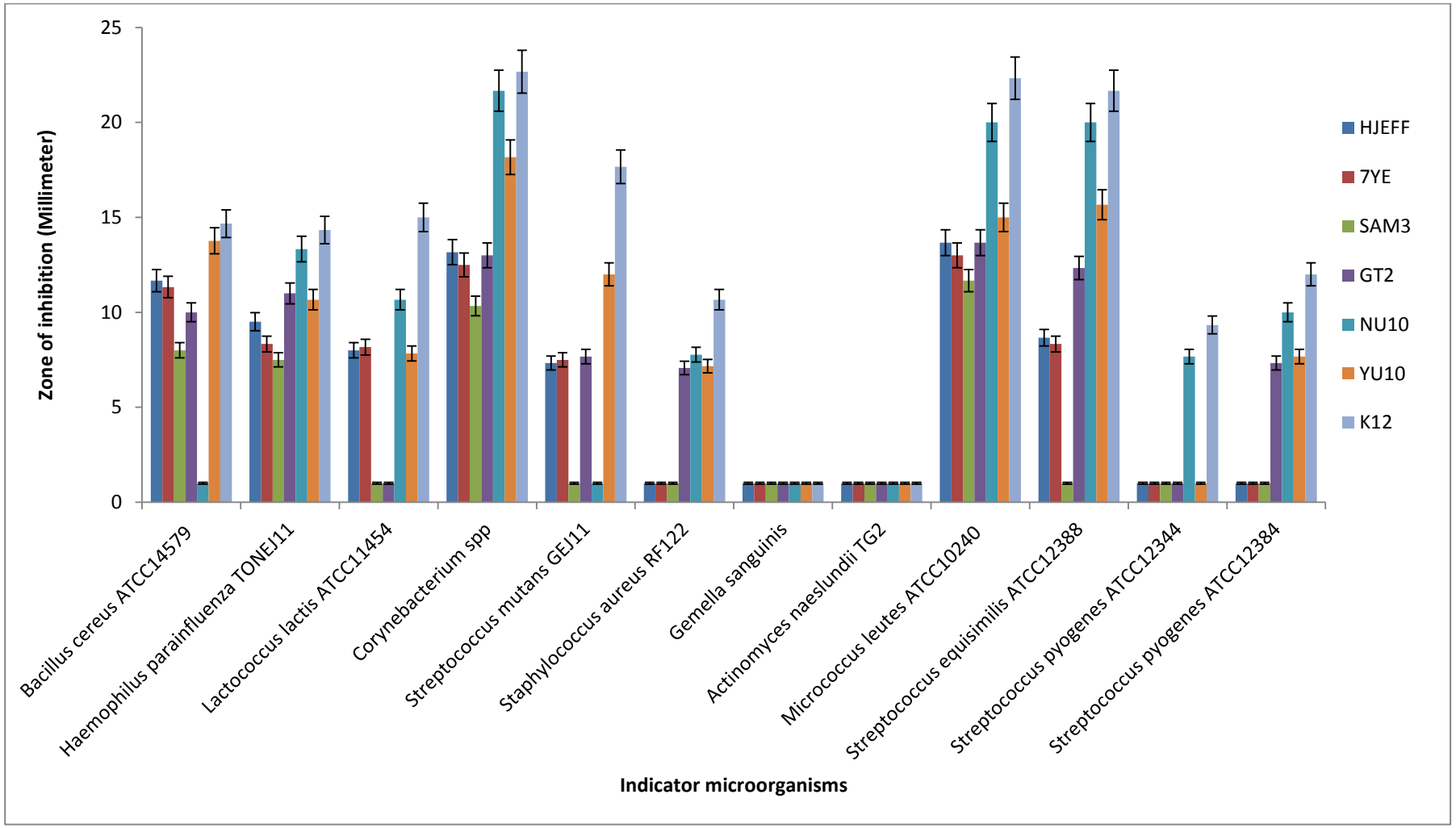


Figure 4.20: Well diffusion assay of BLIS extracted from different *S. salivarius* strains by using acidic methanol extraction of the cells.

Corynebacterium seemed to be one of the most sensitive strains to the acidic methanol extraction, while *Lactococcus lactis* and *Bacillus cereus* showed some resistance to extracts from strains GT2 and NU10.

Gemella sanguinis and *Actinomyces naslundii* were not inhibited by the acidic methanol extracts of all *S. salivarius* strains. While *Streptococcus mutans* was sensitive to extracts from strains K12, YU10 and GT2.

Both strains *Micrococcus luteus* ATCC10240 and *Streptococcus equisimilis* were inhibited strongly by all acidic methanol extracts especially those extracted from the cell surfaces of strains K12 and NU10. Concentrated BLIS extracted from strains K12, NU10, YU10 and GT2 inhibited *Streptococcus pyogenes*.

4.6 Production of BLIS NU10 by freeze thaw extraction

This is a standard method to recover salivaricin A and salivaricin B from *S. salivarius* K12. The production media used to produce the inhibitory peptides from strain K12 was modified to produce enhanced quantity of the putative peptide from strain NU10. The modified media used in this method was M17 broth supplemented with 1% sucrose, 0.1% calcium carbonate and 0.7% agarose. The agarose was selected instead of bacteriological agar because it was more efficient during freeze thaw extraction. After autoclaving and before pouring the media into petri dishes the media was cooled down till 50^{o}C when 4% of *S. salivarius* NU10 was added to the media and mixed by gentle stirring. 1% of crude BLIS NU10 was added before pouring. After the agarose was set the plates (approximately 100 plates) were incubated anaerobically at 35^{o}C for 22 hours. This method enhanced the

production of the putative peptide after 65% ammonium sulphate precipitation from 8 AU.ml^{-1} against *Micrococcus luteus* to 128 AU.ml^{-1} against the same bacteria.

Table 4.6: Well diffusion assay of BLIS produced by freeze thaw extraction.

Indicator strain	Inhibitory activity recovered from *S. salivarius* strains with freeze thaw extraction					
	HJEFF	**7YE**	**GT2**	**K12**	**NU10**	**YU10**
Micrococcus luteus	-	-	9	15	12	11
Corynebacterium spp	-	-	8	16	15	15
Streptococcus equisimilis	-	-	-	12	10	10

Inhibition zones were measured in millimeter.

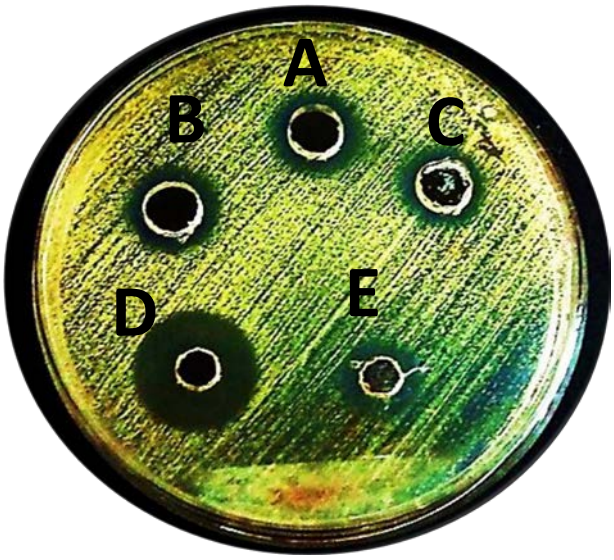


Figure 4.21: Well diffusion assay for BLIS preparation achieved by different methods. A,B,C are wells filled with BLIS achieved by freeze thaw extraction from strains YU10, NU10 and GT2 strains respectively. D is the desalted BLIS recovered from HJEFF strain using vivaspin column after ammonium sulphate precipitation. E is the effluent of the desalting process.

4.7 Purification of BLIS-NU10 produced by freeze thaw extraction:

After the crude BLIS NU10 was extracted by freeze thaw extraction 50% ammonium sulphate saturation was applied to concentrate the BLIS activity. The resulting crude BLIS NU10 was desalted using Sephadex G 25 column. Figure 4.22 shows the elution profile of BLIS NU10 from gel filtration column.

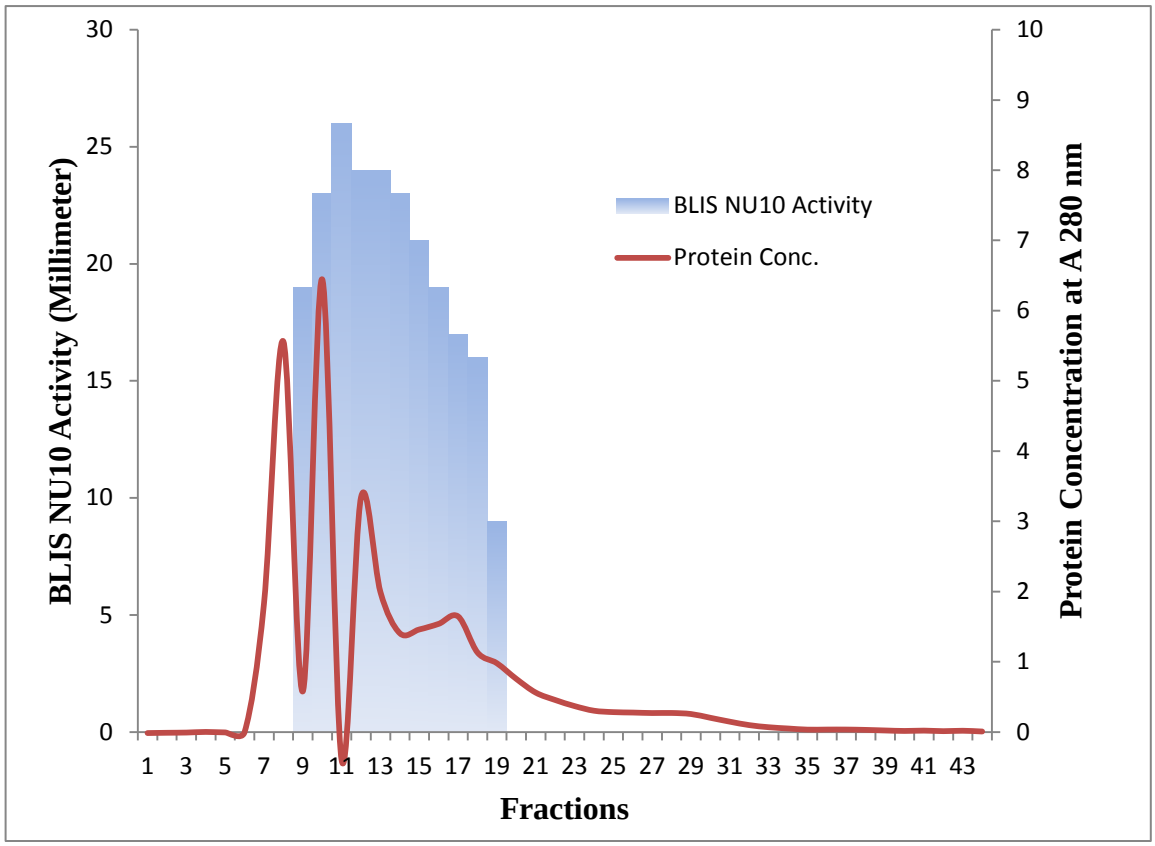


Figure 4.22: Gel filtration chromatography of BLIS NU10 using Sephadex G-25. Gel filtration using Sephadex G25 column equilibrated with 25 mM phosphate buffer pH 5.8. 5 ml fractions were collected at a flow rate of 3 ml/min. The protein concentration was measured by NanoDrop 2000 (Thermo Scientific) at 280 nm. All the fractions were tested by well diffusion assay using *Micrococcus luteus* ATCC® 10240 as indicator bacteria.

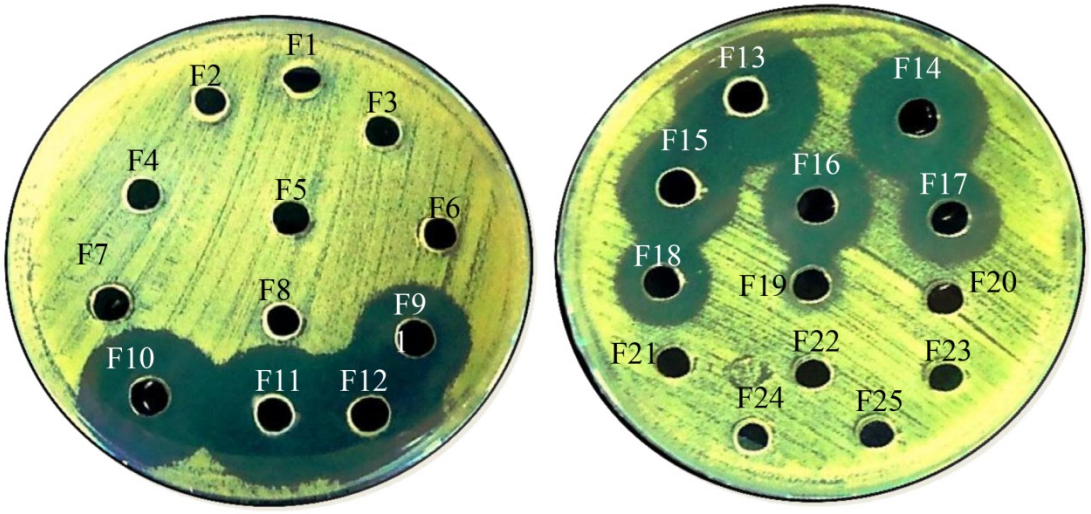


Figure 4.23: Well diffusion assay of fractions eluted from Sephadex G-25 column during gel filtration process. Antimicrobial activity of the fractions eluted from Sephadex G25 column using 25mM Phosphate buffer pH 5.8. 5ml fractions were collected at a flow rate of 3 ml/min and 50 µl of each fraction was loaded into 6mm well that had been cut into the CAB assay agar freshly seeded with *Micrococcus luteus* ATCC 10240 by using cotton swab. The plates were incubated at 37^{o}C for 18 hours and zone of inhibition was noticed as an effect of the desalted antimicrobial peptide.

Table 4.7: purification of BLIS NU10 produced by *Streptococcus salivarius* NU10

Step	Volume (ml)	Activity (AU/ml)	Total protein (mg)	Total activity ($AU.10^3$)	Specific activity (AU/mg)	Yield (%)	Purification (fold)
Freeze thaw culture supernatant	1000	800	10000	800	80	100	1
Ammonium sulphate precipitation	100	3200	4000	320	40	40	1
SEPHADEX G-25 gel filtration	30	3200	320	96	300	12	3.75
Amberlite XAD-2 chromatography	15	1600	50	24	480	3	6
Sep-pak C18	8	3200	0.005	25	5×10^6	0.032	640

The XAD-2 chromatography and C18 chromatography following gel filtration gave pure NU10 peptide as it shown in table 4.7. Following these steps achieved pure NU10 peptide. When the freeze thaw culture supernatant was applied directly to XAD-2 column only small quantity of peptide was recovered. That is why steps of ammonium sulphate precipitation and Gel filtration were applied to concentrate the inhibitory activity before loading into XAD-2 column. XAD-2 chromatography was a great technique to remove all hydrophobic proteins from the crude peptide. And it was very easy to recover the activity using acidic methanol of 95% concentration. Sep-Pak C18 resin was ideal to capture the NU10 peptide and fractionate it by applying gradient concentrations of methanol.

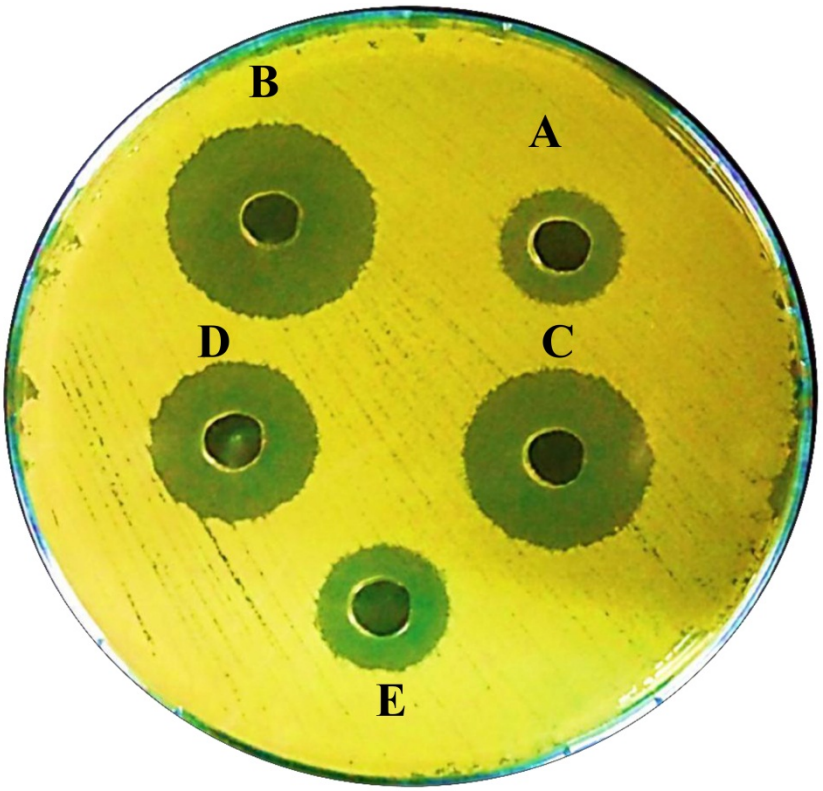


Figure 4.24: Well diffusion assay of different preparations of BLIS NU10. A: freeze thaw crude, B: 65% ammonium sulphate precipitation, C: Amberlite XAD-2, D: gel filtration G-25, E: Sep-Pak C18 purification. Indicator bacteria: *Micrococcus luteus* ATCC® 10240.

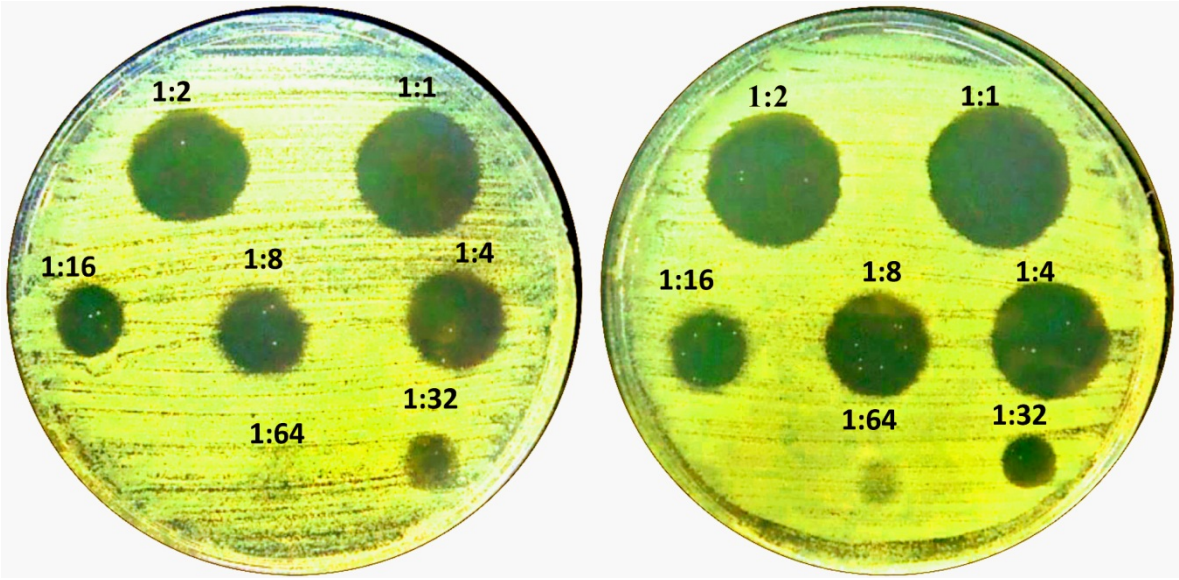


Figure 4.25: Spot on lawn assay of both crude and purified BLIS NU10 isolated from *S. salivarius* NU10. (Left) BLIS NU10 (crude) activity after freeze thaw extraction and 65% ammonium sulphate precipitation.(Right) BLIS NU10 (semi pure) activity after freeze thaw extraction, 65% ammonium sulphate precipitation, followed by Sephadex G25 XAD-2 chromatography and C18 Reverse Phase Sep-pack cartridge solid phase extraction. The samples were diluted as two fold serial dilution and spotted as 20µl onto the surface of CAB freshly seeded with *Micrococcus luteus* ATCC® 10240. After incubation at 37^{o}C for 18 hours a zone of inhibition were noticed and the arbitrary units of the inhibitor per ml were counted.

4.8 Bacteriostatic, bactericidal and bacteriolytic activity

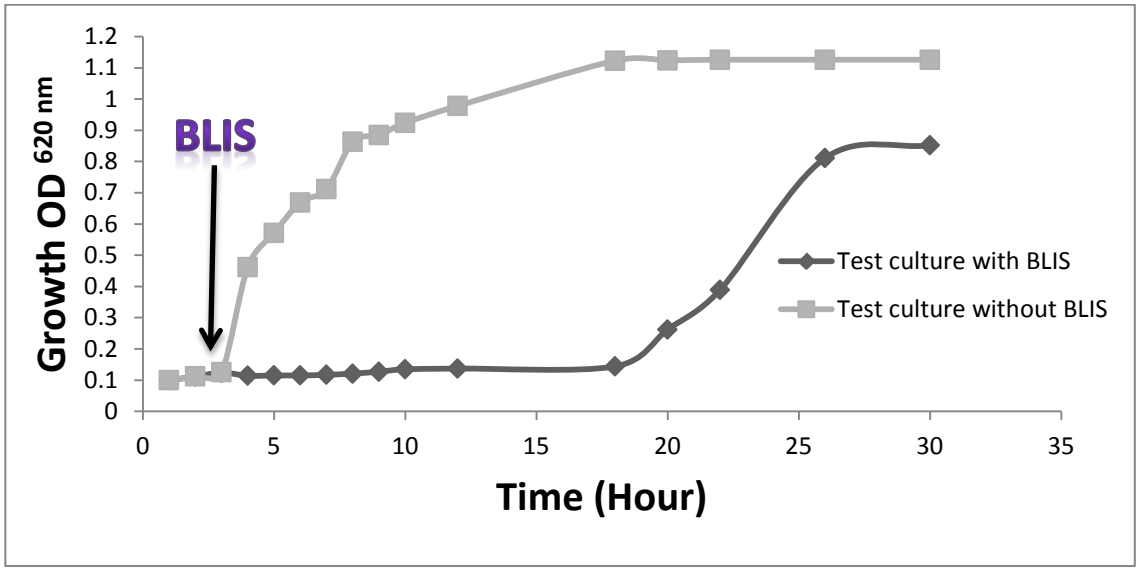


Figure 4.26: Bacteriostatic mode of action of BLIS produced by *Streptococcus salivarius* YU10, *Micrococcus luteus* ATCC10240 was used as a test culture.

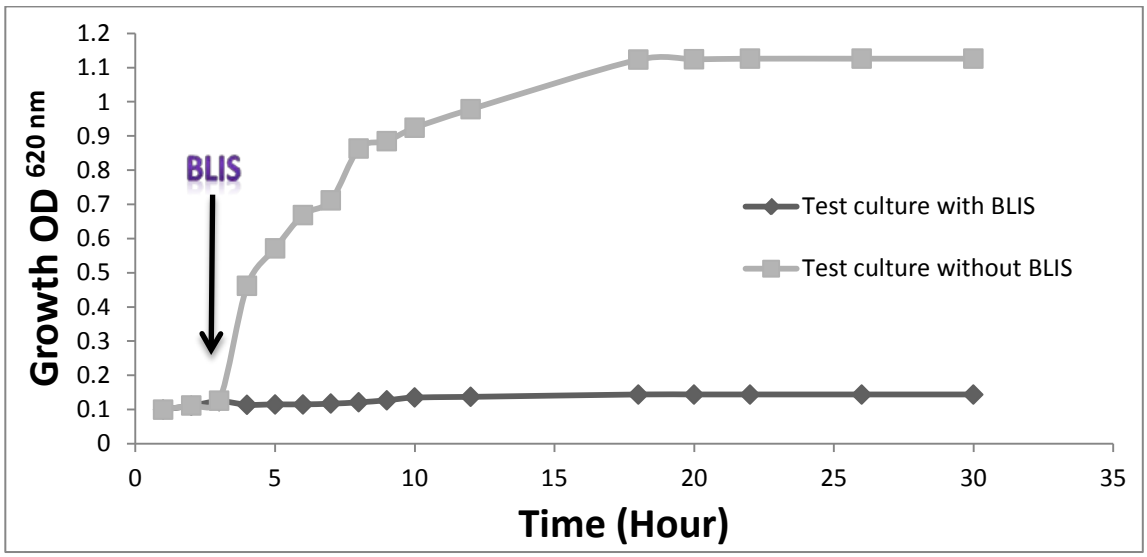


Figure 4.27: Bactericidal mode of action of BLIS produced by *Streptococcus salivarius* NU10, *Micrococcus luteus* ATCC10240 was used as a test culture.

Bacteriostatic and bactericidal mode of action of BLIS preparation was studied depending on the growth kinetics of the indicator strain after incubation with BLIS extracted from strains NU10 and YU10. After incubation with BLIS YU10, *Micrococcus luteus* ATCC10240 was able to grow again after 20 hours of incubation which indicated that the mode of action of BLIS YU10 is bacteriostatic rather than bactericidal. BLIS NU10 showed bactericidal mode of action where *Micrococcus luteus* ATCC10240 could not grow even after incubation with BLIS NU10 for 30 hours.

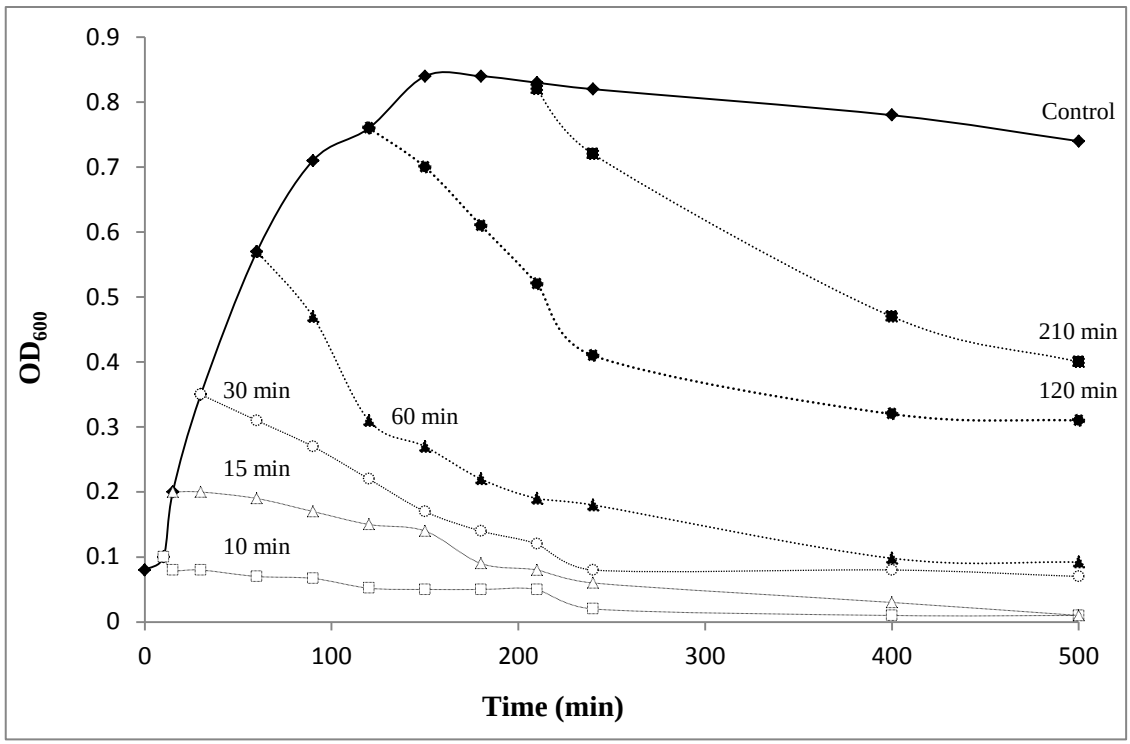
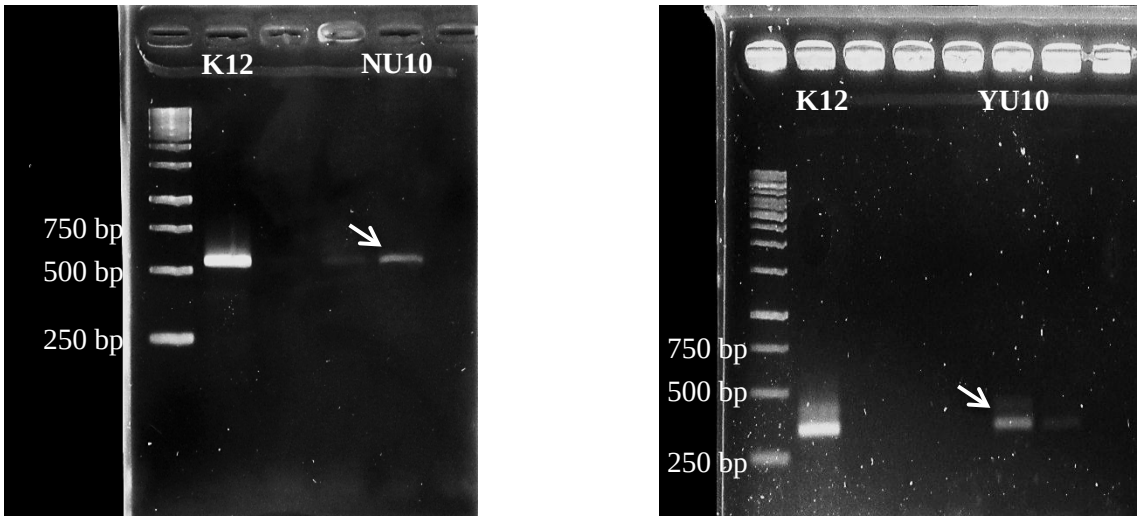


Figure 4.28: Bacteriolytic Effect of salivaricin BLIS-NU10 on growing culture of *Micrococcus luteus* ATCC10240 when added at different phases of growth. BLIS-NU10 was added to freshly inoculated culture after 10, 15, 30, 60, 120 and 210 minutes at 37^{o}C. Bacterial growth without adding salivaricin was designated as control. The bacterial growth was recorded by measuring OD_{600}.

4.9 Lantibiotic structural genes



A **B**

Figure 4.29: Distribution of salivaricin A and salivaricin B encoding structural genes in the best two positive producers. *salA* and *sboB* genes distribution in *S. salivarius* isolates (A) Gel electrophoresis to detect salivaricin B structural gene after PCR reaction. (B) Gel electrophoresis to detect salivaricin A structural gene after PCR reaction. K12 (positive control) showed a thick bands for salivaricin A and salivaricin B encoding genes detection. The gene expression in K12 strain resulted in thick bands. Strain YU10 appeared to be positive for salivaricin A gene and strain NU10 was positive for salivaricin B while K12 was positive for both genes.

Table 4.8: Lantibiotic structural genes:

S. salivarius	*salA*	*sboB*	*sivA*
NU10	±†	+	+
YU10	+	-	+

† Faint band of *salA* gene was detected in the PCR product of NU10.

DNA sequencing of the *sboB* gene encoding salivaricin B production by *S. salivarius* strain NU10:

GGTGTGAATTTCCTTCAAGAATTGACTCTTGAAGAAATTGACAACGTTCTTGGT

GCTGGTGGTGGAGTAATCCAAACCATTTCACACGAATGTCGCATGAACTCATG

GCAGTTCTTGTTTACTTGTTGCTCTTAATTACGTTCTATAAGCCTAAATTTTAGC

ACGTAATTTGACTTTTAAGAATTTTAAGGGAGATATATTTAAAATAATATCTTC

TAGAAATTCTAATAGTATCACAATCTAACAATGAGAGGTGTCTCTACTCTTGGT

TTTATTCATAGAAATCTTGTTCATCATGAAAATAGGTGGTCAAAATGGAAAATA

CCGAGAAAAATAAATTATTTAACAAATTTACTAGAAATTTTATCCAAACAAACT

TCTCAGAGTATGATATGCAAAACTGTGAGATAGAATTTTTAGAAGATAAAGTTT

TAGATATTTATATGAATACACTAATCCGTTTGATAAACGAAAAAACAAAATAA

GGAATTTAAAGGGCCCAACTTCGGAAGAGCGGTATGAATATTTTA

The DNA sequence of *sboB* gene was translated using expasy translate tool (http://web.expasy.org/translate/) and shown to have the following amino acid sequencing:

GVNFLQELTLEEIDNVLGAGGGVIQTISHECRMNSWQFLFTCCS

When this sequence blasted on http://www.uniprot.org/ the identity was similar to *sboB* gene product. Strain NU10 and K12 were the only isolates harbour *sboB* gene. However salivaricin B lantibiotic was not detected in the purified BLIS-NU10. Surprisingly salivaricin B was not detected when pure BLIS-NU10 was subjected to MALDI-TOF analysis.

DNA sequencing of the *sivA* gene encoding salivaricin 9 production by *S. salivarius* strain NU10:

TATATCCATGAGCTCTCCTAGTGAAATAATTTGAATTTTCCATATTTATAGTATA
TCAAAAATATCAAGTAAAATGTAAGCTCACTAAATTCTTTAGGTTTTCTTAAGG
TAAATTTTGACATTTCTTTATCTTGTACTTATAAAATGTAATTGTAGCTAGATAA
AGATGCTTAATATTGATTTAAATTAAAAAAGGAGAAATATCCATGAAATCAAC
AAATAATCAAAGTATCGCAGAAATTGCAGCAGTAAACTCACTACAAGAAGTAA
GTATGGAGGAACTAGACCAAATTATTGGTGCCGGAAACGGAGTGGTTCTTACT
CTTACTCATGAATGTAACCTAGCAACTTGGACAAAAAAACTAAAATGTTGCTA
ATTATTTAACAAGAAAATTTTACTTAAGCATTCAAGTATTTTTATCTGTTACATA
TTAAGTTGTGTTAATTAACTTAATGGACTTAAAAGTTTGGGGTAAAG

Expassy DNA to protein translation of the *sivA* gene detected in strain NU10:

MKSTNNQSIAEIAAVNSLQEVSMEELDQIIGAGNGVVLTLTHECNLATWTKKLKCC

sivA gene product (salivaricin 9) was the only known lantibiotic to be detected in the MALDI-TOF analysis of purified BLIS-NU10.

4.10 BLIS-NU10 induction assay

Table 4.9: Induction of inhibitor production by *S. salivarius* strains NU10, YU10, K12 and nisin-producing strain ATCC11454 using crude preparations, purified FPLC-fraction of BLIS-NU10 and nisin.

Inhibitor-positive preparation tested for inducing activity	**Preparation induces inhibitor production in *S. salivarius* strains and nisin-producing strain**			
	NU10	**YU10**	**K12**	**ATCC11454** [†]
BLIS-NU10 [α]	Yes	Yes	Yes	No
FPLC fraction BLIS-NU10 [β]	Yes	Yes	No	No
BLIS-YU10 [α]	Yes	Yes	Yes	No
BLIS-K12 [α]	Yes	Yes	Yes	No
Nisin (Sigma)	No	No	No	Yes

[α] BLIS (bacteriocin-like inhibitory substances) representing the crude extract of each producer strain.
[β] FPLC-purified fraction BLIS-NU10 produced by strain NU10.
[†] *Lactococcus lactis* strain (producer of nisin lantibiotic).

BLIS-NU10 shown to be Auto regulated while small amount of the crude peptide was able to enhance the production of BLIS-NU10 when it was incubated with the producer strain. BLIS-NU10 shown to be auto-regulated and this discovery used to enhance the production of the putative peptide. After the cells were incubated with BLIS produced by K12, YU10 or NU10 the small quantity of BLIS peptide helped to induce BLIS production (Figure 4.30). Control sample (washed producer cells + BLIS) was not incubated together with the BLIS inducer, the inducer was added to the producer cell free supernatant just before loading 50µl of the mixture into the well to test for activity. Absence of the inhibitory

action in the control well and clear zone of inhibition in the induced well leads to conclusion that strains K12, YU10 and NU10 induced BLIS-NU10 production.

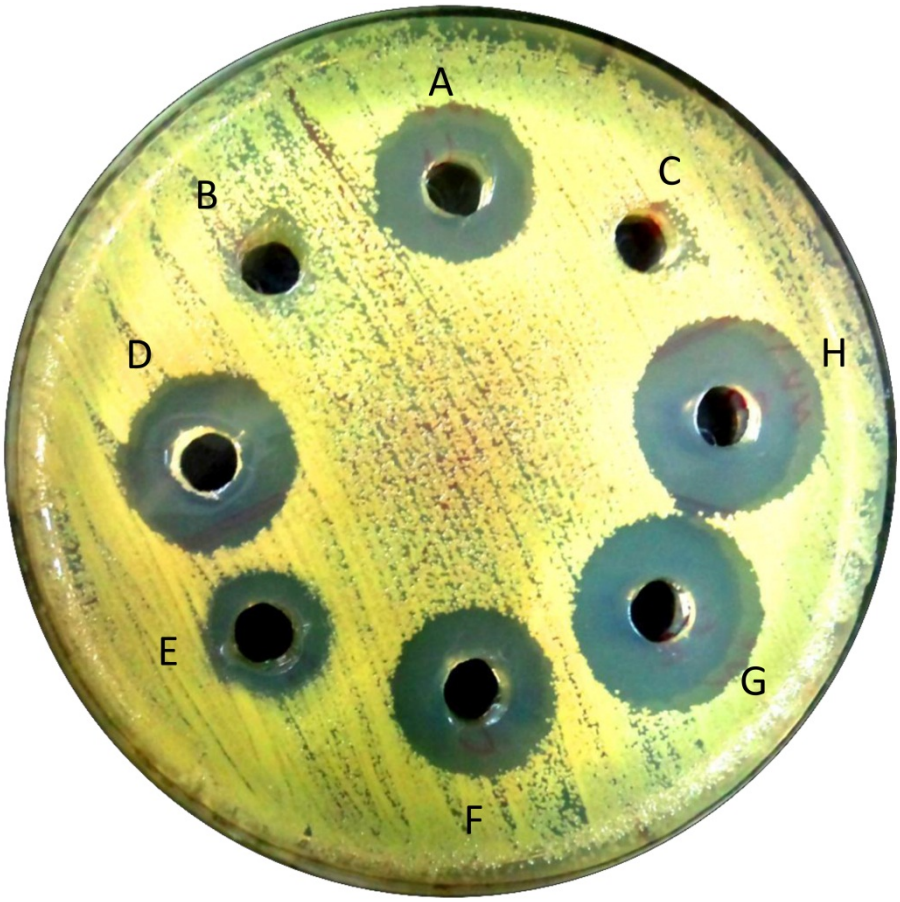


Figure 4.30: well diffusion assay of the induced producer's strains. A: BLIS NU10; B & C: control (supernatant of the producer strain); D: K12 cells + BLIS YU10; E: YU10 cells + BLIS K12; F: YU10 cells + BLIS YU10; G: NU10 cells + BLIS K12; H: NU10 cells + BLIS NU10.

4.11 Production of BLIS-NU10 in liquid medium

To enhance the production of BLIS-NU10 in liquid medium, a new induction assay was developed. The induced culture of *S. salivarius* NU10 showed detectable amount of the inhibitory activity. After 8 hours of the final inoculation the production of BLIS-NU10 started to increase gradually until it reached more than 1200 AU/ml after 16 hours. Once the growth kinetics of strain NU10 reached the stationary phase the level of the inhibitory activity remained stable and consistent.

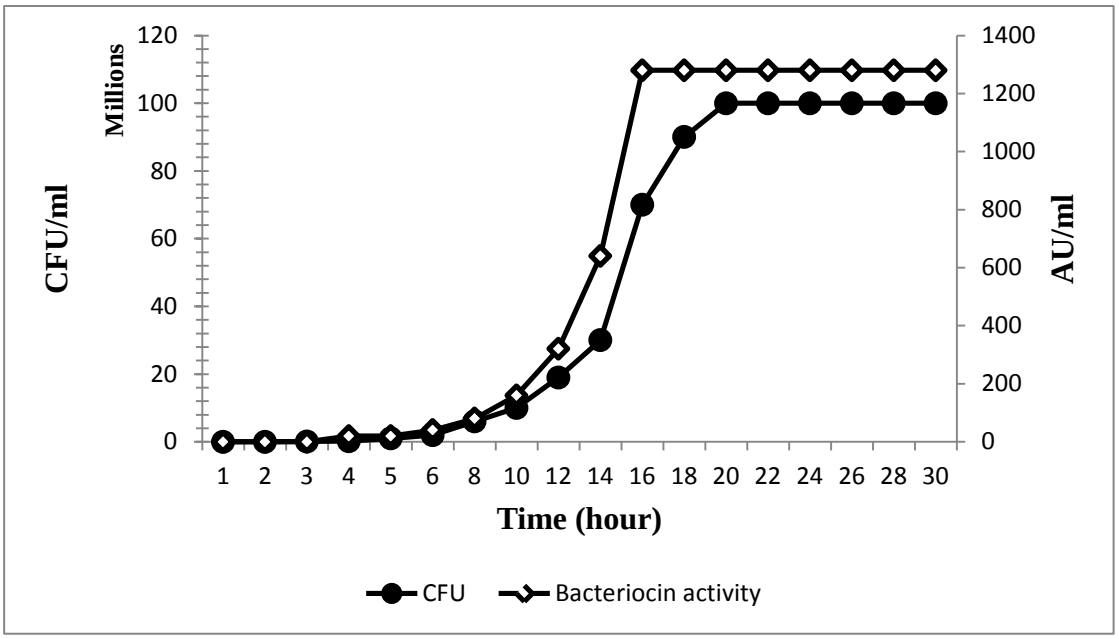


Figure 4.31: growth kinetics of strain NU10 and BLIS-NU10 production.

4.12 Cation exchange Chromatography

BLIS-NU10 shown to be of cation nature for this reason SP FF strong cation exchanger was used to purify the bioactive peptide. This is the first report of using cation

chromatography to purify BLIS-NU10. BLIS-NU10 could not be detected at 280 nm. That is why two additional wavelengths were used to detect the bioactive peptide. This method was ideal to get rid of most of contaminant proteins which was washed out of the column without any binding to the cation exchange column. Before injecting the sample into the FPLC system the column was washed with 20 mM sodium phosphate buffer to equilibrate the column before the positively charged peptide bind to the negatively charged column resin. BLIS-NU10 started to be eluted when the NaCl concentration of the elution buffer reached 23%. Three fractions (1 ml each) showed significant BLIS-NU10 activity when tested in spot on lawn assay using *Micrococcus luteus* as indicator microorganism. BLIS-NU10 was detected at wavelengths of 214 and 207 nm. The active fractions eluted from the column were colorless with no turbidity at all. Dissolving the sample with the binding buffer enhanced the binding process. Sample preparation before cation exchange purification also played a great role in enhancing the binding between the inhibitory activity and SP column. The sample was desalted on XAD-16 column before pursuing to cation exchange chromatography. This cleaning up process was essential to further purify step using AKTA purifier FPLC system. From figure 4.32 it can be seen that BLIS-NU10 could not be detected at 280 nm due to the absence of aromatic amino acids in this lantibiotic. In spot on lawn assay only 10µl or 20µl of each fraction was applied on the surface of indicator lawn, in this case only 3 fractions showed antimicrobial activity but when well diffusion assay was applied whereby 50µl of each sample was loaded into 6mm and this test 6 fractions showed antimicrobial activity including the three fractions from the spot on lawn assay.

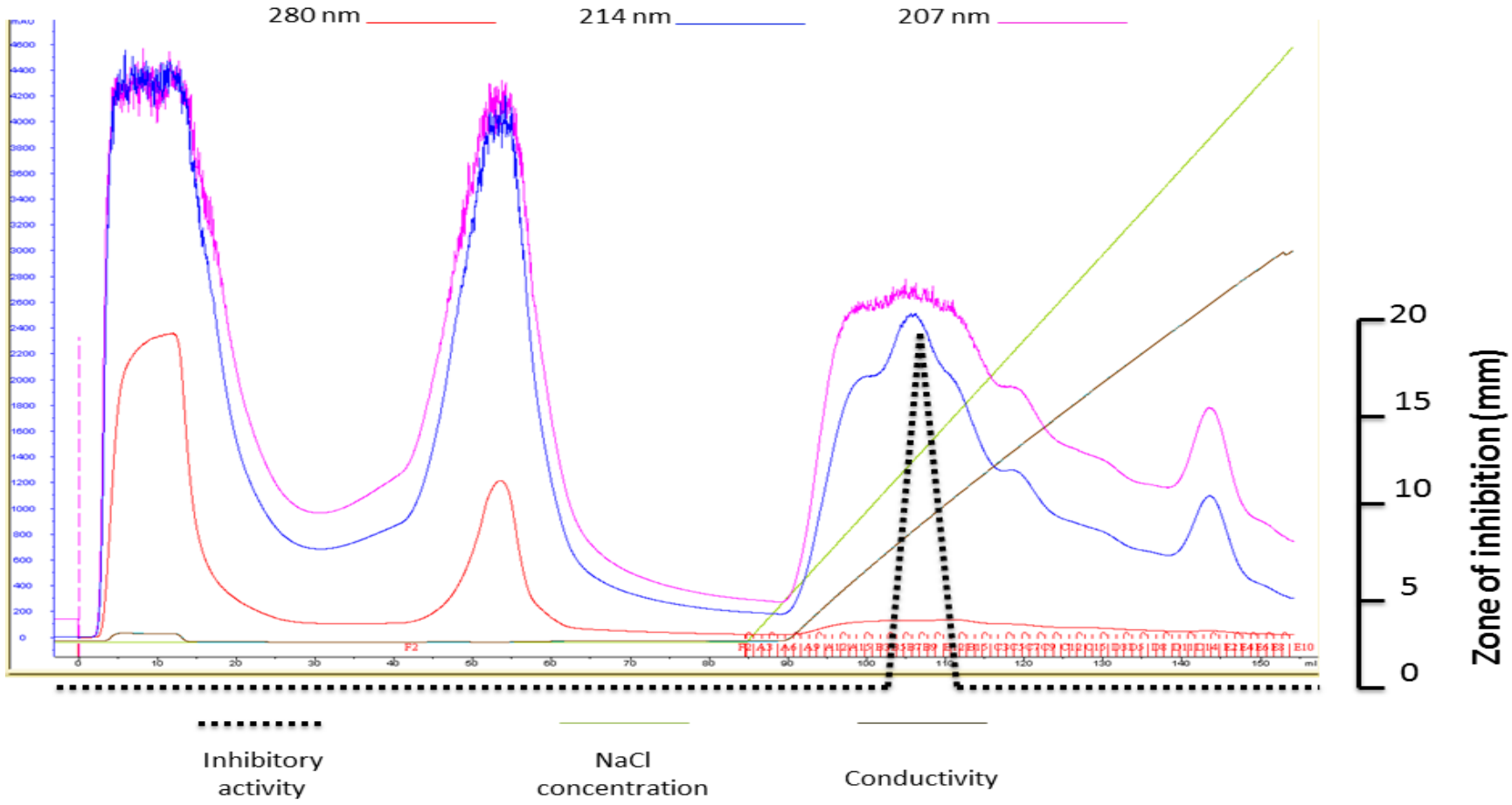


Figure 4.32: Cation exchange chromatography of BLIS-NU10 using SP FF 5ml column.

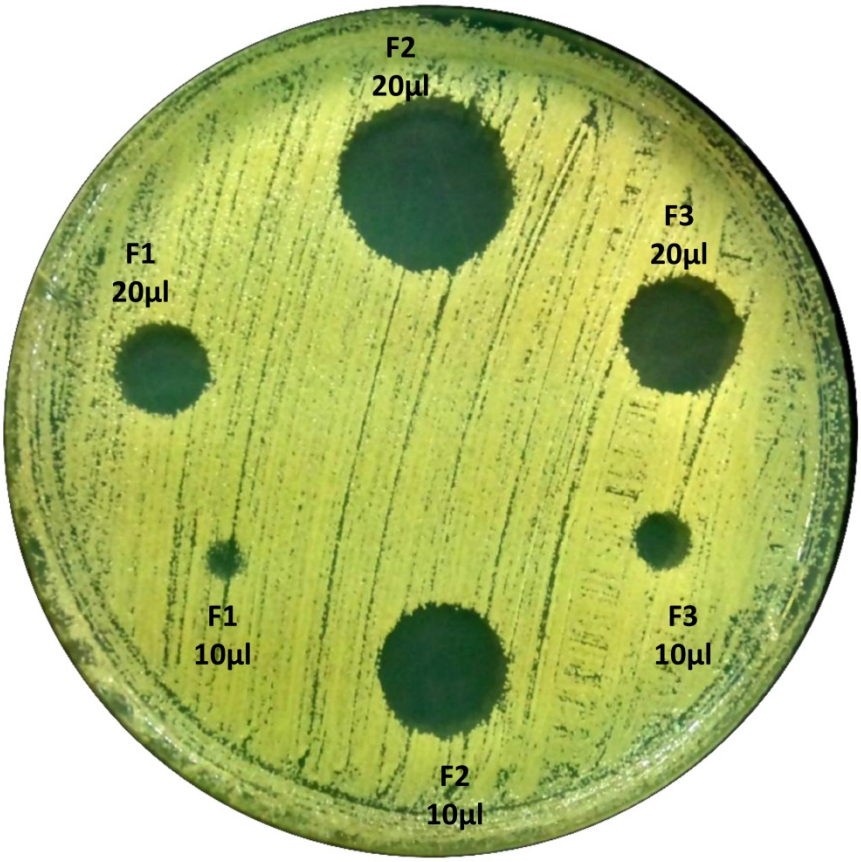


Figure 4.33: Spot on lawn assay of the active pure fractions eluted from SP FF column. F1, F2, and F3: the three active pure fractions of purified BLIS-NU10 using cation exchange chromatography.

4.13 Tris-Tricine SDS PAGE

SDS page was used to check the purity of the active three fractions of BLIS-NU10. The protein standard used was especially for the low molecular weight proteins and all of the three fractions showed to have exactly the same molecular weight of approximately 3,000 Da. Aliquots (50 µl) of the three fractions from the cation exchange FPLC analyzed by 1D-SDS-PAGE (Figure 4.34).

Lane 1 was loaded with 10µl the protein marker. Lanes 2,3 and 4 were loaded with 15µl of each FPLC fraction prepared in sample buffer. Each of the three fractions showed single intense band.

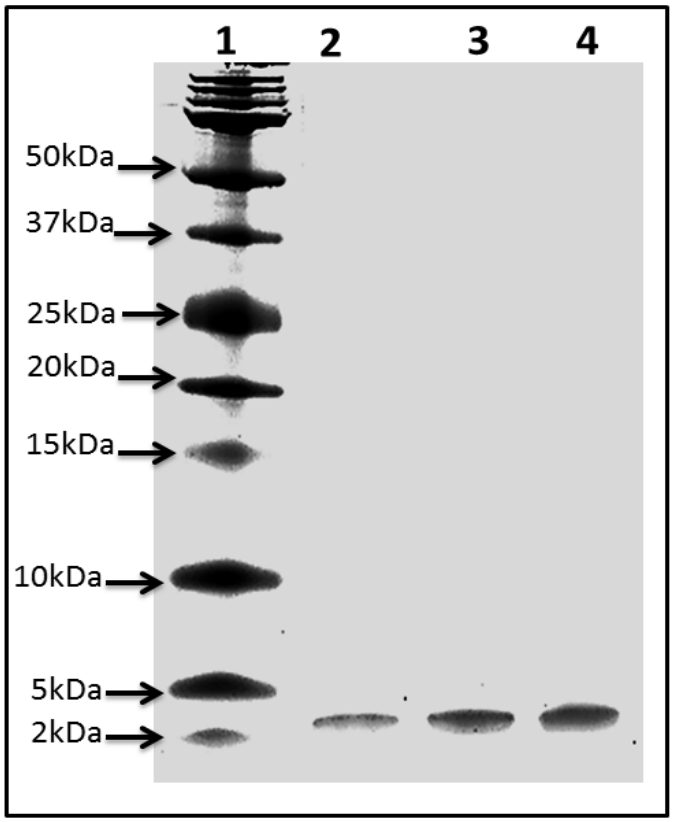


Figure 4.34: SDS-PAGE of cation exchange FPLC active fractions of BLIS produced by strain NU10. Lane 1: Dual Xtra protein marker. Lane 2,3 and 4: FPLC active BLIS-NU10 fractions.

4.14 Characterization of BLIS-NU10

Pure BLIS-NU10 was subjected to LC/MS-ESI and MALDI-TOF mass spectrometry to identify BLIS-NU10. Salivaricin 9 (2560 Da) was detected in the pure BLIS-NU10. Also 2 intensive peaks (2068 Da and 2082 Da) were detected.

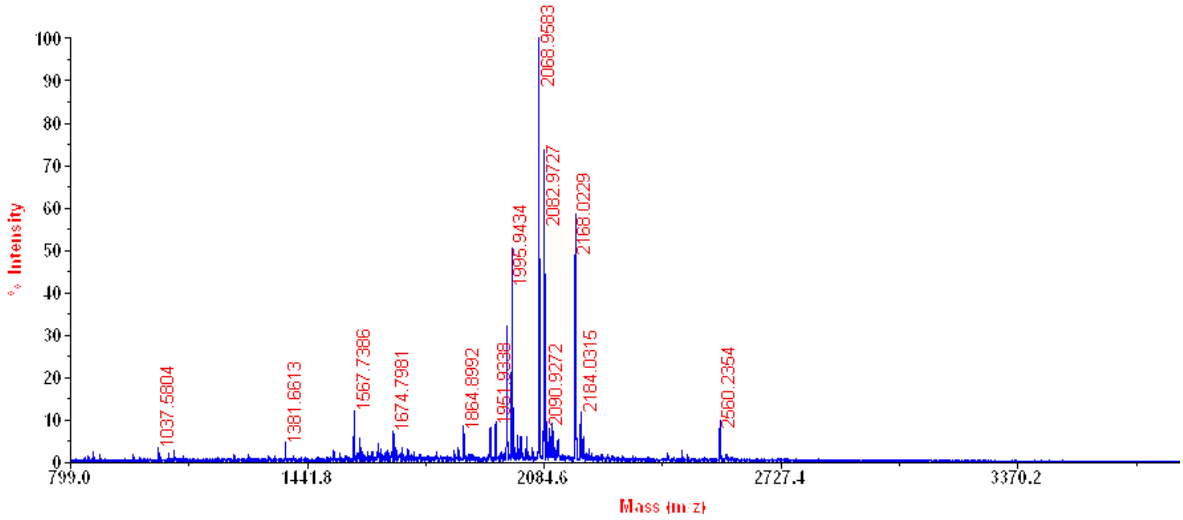


Figure 4.35: MALDI-TOF MS analysis of active fraction of BLIS-NU10 from FPLC fractionation.

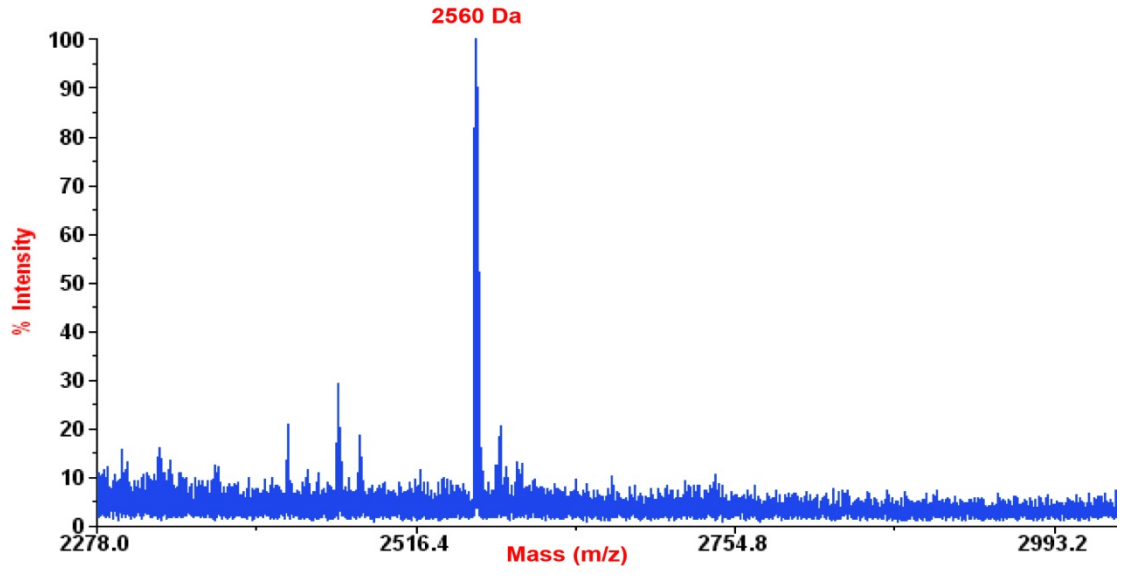


Figure 4.36: MALDI-TOF MS analysis of salivaricin 9 (2560 Da) detected in BLIS-NU10 fraction from FPLC fractionation.

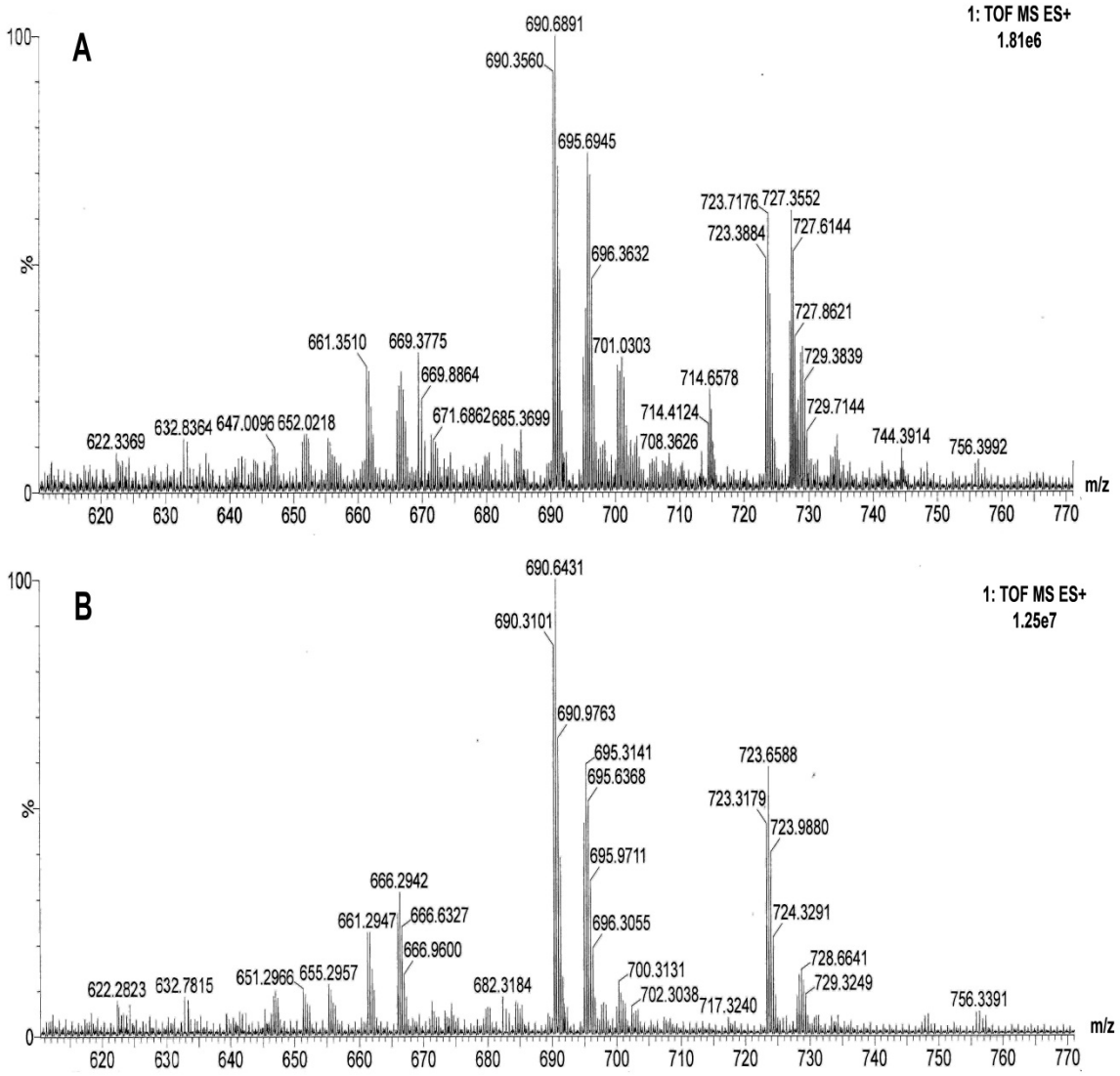


Figure 4.37: LC/MS-ESI TOF analysis of BLIS-NU10. A: pooled active fractions eluted from FPLC system. B: single active fraction eluted from FPLC system (with higher inhibitory activity).

LC/MS-ESI analysis also detected the most intensive peaks which were detected in MALDI-TOF MS analysis with a molecular weight of 2068 and 2082 Da. The De novo amino acid sequencing suggested that these two peptides are rich in proline.

Table 4.10: De novo amino acid sequencing of some detected peptides in pure BLIS-NU10

Peptide detected in pure BLIS-NU10	Amino acids sequencing
Salivaricin 9 (2560 Da)	GNGVVLTLTHECNLATWTKKLKCC
Peptide 1 (2068 Da)	TPPQHGAVQSPLPSPFPPSQ
Peptide 2 (2082 Da)	TPPNGASGALPHGAAPFNPPQS

When de novo sequenced peptides of molecular weights (2082, 2068) was blasted against data base of Swiss port, no significant results could be achieved.

4.15 Membrane permeabilization assay

Investigation of the mode of action of antimicrobial peptide was as described by Bourbon *et al.,* (2008) with some modifications. 10 mM Phosphate buffer pH 7.2 was ideal to suspend the bacterial cells and do the fluorescence assay because it does not contain any DNA fragments which might bind to the SYTOX Green stain and enhance the background fluorescence. 70% ethanol was used as a positive control as it is known that ethanol can penetrate the bacterial membrane by detergent-like mechanism. The fluorescence in the 70% ethanol microplate well was strong due to fast penetrating and binding between SYTOX green and the inner nucleic acid. The other positive control, K12 bacteriocin, was extracted from *S. salivarius* K12 and this extract contains more than two antimicrobial peptides since the strain can produce salivaricin A, salivaricin B and others. The SYTOX fluorescence was also strong in this well but it was also noticed that the membrane penetration started very fast once the K12 extract was added to the bacteria. We can see

from Figure 4.38 that BLIS-NU10 isolated from *S. salivarius* strain NU10 at different concentrations had penetrated the cell membrane of *Micrococcus luteus* ATCC 10240. Even 0.00625 g ml^{-1} of NU10 peptide could penetrate the cell membrane and cause permeabilization of the indicator bacteria in which the fluorescence signal for this well was significantly higher than the negative control (indicator bacteria without peptide added). This negative control well showed low and consistent fluorescence signal. Tetracycline was added to the indicator bacteria in the final well to see if any obvious fluorescence could be detected after treating the bacteria with 0.1 mg ml^{-1} of tetracycline and the fluorescence was below the negative control and this is evidence that tetracycline did not penetrate the cell membrane and no permeabilization action occurred. After the real time PCR run was completed, the bacteria was collected from the wells and washed with phosphate buffer and then diluted 1:100 with the same buffer and streaked on CAB plates before incubation for 18 hours. Bacteria collected from negative control wells could grow perfectly while bacteria collected from 70% ethanol and tetracycline wells were not able to grow. *Micrococcus luteus* cells treated with tetracycline did not exhibit significant fluorescence during Real-Time PCR run and when the tetracycline treated cells were subcultured on fresh medium after the Real-Time PCR run was complete, there was no bacterial growth indicating that Tetracycline mechanism of action is not membrane permeabilization action but protein synthesis inhibition. The Permeabilization assay was repeated using different indicator microorganisms to confirm the membrane penetration activity toward targeted cells. Figure 4.39 shows the permeabilization activity using *Streptococcus equisimilis* and *Corynebacterium spp* as indicator strains.

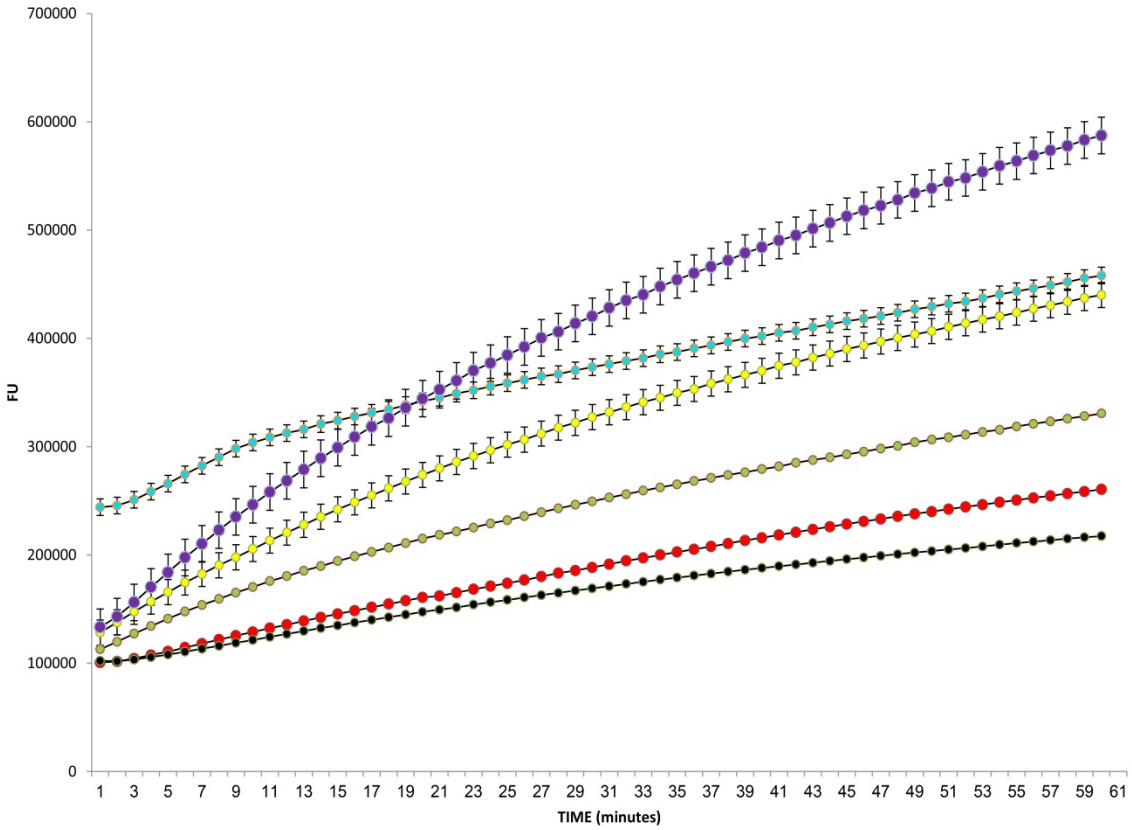


Figure 4.38: Permeabilization of *Micrococcus luteus* ATCC® 10240 membrane by BLIS-NU10. The bacterial cells suspension was incubated with different concentration of BLIS-NU10 with 5 µM SYTOX Green. ●, ethanol 70%; ●BLIS-K12; ●BLIS-NU10 0.05 g ml^{-1}; ● BLIS-NU10 0.025 g ml^{-1}; ●negative control; ● tetracycline 0.1 mg m^{-1}. Binding of fluorescent probe to intracellular nucleic acid was determined at 521nm.

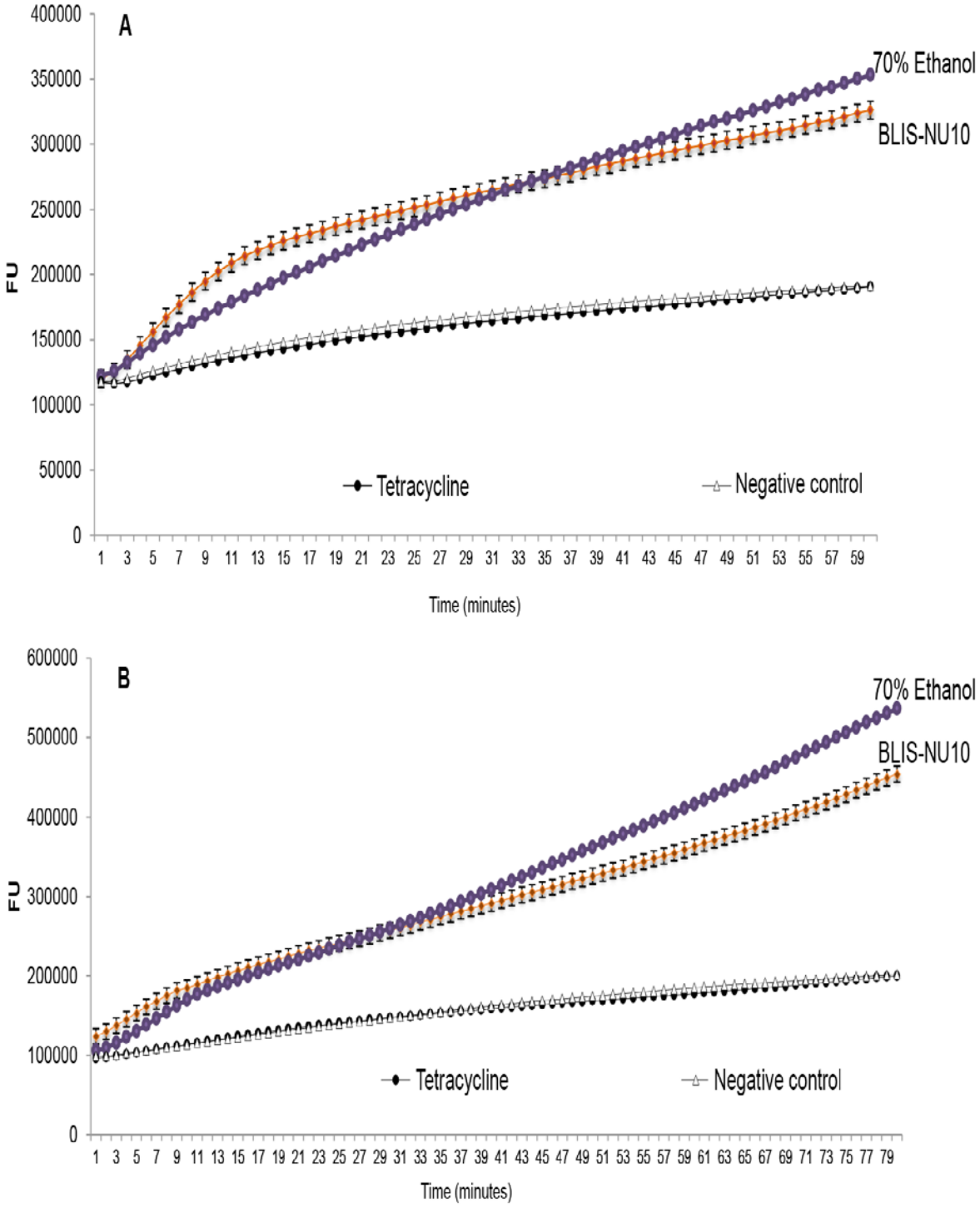


Figure 4.39: Permeabilization of targeted cell membrane by BLIS-NU10. A: indicator strain used *Streptococcus equisimilis*. B: indicator strain used *Corynebacterium* spp.

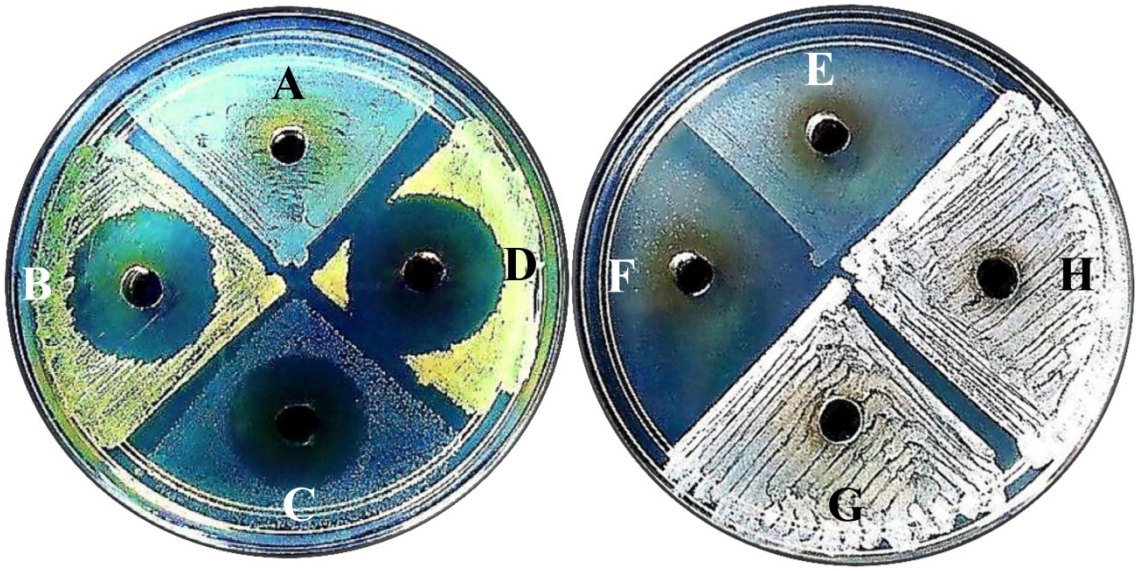


Figure 4.40: Well diffusion assay of purified BLIS-NU10 peptide isolated from *S. salivarius* NU10. 50µl of the purified peptide (0.05 g ml^{-1}) added to the wells (6 mm) have been cut into CAB media. A: *Streptococcus gordonii* ST2, B: *Micrococcus luteus* ATCC 10240, C: *Streptococcus equisimilis* ATCC 12388, D: *Corynebacterium* spp GHG17, E: *Lactococcus lactis* ATCC11454, F: *Streptococcus pyogenes* ATCC 12344, G: *Haemophilus parainfluenza* TONE J11 and H: *Actinomyces naeslundii* TG2.

4.16 Scanning Electron Microscopy

Scanning electron microscopy helped to confirm that the mechanism of action was done by permeabilization of the cell membrane. Six hours of bacterial incubation time with BLIS-NU10 was enough to penetrate the cell membrane as shown in figure 4.41 that the bacteria was affected by BLIS-NU10 and some pores were formed in the cell membrane causing bacterial contents to ooze through the pores as the first step of the peptide penetrating the cell membrane. This is a common mechanism of action for lantibiotics. After that the bacteria will lose all the inner contents and start to collapse and die. Some cells were badly affected by BLIS-NU10 and after the inner material went out through the pores, the cells were ruptured.

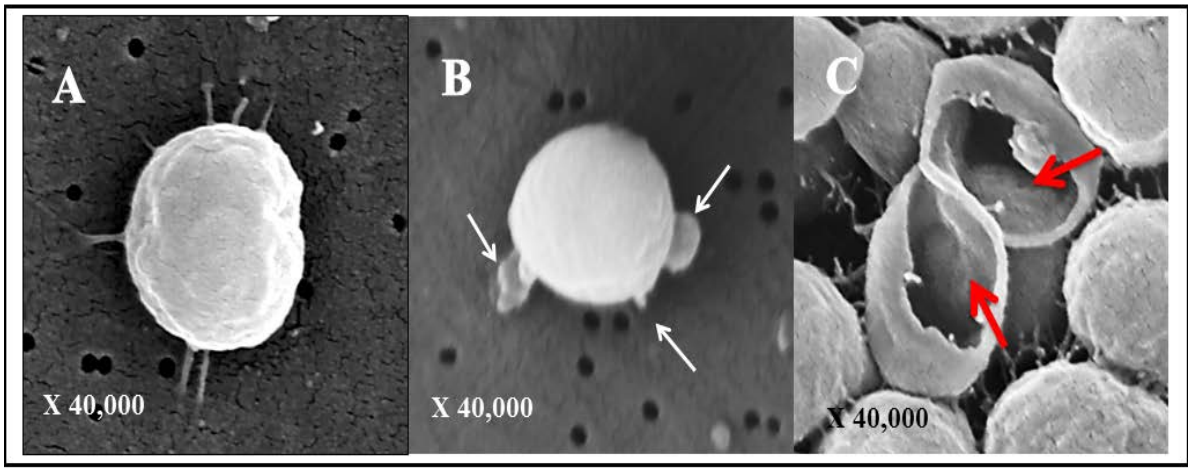


Figure 4.41: SEM sections show the mechanism of action of purified BLIS-NU10 isolated from *S. salivarius* NU10. A: a healthy *Micrococcus luteus* ATCC 10240 cell without treatment used as a control in this experiment. B: release of internal materials through pores formed by NU10 peptide. C: a ruptured *Micrococcus luteus* ATCC 10240 cell after incubation with purified NU10 peptide at 37^{o}C for 6 hours.

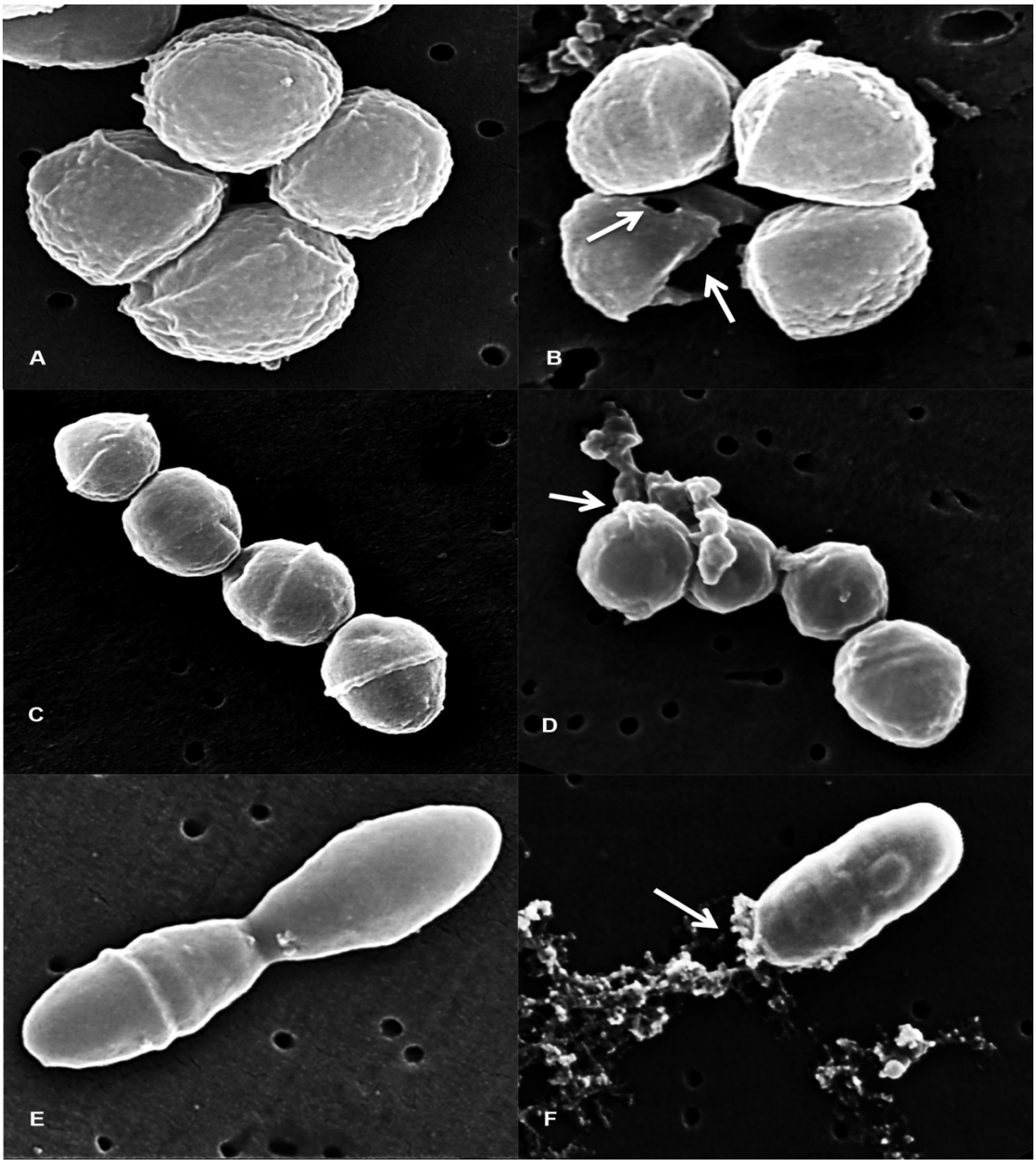


Figure 4.42: SEM sections show the mechanism of action of purified BLIS-NU10 isolated from *Streptococcus salivarius* NU10 using different indicator strains.

Indicator strain	Control (untreated)	Treated with BLIS-NU10
Micrococcus luteus	A	B
Streptococcus equisimilis	C	D
Corynebacterium spp	E	F

4.17 BLIS-NU10 stability (thermo-stability/ pH/ proteinase K/ chemicals)

Table 4.11: Stability profile of BLIS-NU10

Stability	Concentration	Inhibition zone
Temperature (^{o}C)		
4, 20, 30, 37, 40, 50, 60, 70, 80 for 1 hour		++++
90, 100 for 30 min		+++
121 for 20 min		-
pH value		
2- 7		++++
8-10		+++
11-12		-
Enzymes	1 mg ml^{-1}	
Proteinase		-
Proteinase K		-
Peptidase		-
Lyticase		++++
Catalase		++++
Detergents / Chemicals	1% (W/V)	
Tween 80		++++
Tween 20		++++
Tritone X100		++
β-merchaptoethanol		++++
SDS		++++
EDTA		++++
Urea		++++
NaCl		++++

(++++): inhibition zone >20 mm, (+++): inhibition zone =20 mm, (++): inhibition zone < 20 mm, (-): no bacterial inhibition. *Micrococcus luteus* was used as indicator. Salivaricin 9 titer: 800 AU/ml.

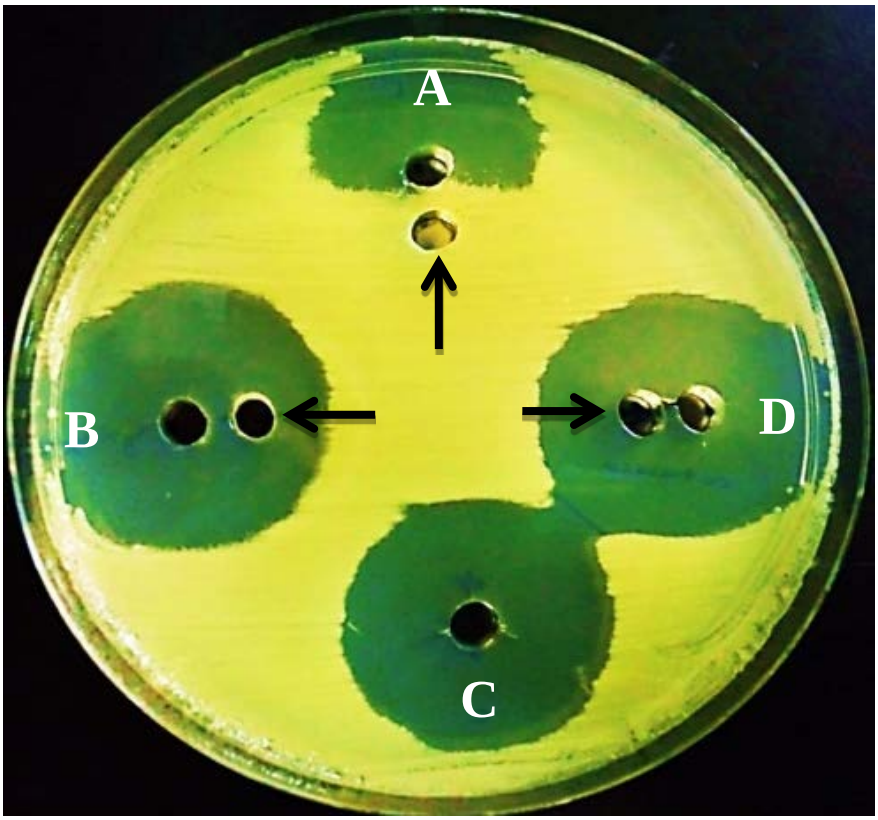


Figure 4.43: Enzyme stability test of BLIS-NU10. Black arrows indicates where the enzymes where loaded close to where BLIS-NU10 was loaded. A: proteinase K, B: catalase, C: control and D: lyticase.

Chapter Five

Discussion

5.1 *Streptococcus salivarius* isolation

S. salivarius is a predominant coloniser of the oral cavity in humans. Some *S. salivarius* produce small molecules called BLIS to aid their competition within the oral ecosystem which comprises of a large number of diverse communities of microorganisms. Most of the BLIS molecules produced by *S. salivarius* are lantibiotics or lanthionine-containing peptides. All Malaysian subjects in this study were shown to have *S. salivarius* in their oral cavity. Some of these isolates were swabbed from the tongue of subjects with periodontal problems e.g. dental caries, gum disease and halitosis. Two of the *S. salivarius* isolated from these subjects (SAM3 and IND9) failed to produce significant BLIS activity and in further investigations in this present study it was found that most of the activity totally disappeared when the isolates were grown on medium supplemented with calcium carbonate (refer to the data as table or figure from Results). This indicates that their limited activity is due to the production of lactic acid. However, some of the *S. salivarius* isolated from healthy Malaysian subjects (NU10 and YU10) showed significant BLIS production, especially when blood-containing medium was used in the production study.

For primary isolation, MSA or M17 supplemented with 20% sucrose media were used since *S. salivarius* colonies appear as dome-shaped puffy colonies when the growth medium is supplemented with sucrose. This unique characteristic is due to levan production. In further investigation of BLIS-producing isolate YU10, the bacteria were shown to produce a significant amount of 103.9 kDa levansucrase enzyme (Figure 4.12) when grown in M17 supplemented with 1% sucrose and 0.1 % calcium carbonate. This enzyme polymerizes the fructose moiety of sucrose into fructans which possess levan structure (Ebisu *et al.*, 1975).

5.2 BLIS production by *Streptococcus salivarius* isolated from Malaysian subjects

S. salivarius strain HJEFF was the only strain that showed some BLIS production when it was growing in MHB. No BLIS activity could be detected from the cell free supernatant (CFS) but anti-*Bacillus cereus* and anti-*Haemophilus parainfluenzae* activity could be detected from the ammonium sulphate precipitate of the CFS. Surprisingly no BLIS production was evident when more enriched media was used in the production study. In the deferred antagonism assay some strains, namely HJEFF, 7YE and GT2, showed ~~a~~ very weak BLIS production when BACa was used as a production medium but when TSYECa was used instead of BACa the BLIS activity was increased significantly. The production of BLIS activity in TSYECa medium and its reduced production in BACa medium was also reported in a study of salivaricin M production. Unlike most of the bacteriocins produced by *S. salivarius,* salivaricin M was not produced efficiently in blood-enriched medium and there appeared to be strict regulation of its locus expression (Wescombe *et al.*, 2012). Salivaricin M produced by *S. salivarius* strain M18 (BLIS Technologies) is a chromosomally encoded lantibiotic (unlike most lantibiotics produced by *S. salivarius*) which is active against *Streptococcus mutans*. The very first characterized lantibiotic produced by *S. salivarius* strain 20P3 is salivaricin A (2315 Da). Salivaricin A production is encoded by *salA,* located on a megaplasmid. Salivaricin A has 5 variants namely A1, A2, A3, A4 and A5 with molecular weights of 2327 Da, 2368 Da, 2319 Da, 2342 Da and 2329 Da respectively. The mode of action of salivaricin A appeared to be bacteriostatic rather than bactericidal. Strain YU10, isolated from a Malaysian subject appeared to have the structural gene *salA* and the antimicrobial activity of the lantibiotic produced by this strain was reported in this study. Techniques used to extract the bioactive peptide from this strain

were successful when freeze thawed extraction from the agar culture and acidic methanol extraction of the centrifuged and isolated cells were applied. No detectable BLIS activity could be recovered from this strain when the bacteria were grown in liquid medium. In further investigation, strain YU10 appeared to harbour both *sivA* and *slnA* (structural genes encoding salivaricin 9 and salivaricin G32 respectively) in addition to salA which makes strain YU10 a good BLIS producing strain (Tagg, 2013). BLIS produced by strain YU10 was bacteriostatic in action and this mode of action was similar to that of salivaricin A. Strain NU10 was also isolated from one Malaysian subject and showed BLIS production in deferred and simultaneous antagonism tests. Out of six isolates, both strains NU10 and YU10 showed good BLIS production especially when the deferred antagonism test was done on BACa medium and that is linked to the need blood medium to secrete the antimicrobial peptide into the agar-supplemented medium. Strain NU10 showed erratic BLIS production. When this strain was used as BLIS producer in deferred and simultaneous antagonism tests, it secreted BLIS in BACa medium when it was grown in both aerobic and anaerobic conditions. It has been noticed that when NU10 was grown in anaerobic condition, the BLIS production was increased significantly. Usually BLIS production is optimum in CO_2-enriched atmosphere, especially when strain K12 is used as the BLIS producer (Hyink *et al.*, 2007). When strain NU10 was grown in such a CO_2 enriched condition, it took a longer time than K12 to reach the stationary phase although it could still secrete BLIS into the medium. BLIS production in strain NU10 was unstable and when glucose or lactose was used instead of sucrose as a carbon source, almost no BLIS production could be detected. In an attempt to enhance BLIS production in strain NU10, 1% of sucrose was added to M17 medium supplemented with 0.1% calcium carbonate instead of 0.5% of sucrose as used in past and published procedures to generate BLIS in

K12 (Hyink *et al*., 2007). In this study increasing the sucrose percentage from 0.5% to 1% resulted in a more homogenous and turbid bacterial culture increasing the CFU as well.

5.3 BLIS-NU10 purification

Attempts to purify the inhibitory molecules produced by strain NU10 began by first concentrating the BLIS activity of the freeze thawed extract by adding solid ammonium sulphate. The lowest saturation that precipitated the inhibitory activity was 40%, but to recover significant amounts of BLIS, 65% saturated ammonium sulphate was required. Ammonium sulphate precipitation was found to be the ideal method to precipitate and concentrate BLIS-NU10 based on the current investigations. Gel filtration was applied using SEPHADEX G25 column. This combination of ammonium sulphate and gel filtration was reported previously for purification of zoocin A produced by *Streptococcus zooepidemicus* strain 4881 and viridin B produced by *Streptococcus mitis* (Apelgren and Dajani, 1979). This preparation of crude BLIS-NU10 was loaded into XAD-2 column to get rid of hydrophobic molecules and impurities. XAD-2 was previously used to prepare salivaricin A and B from freeze thawed extract of K12 culture (Hyink *et al.*, 2007). Sep-pak C18 cartridge increased the purity from 6 to 640 fold which makes this technique effective in BLIS-NU10 purification. Sep-pak C18 cartridge was used previously to achieve semi purified streptin (type A1 lantibiotic produced by *Streptococcus pyogenes* strain M25) (Wescombe and Tagg, 2003).

5.4 BLIS-NU10 and BLIS-YU10 mode of action

Salivaricin A, the first lantibiotic to be characterized from *S. salivarius*, has no dehydrated residues in its biologically active propeptide form and it exhibits bacteriostatic mode of action towards the targeted cells rather than bactericidal (Hyink *et al.*, 2007). BLIS-YU10 appeared to exhibit a bacteriostatic mode of action when the BLIS was added to a log phase culture of the indicator microorganism the bacterial growth being inhibited and no increase in OD measurement observed. However, after a few hours of incubation the bacteria started to grow again in the presence of BLIS-YU10. Purified BLIS-NU10 produced by strain NU10 showed a bactericidal mode of action and the indicator microorganism failed to grow in the presence of BLIS-NU10 after 30 hours of incubation. BLIS-NU10 was added to different phases of targeted bacterial growth and each time BLIS-NU10 effectively reduced the OD readings when it was added. Such bacteriolytic activity was reported as a mode of action for both bovicin and nisin (de Carvalho *et al.*, 2007)

5.5 Bacteriocin induction activity and BLIS-NU10 production in liquid medium

It has been reported that some bacteriocins including salivaricin A, B (Hyink *et al.*, 2007; Ross *et al.*, 1993) and mutacin (Nicolas *et al.*, 2011) are controlled by quorum sensing mechanisms and they are better expressed when the producer bacteria is grown on solid media where the bacteria can be found in high density. This production strategy will result in significant bacteriocin expression in that particular medium comparing with low density of bacteria growing in liquid media where no detectable bacteriocin production can be observed (Kleerebezem *et al.*, 1997; Qi *et al.*, 2000). Usually to produce these classes of

bacteriocins, a freeze thaw method is used while the bacteria are grown on solid or semi-solid media containing agar or agarose. In this study, a new method was developed to produce the bacteriocin in liquid medium. When the production experiment was applied for the first time using *S. salivarius* NU10 as a producer, no inhibitory activity could be detected from the cell free supernatant of the culture. Most lantibiotics biosynthesis can be auto regulated by signal transduction system like bovicin HJ50 produced by *Streptococcus bovis* (Ni *et al.*, 2011) and salivaricin A produced by *S. salivarius* 20P3 (Upton *et al.*, 2001) and nisin produced by *Lactococcus lactis* (Kuipers *et al.*, 1995). To investigate in what stage of the bacterial growth the optimum quantity of BLIS-NU10 is being produced, a liquid medium system was applied to count the CFU followed by the peptide titer. The induction procedure showed that BLIS-NU10 is auto-regulated. As the bacteria continues to grow in the medium in the presence of the introduced BLIS-NU10 molecules for the induction, the expression of BLIS-NU10 in liquid medium was increased significantly by 1200 AU/mL (Figure 4.31). Feeding the induced culture with fresh medium helped to scale up the productivity while the culture itself worked as inoculum and inducer at the same time.

5.6 Cation exchange chromatography as BLIS purification strategy

Using cation exchange chromatography to purify lantibiotics has been described previously (Furmanek *et al.*, 1999; Sahl, 1994). Salivaricin B purification described previously (Hyink *et al.*, 2007) by using XAD-2 chromatography and RPHPLC system has been used to separate specific lantibiotics namely salivaricin A2 and B produced by strain *S. salivarius strain* K12. The current study is the first report of BLIS-NU10 purification using SP

sepharose column as a cation exchange technique. Using cation exchange chromatography resulted in enhanced yield of BLIS-NU10 which was recovered after XAD-16 chromatography and preferred over XAD-2 chromatography due to its higher capacity to bind the lantibiotic. XAD-2 particles had been used previously to adsorb salivaricin A molecule (Ross *et al.*, 1993). Amberlite XAD-4 was used to adsorb nisin molecules (Tolonen *et al.*, 2004). XAD-16 hydrophobic resin was a critical step in the current protocol to achieve clear and desalted crude peptide. XAD-16 Amberlite was used previously to adsorb LtnA1 and LtnA2 that formed the two components of lacticin 3147 (Morgan *et al.*, 2005). FPLC system showed capability to separate BLIS-NU10 from crude preparations achieved from XAD-16 chromatography and this purification protocol resulted in single protein intensive band when Tris-Tricine SDS-PAGE was applied. Most but not all of the impurities were washed out from the column without any binding to the SP Sepharose resin while more than one bound peak was detected after elution with a linear gradient of increasing salt concentration. The bioactivity test of all the unbound and bound fractions showed that 3 active fractions related to one peak were eluted after applying a specific moderate concentration of NaCl ≈ 23%. This finding indicates that BLIS-NU10 is of cationic nature.

5.7 Membrane permeabilization and pores forming mechanism of action

Permeabilization activity of lantibiotics toward gram positive bacteria has been studied previously (Chun and Hancock, 2000). The depolarization of the cytoplasmic membrane of gram positive bacteria was assessed by dequenching of SYTOX green fluorescence by pure BLIS-NU10 in this study. Type A lantibiotics (e.g. nisin, salivaricin and epidermin) are

amphipathic and elongated molecules which mainly act by forming pores into the cytoplasmic membrane of the targeted bacterial cell (Kordel *et al.*, 1989; Moll *et al.*, 1996; van Heusden *et al.*, 2002). Lipid II is an initial factor to induce a transmembrane orientation of the pore forming lantibiotic (van Heusden *et al.*, 2002). At some stages lantibiotics such as nisin can form pores and can inhibit the cell wall biosynthesis when it binds to the peptidoglycan precursor lipid II (Wiedemann *et al.*, 2001). Studying the mechanism of action of BLIS-NU10 showed the same characteristics compared with other lantibiotics while it shares the membrane permeabilization mechanism of action which form pores in the cytoplasmic membrane of the sensitive gram positive bacteria as shown in this study in the SEM images (Figures 4.41 and 4.42) using targeted bacteria incubated with BLIS-NU10. The pores formed after incubation with BLIS-NU10 showed variation when the three indicator strains were used. After 240 minutes of BLIS-NU10 incubation with *Micrococcus luteus* or *Corynebacterium spp,* the SEM image in figure 4.42 showed significant pores in the targeted cell membrane wherein the bacterial cells were ruptured completely resulting in significant changes in cell morphology. However, another sensitive strain namely *Streptococcus equisimilis* showed less susceptibility to BLIS-NU10 compared to *Micrococcus luteus* or *Corynebacterium spp* in deferred antagonism test and permeabilization activity assay. The SEM visualized that no significant changes in cell morphology occurred in this strain after incubation with BLIS-NU10.

5.8 BLIS-NU10 stability

BLIS-NU10 showed heat stability when it was exposed to high temperature (90-100^{o}C). BLIS-NU10 remained active for 9 months when it was stored at 4^{o}C. At low temperature conditions (-20^{o}C) BLIS-NU10 did not lose any of its activity for 9 months. BLIS-NU10 is stable in different values of pH ranging 2-10. However, at extreme alkaline conditions (pH 11-12) BLIS-NU10 lost most if not all its activity while it was noticed that the optimum pH values of BLIS-NU10 stability is pH 2-7 (Table 4.16). However BLIS-NU10 activity showed no decreasing in activity after heating at 80^{o}C for 30 min when all available indicator strains were tested including *Micrococcus luteus*, *Corynebacterium spp* and *Streptococcus equisimilis.* Usually bacteriocins with a molecular weight <5 kDa are heat stable molecules including lantibiotics (Nes *et al.*, 2007). When treated with proteinase K or peptidase, BLIS-NU10 lost its antimicrobial activity which indicates that BLIS-NU10 is of protein nature.

5.9 BLIS-NU10 as a potential probiotic

When strain NU10 was tested as a producer strain in deferred antagonism test using 9 indicator strains (Tagg and Bannister, 1979) to determine what kind of salivaricin strain NU10 can produce, the scheme showed no production characteristics of either salA nor salB (Tagg, personal communication). However, strain NU10 showed to harbour the structural genes of three different lantibiotics (salA, salB and sal9). This finding indicates that although NU10 harbours known structural genes of previously described lantibiotics,

the biologically active product of the structural genes mentioned above might not be expressed during production studies and the only gene product detected in MALDI-TOF MS analysis is salivaricin 9 (Figure 4.36). The mode of action of BLIS-NU10 showed to be bactericidal rather than bacteriostatic. Hence we can conclude that BLIS-NU10 is not salA (the bacteriostatic lantibiotic) (Wescombe *et al.*, 2006). BLIS-NU10 showed to be auto-inducible where it enhanced the inhibitory activity production when incubated with the washed cells of strain NU10. To enhance BLIS-NU10 production, strain NU10 always required to be stimulated by induction assay of the same BLIS molecules. MALDI-TOF MS analysis showed the presence of salivaricin 9 (2560 Da) in the current study. However, in addition to the salivaricin 9 peak, another 3 intensive peaks were detected, each of molecular weight approximately 2000 Da and these peaks showed to be proline-rich peptides which is uncommon among salivaricins. When these peptides were de novo sequenced, they showed no significant similarity of any previously characterized bacteriocins.

Chapter Six

Conclusion

S. salivarius strains were isolated from six Malaysian subjects using sucrose-enriched media. Sucrose was used in the medium as *S. salivarius* metabolize sucrose into levan by levansucrase enzyme which was detected in the cultures of *S. salivarius* grown in such a medium. The isolates were identified using API kits and 16S rDNA gene sequencing for further confirmation. All these isolated strains 16S rDNA genomic sequences were deposited in the NCBI database. Some of the isolates appeared to produce bacteriocin-like inhibitory activity when tested in both simultaneous and deferred antagonism tests. An attempt was applied to produce the inhibitory activity in liquid media. However, strain HJEFF was the only strain that produced limited inhibitory activity when grown in MHB. The ammonium sulphate precipitate of the cell free supernatant of strain HJEFF was of anionic nature inhibiting *Bacillus cereus* and *Haemophilus parainfluenzae*. Strains K12, NU10 and YU10 showed significant inhibition against *Micrococcus luteus, Corynebacterium spp, Streptococcus equisimilis and Lactococcus lactis.* Antimicrobial crude peptide was obtained from strains K12, NU10 and YU10 using acidic methanolic extraction of the producer cells and freeze thaw extraction of the strains using M17SUCa medium. BLIS produced by strain YU10 was bacteriostatic in its mode of action while BLIS obtained from strain NU10 was bactericidal. The distribution of structural genes encoding previously characterized salivaricins was investigated in the Malaysian isolates. Strain YU10 harboured *salA, sivA* and *slnA* encoding salivaricin A, salivaricin 9 and salivaricin G32 respectively. Hence strain YU10 has good potential for application as a probiotic. Strains K12 and NU10 harboured *sboB* (a structural gene encoding salivaricin B) and *sivA* encoding salivaricin 9. When strain NU10 was applied in deferred antagonism assay using 9 standard indicators, it appeared that the inhibitory activity produced by NU10 was not from either salivaricin A or salivaricin B. A new method was used to enhance

BLIS-NU10 production in liquid medium. BLIS-NU10 was shown to be auto-regulated. Cation exchange chromatography was used to purify BLIS-NU10 and the pure fractions were subjected to Tris-Tricine SDS PAGE to demonstrate the molecular weight, which appeared to be about 3,000 Da. Further analysis of the purified BLIS-NU10 using MALDI-TOF MS showed the presence of salivaricin 9 lantibiotic (2560 Da). Other peptides with molecular weights of 2068 Da and 2082 Da detected in BLIS-NU10. When these peptide sequences where blasted against SwissProt and NCBI databases, no significant matches were found. The BLIS-NU10 mechanism of action was studied using SYTOX green dye to investigate the ability of BLIS-NU10 to penetrate the cytoplasmic membrane of the targeted cells. BLIS-NU10 induced cytoplasmic membrane permeabilization of its targeted bacteria. Scanning Electron Microscope images of sensitive bacteria treated with BLIS-NU10 showed that the antimicrobial peptide induces pore formation in the indicator bacterial membrane. BLIS-NU10 exhibited thermo-stability when exposed to a temperature of 100^{o}C for 30 minutes and retained biological activity when subjected to different pH values ranging from 2 to 10. When treated with proteinase K, BLIS-NU10 lost all of its antimicrobial activity. BLIS-NU10 can be a potentially new antimicrobial peptide, but further work may be needed to detect the structural genes encoding BLIS-NU10 production.

APPENDICES

APPENDIX A

26th April 2012

Volunteer information sheet

Re: Study of bacteriocins produced by *Streptococcus salivarius* isolated from Malaysian subjects

Dear volunteer,

We would like to invite you to take part in a research study. In this study, we wish to obtain information about the bacteriocins produced by *Streptococcus salivarius* isolated from Malaysian subject.

As part of the research, your oral samples will be collected in the morning (9-10 am) before consuming any food. Please refrain from oral hygiene measures on the sampling day. You can have teeth brushed on the previous day as usual but brushing and mouth rinse solution should be avoided on the day of sampling. Your oral samples will be collected from the tongue surface by cotton swabs. The sampling procedure is a painless and non-invasive process.

Taking part in this research will not benefit you directly and is entirely voluntary. If you have any questions about the research, please do not hesitate to ask.

Thank you.

……..

Abdelahhad Barbour

M.S.c Candidate

Microbiology Division

Institute of Biological Sciences

Faculty of Science

University of Malaya

Kuala Lumpur

APPENDIX B

Consent form

Re: Study of bacteriocins produced by Streptococcus salivarius isolated from Malaysian subjects

1. I have read the volunteer information sheet
2. I have had the opportunity to ask questions and discuss the research
3. I am satisfied with the answers to the questions
4. I have received enough information about the research
5. I agree to take part in this research

Signature of volunteer: ……..

Name :……

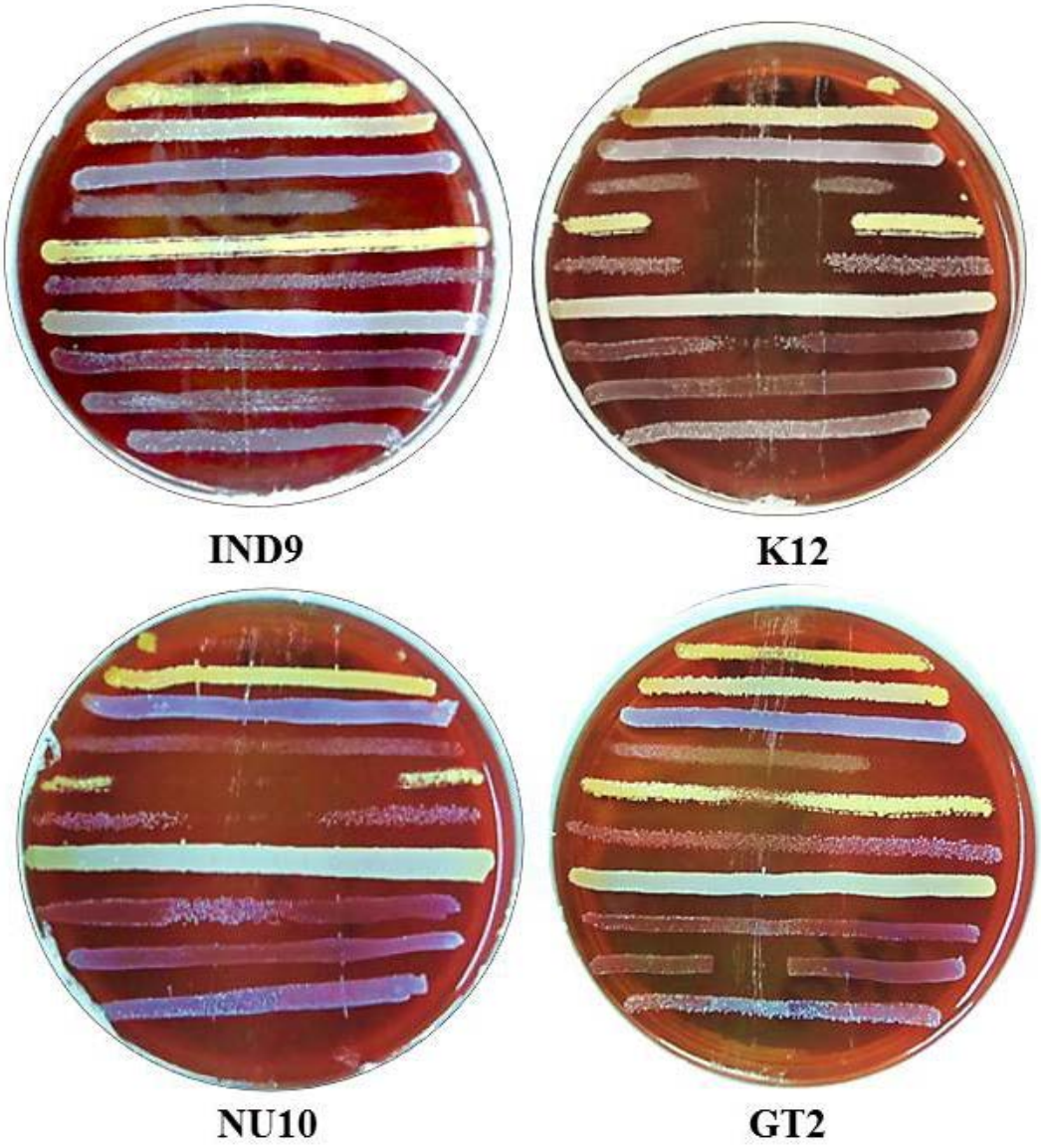
APPENDIX C

Details of subjects included in this study:

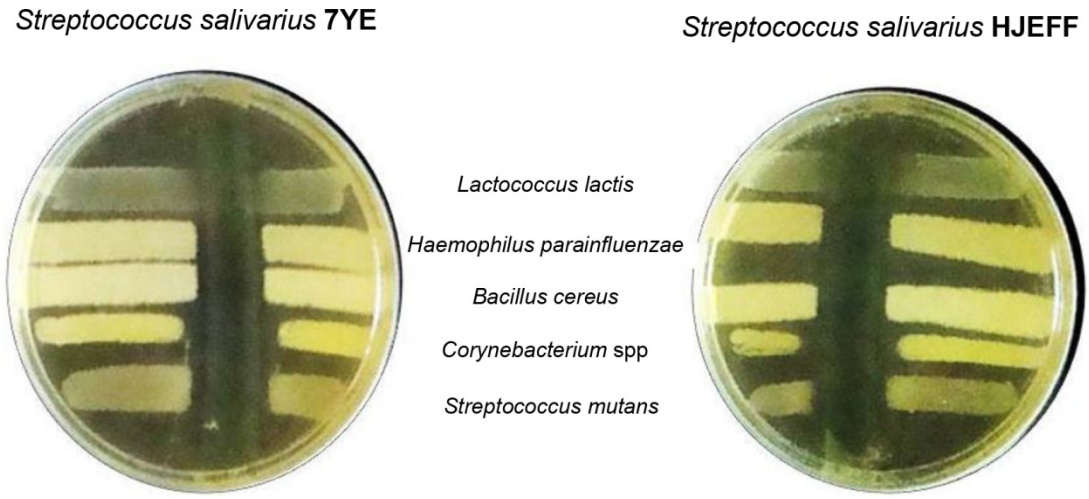
***S. salivarius* isolate**	**Gender**	**Age**	**Race**	**Smoker***	**Dental problems**	**Isolation source**
NU10	Female	25	Malay	No	No	Tongue
YU10	Female	25	Chinese	No	No	Tongue
GT2	Female	25	Chinese	No	No	Tongue
7YE	Male	7	Malay	No	Yes	Saliva
IND9	Female	26	Indian	No	Yes	Saliva
SAM3	Female	35	Indian	No	Yes	Saliva
HJEFF	Male	26	Malay	No	No	Tongue

*Only exclusion criterion used was to exclude smokers

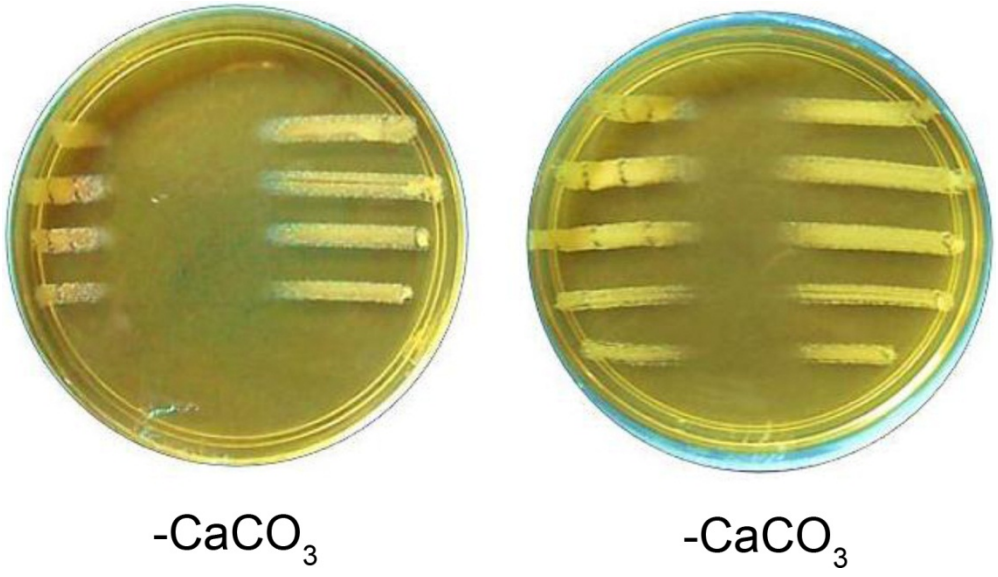
APPENDIX D



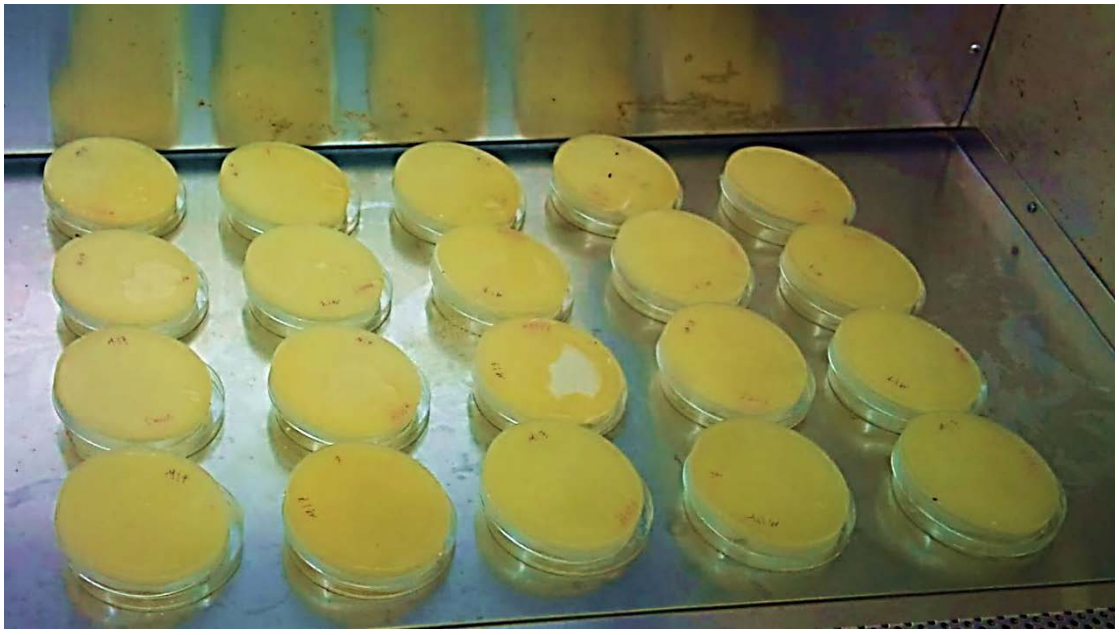
Deferred antagonism assay using different *S. salivarius* strains. The producer strains were grown aerobically. Indicators used from top to bottom: *Micrococcus luteus, Haemophilus parainfluenzae, Bacillus cereus, Lactococcus lactis, Corynebacterium* spp, *Streptococcus Pyogenes, Staphylococcus aureus, Streptococcus equisimilis, Streptococcus mutans* and *Streptococcus gordonii.* When strain K12 was grown aerobically with 5% CO_2, an increment in the inhibitory activity was observed specially against *Streptococcus equisimilis* and *Streptococcus mutans.*



Deferred antagonism assay using TSYECa as a production medium.



Deferred antagonism test using 7YE and HJEFF strains as producers. TSYE medium was used with or without calcium carbonate supplement. *Corynebacterium* spp was used as an indicator in replicates.



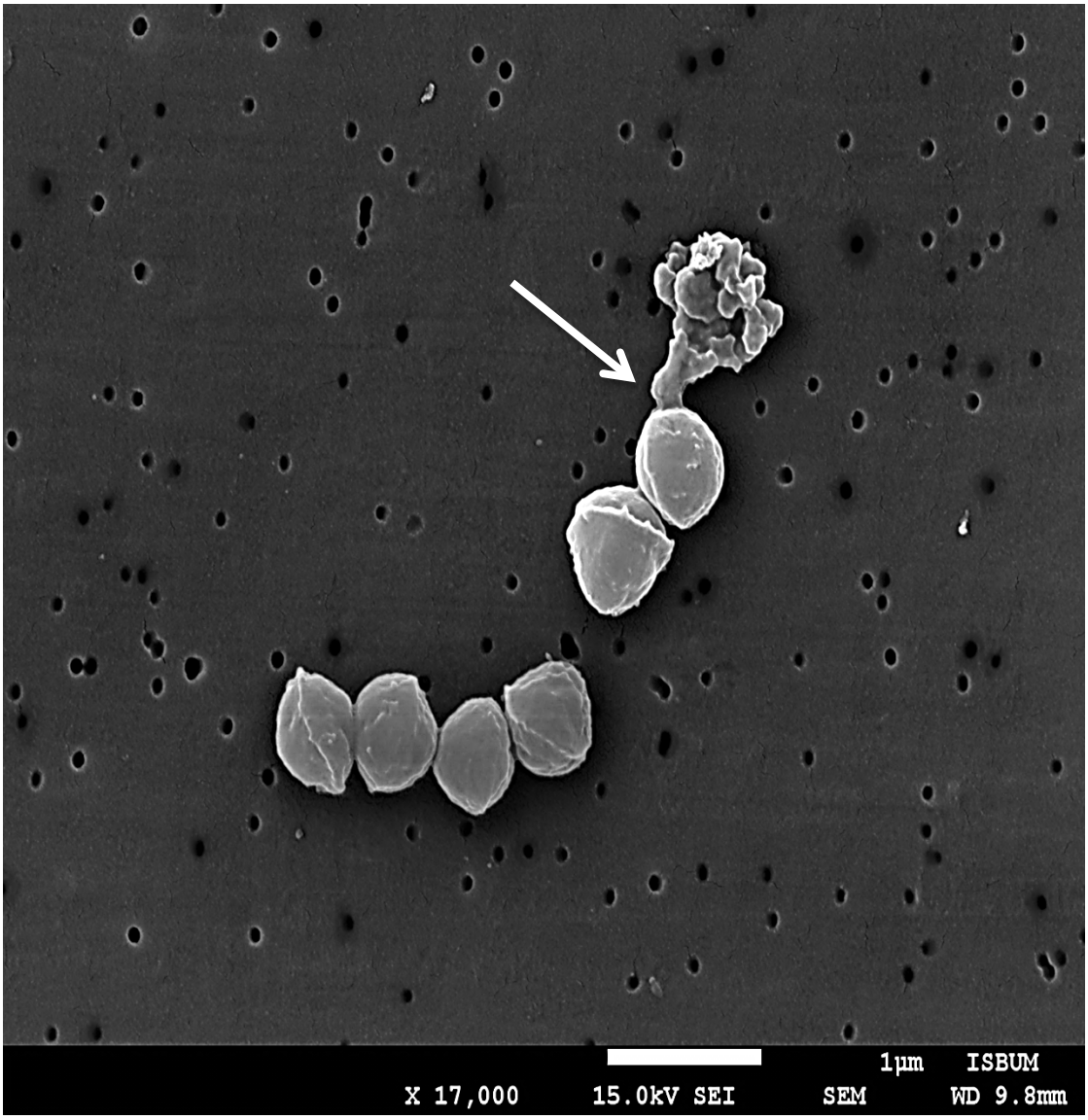
Freeze thaw extraction using M17SUCa as a production medium. The frozen solid culture was thawed at a room temperature.

Precipitated protein obtained from 60% ammonium sulphate precipitation. Solid ammonium sulphate was added to the liquid obtained from freeze thaw extraction and centrifuged at 18,000 x g for 30 min to precipitate the inhibitory activity.

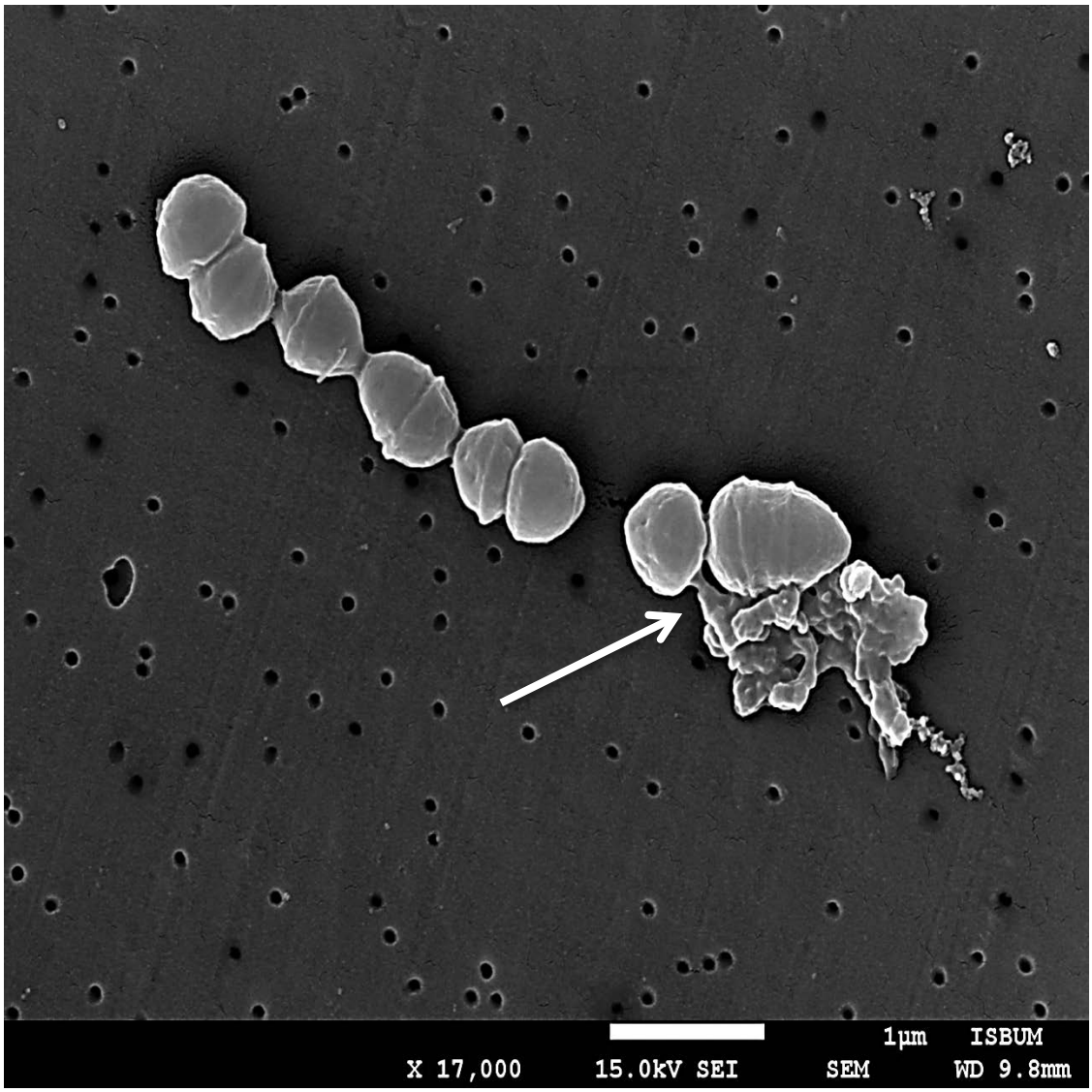
Concentrated crude BLIS-NU10 obtained by freeze thaw extraction using two different production medium namely M17SUCa and THBCa. When tested by well diffusion assay BLIS-NU10 obtained using M7SUCa medium showed positive inhibitory activity when tested against *Corynebacterium* spp.

APPENDIX E

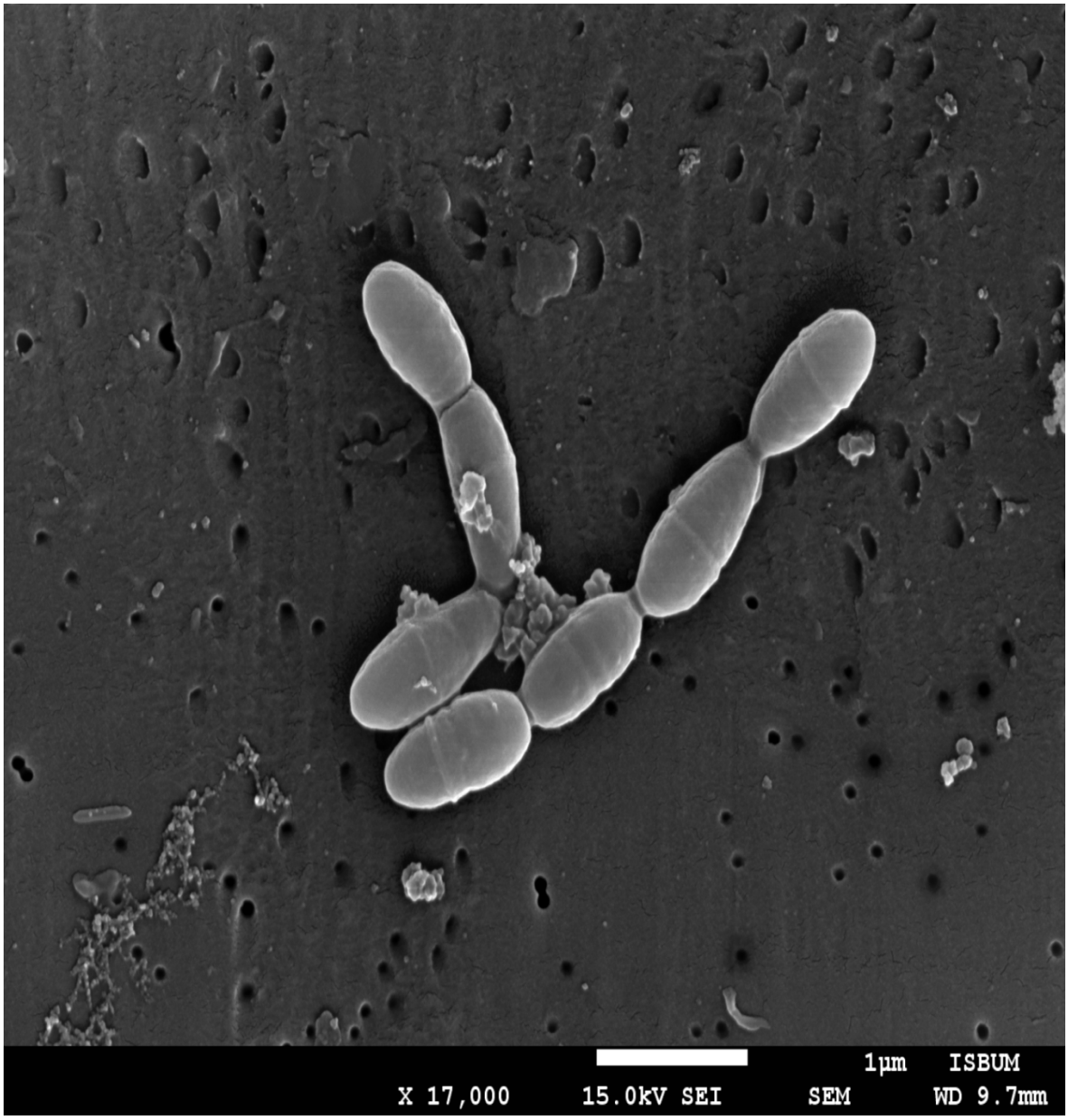
SEM (Morphological changes)



Morphological changes of *Streptococcus equisimilis* treated with pure BLIS-NU10. White arrow indicates the inner bacterial material oozing out through pore formed by BLIS-NU10.



Morphological changes of *Streptococcus equisimilis* treated with pure BLIS-NU10. White arrow indicates the inner bacterial material oozing out through pore formed by BLIS-NU10.



Morphological changes of *Corynebacterium* spp treated with pure BLIS-NU10. White arrow indicates the inner bacterial material oozing out through pore formed by BLIS-NU10.

APPENDIX F

***Streptococcus salivarius* isolates in this study deposited to NCBI gene bank:**

***Streptococcus salivarius* strain NU10 16S ribosomal RNA gene, partial sequence**

GenBank: KC796011.1

LOCUS KC796011 1358 bp DNA linear BCT 06-APR-2013

ORIGIN

```
   1 gctcttcttg gatgagttgc gaacgggtga gtaacgcgta ggtaacctgc cttgtagcgg
  61 gggataacta ttggaaacga tagctaatac cgcataacaa tggatgactc atgtcattta
 121 tttgaaaggg gcaaatgctc cactacaaga tggacctgcg ttgtattagc tagtaggtga
 181 ggtaacggct cacctaggcg acgatacata gccgacctga gagggtgatc ggccacactg
 241 ggactgagac acggcccaga ctcctacggg aggcagcagt agggaatctt cggcaatggg
 301 ggcaaccctg accgagcaac gccgcgtgag tgaagaaggt tttcggatcg taaagctctg
 361 ttgtaagtca agaacgagtg tgagagtgga aagttcacac tgtgacggta gcttaccaga
 421 aagggacggc taactacgtg ccagcagccg cggtaatacg taggtcccga gcgttgtccg
 481 gatttattgg gcgtaaagcg agcgcaggcg gtttgataag tctgaagtta aaggctgtgg
 541 ctcaaccata gttcgctttg gaaactgtca aacttgagtg cagaagggga gagtggaatt
 601 ccatgtgtag cggtgaaatg cgtagatata tggaggaaca ccggtggcga aagcggctct
 661 ctggtctgta actgacgctg aggctcgaaa gcgtggggag cgaacaggat tagataccct
 721 ggtagtccac gccgtaaacg atgagtgcta ggtgttggat cctttccggg attcagtgcc
 781 gcagctaacg cattaagcac tccgcctggg ggagtacgac cgcaaggttg aaactcaaag
 841 gaattgacgg gggcccgcac aagcggtgga gcatgtggtt taattcgaag caacgcgaag
 901 aaccttacca ggtcttgaca tcccgatgct atttctagag atagaaagtt acttcggtac
 961 atcggtgaca ggtggtgcat ggttgtcgtc agctcgtgtc gtgagatgtt gggttaagtc
1021 ccgcaacgag cgcaacccct attgttagtt gccatcattc agttgggcac tctagcgaga
1081 ctgccggtaa taaaccggag gaaggtgggg atgacgtcaa atcatcatgc cccttatgac
1141 ctgggctaca cacgtgctac aatggttggt acaacgagtt gcgagtcggt gacggcaagc
1201 taatctctta aagccaatct cagttcggat tgtaggctgc aactcgccta catgaagtcg
1261 gaatcgctag taatcgcgga tcagcacgcc gcggtgaata cgttcccggg ccttgtacac
1321 accgcccgtc acaccacgag agtttgtaac acccgaag
```

***Streptococcus salivarius* strain YU10 16S ribosomal RNA gene, partial sequence**

GenBank: KC796012.1

LOCUS KC796012 1371 bp DNA linear BCT 06-APR-2013

ORIGIN

```
   1 ggtagccgta acttgctctt cttggatgag ttgcgaacgg gtgagtaacg cgtaggtaac
  61 ctgccttgta gcgggggata actattggaa acgatagcta ataccgcata acaatggatg
 121 actcatgtca tttatttgaa aggggcaaat gctccactac aagatggacc tgcgttgtat
 181 tagctagtag gtgaggtaac ggctcaccta ggcgacgata catagccgac ctgagagggt
 241 gatcggccac actgggactg agacacggcc cagactccta cgggaggcag cagtagggaa
 301 tcttcggcaa tgggggcaac cctgaccgag caacgccgcg tgagtgaaga aggttttcgg
 361 atcgtaaagc tctgttgtaa gtcaagaacg agtgtgagag tggaaagttc acactgtgac
 421 ggtagcttac cagaaaggga cggctaacta cgtgccagca gccgcggtaa tacgtaggtc
 481 ccgagcgttg tccggattta ttgggcgtaa agcgagcgca ggcggtttga taagtctgaa
 541 gttaaaggct gtggctcaac catagttcgc tttggaaact gtcaaacttg agtgcagaag
 601 gggagagtgg aattccatgt gtagcggtga aatgcgtaga tatatggagg aacaccggtg
 661 gcgaaagcgg ctctctggtc tgtaactgac gctgaggctc gaaagcgtgg ggagcgaaca
 721 ggattagata ccctggtagt ccacgccgta aacgatgagt gctaggtgtt ggatcctttc
 781 cgggattcag tgccgcagct aacgcattaa gcactccgcc tggggagtac gaccgcaagg
 841 ttgaaactca aaggaattga cgggggcccg cacaagcggt ggagcatgtg gtttaattcg
 901 aagcaacgcg aagaacctta ccaggtcttg acatcccgat gctatttcta gagatagaaa
 961 gttacttcgg tacatcggtg acaggtggtg catggttgtc gtcagctcgt gtcgtgagat
1021 gttgggttaa gtcccgcaac gagcgcaacc cctattgtta gttgccatca ttcagttggg
1081 cactctagcg agactgccgg taataaaccg gaggaaggtg gggatgacgt caaatcatca
1141 tgcccccttat gacctgggct acacacgtgc tacaatggtt ggtacaacga gttgcgagtc
1201 ggtgacggca agctaatctc ttaaagccaa tctcagttcg gattgtaggc tgcaactcgc
1261 ctacatgaag tcggaatcgc tagtaatcgc ggatcagcac gccgcggtga atacgttccc
1321 gggccttgta cacaccgccc gtcacaccac gagagtttgt aacacccgaa g
```

***Streptococcus salivarius* strain Gt2 16S ribosomal RNA gene, partial sequence**

GenBank: KC796010.1

LOCUS KC796010 1039 bp DNA linear BCT 06-APR-2013

ORIGIN

```
   1 tgaagttgcg gcggctatac atgcaagtag aacgctgaag agaggagctt gctcttcttg
  61 gatgagttgc gaacgggtga gtaacgcgta ggtaacctgc cttgtagcgg gggataacta
 121 ttggaaacga tagctaatac cgcataacaa tggatgacac atgtcattta tttgaaaggg
 181 gcaattgctc cactacaaga tggacctgcg ttgtattagc tagtaggtga ggtaacggct
 241 cacctaggcg acgatacata gccgacctga gagggtgatc ggccacactg ggactgagac
 301 acggcccaga ctcctacggg aggcagcagt agggaatctt cggcaatggg ggcaaccctg
 361 accgagcaac gccgcgtgag tgaagaaggt tttcggatcg taaagctctg ttgtaagtca
 421 agaacgagtg tgagagtgga aagttcacac tgtgacggta gcttaccaga aagggacggc
 481 taactacgtg ccagcagccg cggtaatacg taggtcccga gcgttgtccg gatttattgg
 541 gcgtaaagcg agcgcaggcg gtttgataag tctgaagtta aaggctgtgg ctcaaccata
 601 gttcgctttg gaaactgtca aacttgagtg cagaagggga gagtggaatt ccatgtgtag
 661 cggtgaaatg cgtagatata tggaggaaca ccggtggcga aagcggctct ctggtctgta
 721 actgacgctg aggctcgaaa gcgtggggag cgaacaggat tagataccct ggtagtccac
 781 gccgtaaacg atgagtgcta ggtgttggat cctttccggg attcagtgcc gcagctaacg
 841 cattaagcac tccgcctggg gagtacgacc gcaaggttga aactcaaagg aattgacggg
 901 ggcccgcaca agcggtggag catgtggttt aattcgaagc aacgcgagaa ccttaccagg
 961 tcttgacatc ccgatgctat ttctagagat agaaagttac ttcggtacat cggtgacagg
1021 tggtgcatgg ttgtcgtca
```

***Streptococcus salivarius* strain 7YE 16S ribosomal RNA gene, partial sequence**

GenBank: KC796009.1

LOCUS KC796009 1025 bp DNA linear BCT 06-APR-2013

ORIGIN

```
   1 cgtgggggggg gtgctaatac atgcaagtag aacgctgaag agaggagctt gctcttcttg
  61 gatgagttgc gaacgggtga gtaacgcgta ggtaacctgc cttgtagcgg gggataacta
 121 ttggaaacga tagctaatac cgcataacaa tggatgactc atgtcattta tttgaaaggg
 181 gcaattgctc cactacaaga tggacctgcg ttgtattagc tagtaggtga ggtaacggct
 241 cacctaggcg acgatacata gccgacctga gagggtgatc ggccacactg ggactgagac
 301 acggcccaga ctcctacggg aggcagcagt agggaatctt cggcaatggg ggcaaccctg
 361 accgagcaac gccgcgtgag tgaagaaggt tttcggatcg taaagctctg ttgtaagtca
 421 agaacgagtg tgagagtgga aagttcacac tgtgacggta gcttaccaga aagggacggc
 481 taactacgtg ccagcagccg cggtaatacg taggtcccga gcgttgtccg gatttattgg
 541 gcgtaaagcg agcgcaggcg gtttgataag tctgaagtta aaggctgtgg ctcaaccata
 601 gttcgctttg gaaactgtca aacttgagtg cagaagggga gagtggaatt ccatgtgtag
 661 cggtgaaatg cgtagatata tggaggaaca ccggtggcga aagcggctct ctggtctgta
 721 actgacgctg aggctcgaaa gcgtggggag cgaacaggat tagataccct ggtagtccac
 781 gccgtaaacg atgagtgcta ggtgttggat cctttccggg attcagtgcc gcagctaacg
 841 cattaagcac tccgcctggg gagtacgacc gcaagtgttg aaactcaaag gaattgacgg
 901 gggcccgcac aagcggtgga gcatgtggtt taattcgaag caacgcgaag aaccttacca
 961 ggtctgacat cccgatgcta tttctagaga tagaaagtta cttcggtaca tcggtgacag
1021 tgttg
```

***Streptococcus salivarius* strain SAM3 16S ribosomal RNA gene, partial sequence**

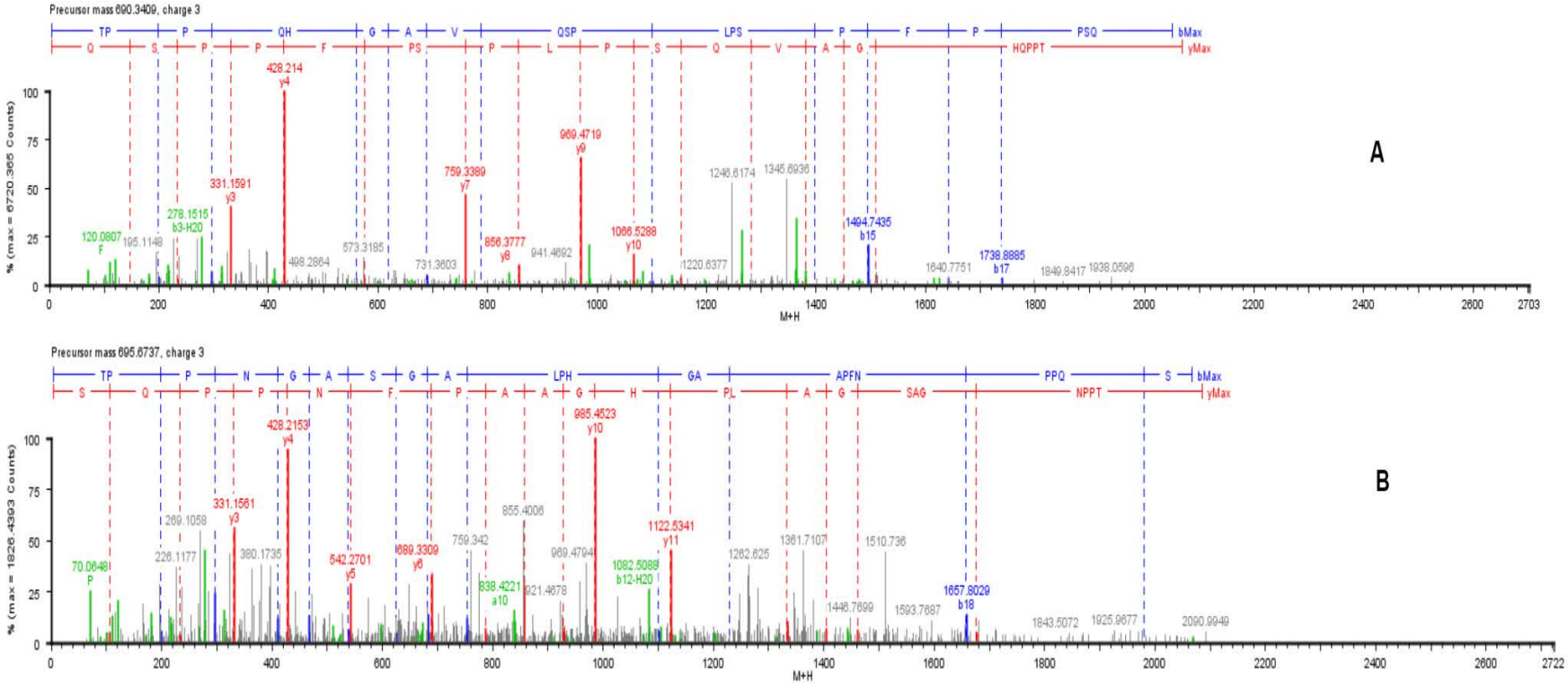
GenBank: KC796008.1

LOCUS KC796008 1021 bp DNA linear BCT 06-APR-2013

ORIGIN

```
   1 ggaagggcgg gtgctataca tgcaagtaga acgctgaaga gaggagcttg ctcttcttgg
  61 atgagttgcg aacgggtgag taacgcgtag gtaacctgcc ttgtagcggg ggataactat
 121 tggaaacgat agctaatacc gcataacaat ggatgactca tgtcatttat ttgaaagggg
 181 caattgctcc actacaagat ggacctgcgt tgtattagct agtaggtgag gtaacggctc
 241 acctaggcga cgatacatag ccgacctgag agggtgatcg gccacactgg gactgagaca
 301 cggcccagac tcctacggga ggcagcagta gggaatcttc ggcaatgggg gcaaccctga
 361 ccgagcaacg ccgcgtgagt gaagaaggtt ttcggatcgt aaagctctgt tgtaagtcaa
 421 gaacgagtgt gagagtggaa agttcacact gtgacggtag cttaccagaa agggacggct
 481 aactacgtgc cagcagccgc ggtaatacgt aggtcccgag cgttgtccgg atttattggg
 541 cgtaaagcga gcgcaggcgg tttgataagt ctgaagttaa aggctgtggc tcaaccatag
 601 ttcgctttgg aaactgtcaa acttgagtgc agaaggggag agtggaattc catgtgtagc
 661 ggtgaaatgc gtagatatat ggaggaacac cggtggcgaa agcggctctc tggtctgtaa
 721 ctgacgctga ggctcgaaag cgtggggagc gaacaggatt agataccctg gtagtccacg
 781 ccgtaaacga tgagtgctag gtgttggatc ctttccggga ttcagtgccg cagctaacgc
 841 attaagcact ccgcctgggg agtacgaccg caaggttgaa actcaaagga attgacgggg
 901 gcccgcacaa gcggtggagc atgtggttta attcgaagca acgcgaagaa ccttaccagg
 961 tcttgacatc ccgatgctat ttctagagat agaaagttac ttcggtacat cggtgacagt
1021 g
```

APPENDIX G



De novo amino acid sequencing of the major peaks detected in pure BLIS-NU10. A: 2068.0376Da; B: 2084.00

Bibliography

Aas, J. A., Paster, B. J., Stokes, L. N., Olsen, I. and Dewhirst, F. E. (2005). Defining the normal bacterial flora of the oral cavity. *J Clin Microbiol, 43*(11), 5721-5732. doi: 10.1128/JCM.43.11.5721-5732.2005

Abee, T., Krockel, L. and Hill, C. (1995). Bacteriocins: modes of action and potentials in food preservation and control of food poisoning. *Int J Food Microbiol, 28*(2), 169-185.

Al-Mahrous, M. M. and Upton, M. (2011). Discovery and development of lantibiotics; antimicrobial agents that have significant potential for medical application. *Expert Opin Drug Discov, 6*(2), 155-170. doi: 10.1517/17460441.2011.545387

Altena, K., Guder, A., Cramer, C. and Bierbaum, G. (2000). Biosynthesis of the lantibiotic mersacidin: organization of a type B lantibiotic gene cluster. *Appl Environ Microbiol, 66*(6), 2565-2571.

Anderssen, E. L., Diep, D. B., Nes, I. F., Eijsink, V. G. and Nissen-Meyer, J. (1998). Antagonistic activity of *Lactobacillus plantarum* C11: two new two-peptide bacteriocins, plantaricins EF and JK, and the induction factor plantaricin A. *Appl Environ Microbiol, 64*(6), 2269-2272.

Apelgren, L. D. and Dajani, A. S. (1979). Purification of a streptococcal bacteriocin (viridin B) and its separation from alpha-hemolysin. *Antimicrob Agents Chemother, 15*(3), 436-439.

Bierbaum, G. and Sahl, H. G. (1993). Lantibiotics--unusually modified bacteriocin-like peptides from gram-positive bacteria. *Zentralbl Bakteriol, 278*(1), 1-22.

Bierbaum, G. and Sahl, H. G. (2009). Lantibiotics: mode of action, biosynthesis and bioengineering. *Curr Pharm Biotechnol, 10*(1), 2-18.

Bill, N. J. and Washington, J. A. (1975). Bacterial interference by *Streptococcus salivarius*. *Am J Clin Pathol, 64*(1), 116-120.

Birri, D. J., Brede, D. A. and Nes, I. F. (2012). Salivaricin D, a novel intrinsically trypsin-resistant lantibiotic from *Streptococcus salivarius* 5M6c isolated from a healthy infant. *Appl Environ Microbiol, 78*(2), 402-410. doi: 10.1128/AEM.06588-11

Blackburn, P., J. Polak, S. A. Gusik, and S. D. Rubing. (1989). Nisin composition for use as enhanced broad range bactericides. *International Patent Application publication WO 89112399*.

Bourbon, C., Bry, C., Roggemans, C., Soulard, C., Thizon, C. and Garbay, B. (2008). Use of a real-time polymerase chain reaction thermocycler to study bacterial cell permeabilization by antimicrobial peptides. *Anal Biochem, 381*(2), 279-281. doi: 10.1016/j.ab.2008.07.005

Breukink, E., van Kraaij, C., Demel, R. A., Siezen, R. J., Kuipers, O. P. andde Kruijff, B. (1997). The C-terminal region of nisin is responsible for the initial interaction of nisin with the target membrane. *Biochemistry, 36*(23), 6968-6976. doi: 10.1021/bi970008u

Brotz, H., Bierbaum, G., Markus, A., Molitor, E. and Sahl, H. G. (1995). Mode of action of the lantibiotic mersacidin: inhibition of peptidoglycan biosynthesis via a novel mechanism? *Antimicrob Agents Chemother, 39*(3), 714-719.

Brunden, K. R., Cramer, W. A. and Cohen, F. S. (1984). Purification of a small receptor-binding peptide from the central region of the colicin E1 molecule. *J Biol Chem, 259*(1), 190-196.

Burton, J. P., Wescombe, P. A., Moore, C. J., Chilcott, C. N. and Tagg, J. R. (2006). Safety assessment of the oral cavity probiotic *Streptococcus salivarius* K12. *Appl Environ Microbiol, 72*(4), 3050-3053. doi: 10.1128/AEM.72.4.3050-3053.2006

Carlsson, J., Grahnen, H., Jonsson, G. and Wikner, S. (1970). Early establishment of *Streptococcus salivarius* in the mouth of infants. *J Dent Res, 49*(2), 415-418.

Castellano, P., Belfiore, C., Fadda, S. and Vignolo, G. (2008). A review of bacteriocinogenic lactic acid bacteria used as bioprotective cultures in fresh meat produced in Argentina. *Meat Sci, 79*(3), 483-499. doi: 10.1016/j.meatsci.2007.10.009

Cheigh, C. I., Park, H., Choi, H. J. and Pyun, Y. R. (2005). Enhanced nisin production by increasing genes involved in nisin Z biosynthesis in *Lactococcus lactis* subsp. *lactis* A164. *Biotechnol Lett, 27*(3), 155-160. doi: 10.1007/s10529-004-7661-3

Chikindas, M. L., Novak, J., Driessen, A. J., Konings, W. N., Schilling, K. M. and Caufield, P. W. (1995). Mutacin II, a bactericidal antibiotic from *Streptococcus mutans*. *Antimicrob Agents Chemother, 39*(12), 2656-2660.

Chun, W. and Hancock, R. E. (2000). Action of lysozyme and nisin mixtures against lactic acid bacteria. *Int J Food Microbiol, 60*(1), 25-32.

Cotter, P. D., Hill, C. and Ross, R. P. (2005). Bacterial lantibiotics: strategies to improve therapeutic potential. *Curr Protein Pept Sci, 6*(1), 61-75.

Cotter, P. D., Hill, C. and Ross, R. P. (2005). Bacteriocins: developing innate immunity for food. *Nat Rev Microbiol, 3*(10), 777-788. doi: 10.1038/nrmicro1273

De Carvalho, A. A., Mantovani, H. C. and Vanetti, M. C. (2007). Bactericidal effect of bovicin HC5 and nisin against *Clostridium tyrobutyricum* isolated from spoiled mango pulp. *Lett Appl Microbiol, 45*(1), 68-74. doi: 10.1111/j.1472-765X.2007.02150.x

De Kwaadsteniet, M., Ten Doeschate, K. and Dicks, L. M. (2008). Characterization of the structural gene encoding nisin F, a new lantibiotic produced by a *Lactococcus lactis*

subsp. lactis isolate from freshwater catfish (*Clarias gariepinus*). *Appl Environ Microbiol, 74*(2), 547-549. doi: 10.1128/AEM.01862-07

De Vos, W. M. (1996). Metabolic engineering of sugar catabolism in lactic acid bacteria. *Antonie Van Leeuwenhoek, 70*(2-4), 223-242.

Delves-Broughton, J., Blackburn, P., Evans, R. J. and Hugenholtz, J. (1996). Applications of the bacteriocin, nisin. *Antonie Van Leeuwenhoek, 69*(2), 193-202.

Ebisu, S., Kato, K., Kotani, S. and Misaki, A. (1975). Structural differences in fructans elaborated by *Streptococcus mutans* and *Streptococcus. salivarius*. *J Biochem, 78*(5), 879-887.

Eijsink, V. G., Skeie, M., Middelhoven, P. H., Brurberg, M. B. and Nes, I. F. (1998). Comparative studies of class IIa bacteriocins of lactic acid bacteria. *Appl Environ Microbiol, 64*(9), 3275-3281.

Fimland, G., Blingsmo, O. R., Sletten, K., Jung, G., Nes, I. F. and Nissen-Meyer, J. (1996). New biologically active hybrid bacteriocins constructed by combining regions from various pediocin-like bacteriocins: the C-terminal region is important for determining specificity. *Appl Environ Microbiol, 62*(9), 3313-3318.

Fimland, G., Johnsen, L., Dalhus, B. and Nissen-Meyer, J. (2005). Pediocin-like antimicrobial peptides (class IIa bacteriocins) and their immunity proteins: biosynthesis, structure, and mode of action. *J Pept Sci, 11*(11), 688-696. doi: 10.1002/psc.699

Flores, S. H. and Alegre, R. M. (2001). Nisin production from *Lactococcus lactis* A.T.C.C. 7962 using supplemented whey permeate. *Biotechnol Appl Biochem, 34*(Pt 2), 103-107.

Furmanek, B., Kaczorowski, T., Bugalski, R., Bielawski, K., Bohdanowicz, J. and Podhajska, A. J. (1999). Identification, characterization and purification of the lantibiotic staphylococcin T, a natural gallidermin variant. *J Appl Microbiol, 87*(6), 856-866.

Garcera, M. J., Elferink, M. G., Driessen, A. J. and Konings, W. N. (1993). In vitro pore-forming activity of the lantibiotic nisin. Role of protonmotive force and lipid composition. *Eur J Biochem, 212*(2), 417-422.

Georgalaki, M. D., Van Den Berghe, E., Kritikos, D., Devreese, B., Van Beeumen, J., Kalantzopoulos, G., De Vuyst, L. and Tsakalidou, E. (2002). Macedocin, a food-grade lantibiotic produced by *Streptococcus macedonicus* ACA-DC 198. *Appl Environ Microbiol, 68*(12), 5891-5903.

Gilmore, M. S., Skaugen, M. and Nes, I. (1996). *Enterococcus faecalis* cytolysin and lactocin S of *Lactobacillus sake*. *Antonie Van Leeuwenhoek, 69*(2), 129-138.

Gonzalez-Toledo, S. Y., Dominguez-Dominguez, J., Garcia-Almendarez, B. E., Prado-Barragan, L. A. and Regalado-Gonzalez, C. (2010). Optimization of nisin

production by *Lactococcus lactis* UQ2 using supplemented whey as alternative culture medium. *J Food Sci, 75*(6), M347-353. doi: 10.1111/j.1750-3841.2010.01670.x

Gross, E. and Morell, J. L. (1971). The structure of nisin. *J Am Chem Soc, 93*(18), 4634-4635.

Hammami, R., Zouhir, A., Le Lay, C., Ben Hamida, J. and Fliss, I. (2010). BACTIBASE second release: a database and tool platform for bacteriocin characterization. *BMC Microbiol, 10*, 22. doi: 10.1186/1471-2180-10-22

Heng, N. C., Ragland, N. L., Swe, P. M., Baird, H. J., Inglis, M. A., Tagg, J. R. and Jack, R. W. (2006). Dysgalacticin: a novel, plasmid-encoded antimicrobial protein (bacteriocin) produced by *Streptococcus dysgalactiae* subsp. *equisimilis. Microbiology, 152*(Pt 7), 1991-2001. doi: 10.1099/mic.0.28823-0

Heritage, J., Evans, E. G. V. and Killington, R. A. (1996). *Introductory microbiology*. Cambridge, Eng. ; New York: Cambridge University Press.

Herzner, A. M., Dischinger, J., Szekat, C., Josten, M., Schmitz, S., Yakeleba, A., Reinartz, R., Jansen, A., *et al.* (2011). Expression of the lantibiotic mersacidin in *Bacillus amyloliquefaciens* FZB42. *PLoS One, 6*(7), e22389. doi: 10.1371/journal.pone.0022389

Hillman, J. D., Novak, J., Sagura, E., Gutierrez, J. A., Brooks, T. A., Crowley, P. J., Hess, M., Azizi, A., *et al.* (1998). Genetic and biochemical analysis of mutacin 1140, a lantibiotic from *Streptococcus mutans*. *Infect Immun, 66*(6), 2743-2749.

Holo, H., Jeknic, Z., Daeschel, M., Stevanovic, S. and Nes, I. F. (2001). Plantaricin W from *Lactobacillus plantarum* belongs to a new family of two-peptide lantibiotics. *Microbiology, 147*(Pt 3), 643-651.

Huot, E., Barrena-Gonzalez, C. and Petitdemange, H. (1996). Comparative effectiveness of nisin and bacteriocin J46 at different pH values. *Lett Appl Microbiol, 22*(1), 76-79.

Hyink, O., Balakrishnan, M. and Tagg, J. R. (2005). *Streptococcus rattus* strain BHT produces both a class I two-component lantibiotic and a class II bacteriocin. *FEMS Microbiol Lett, 252*(2), 235-241. doi: 10.1016/j.femsle.2005.09.003

Hyink, O., Wescombe, P. A., Upton, M., Ragland, N., Burton, J. P. and Tagg, J. R. (2007). Salivaricin A2 and the novel lantibiotic salivaricin B are encoded at adjacent loci on a 190-kilobase transmissible megaplasmid in the oral probiotic strain *Streptococcus salivarius* K12. *Appl Environ Microbiol, 73*(4), 1107-1113. doi: 10.1128/AEM.02265-06

Islam, M. R., Nagao, J., Zendo, T. and Sonomoto, K. (2012). Antimicrobial mechanism of lantibiotics. *Biochem Soc Trans, 40*(6), 1528-1533. doi: 10.1042/BST20120190

Jack, R. W. and Jung, G. (2000). Lantibiotics and microcins: polypeptides with unusual chemical diversity. *Curr Opin Chem Biol, 4*(3), 310-317.

Jack, R. W., Tagg, J. R. and Ray, B. (1995). Bacteriocins of gram-positive bacteria. *Microbiol Rev, 59*(2), 171-200.

Johanson, W. G., Jr., Blackstock, R., Pierce, A. K. and Sanford, J. P. (1970). The role of bacterial antagonism in pneumococcal colonization of the human pharynx. *J Lab Clin Med, 75*(6), 946-952.

Kang, J. G., Kim, S. H. and Ahn, T. Y. (2006). Bacterial diversity in the human saliva from different ages. *J Microbiol, 44*(5), 572-576.

Kleerebezem, M. and Hugenholtz, J. (2003). Metabolic pathway engineering in lactic acid bacteria. *Curr Opin Biotechnol, 14*(2), 232-237.

Kleerebezem, M., Quadri, L. E., Kuipers, O. P. and de Vos, W. M. (1997). Quorum sensing by peptide pheromones and two-component signal-transduction systems in Gram-positive bacteria. *Mol Microbiol, 24*(5), 895-904.

Klocke, M., Mundt, K., Idler, F., Jung, S. and Backhausen, J. E. (2005). Heterologous expression of enterocin A, a bacteriocin from *Enterococcus faecium*, fused to a cellulose-binding domain in *Escherichia coli* results in a functional protein with inhibitory activity against Listeria. *Appl Microbiol Biotechnol, 67*(4), 532-538. doi: 10.1007/s00253-004-1838-5

König, Helmut, Unden, Gottfried and Fröhlich, Jürgen. (2009). *Biology of microorganisms on grapes, in must and in wine*. Berlin: Springer.

Kordel, M., Schuller, F. and Sahl, H. G. (1989). Interaction of the pore forming-peptide antibiotics Pep 5, nisin and subtilin with non-energized liposomes. *FEBS Lett, 244*(1), 99-102.

Kuipers, O. P., Beerthuyzen, M. M., de Ruyter, P. G., Luesink, E. J. and de Vos, W. M. (1995). Autoregulation of nisin biosynthesis in *Lactococcus lactis* by signal transduction. *J Biol Chem, 270*(45), 27299-27304.

Lee, J. H., Li, X. and O'Sullivan, D. J. (2011). Transcription analysis of a lantibiotic gene cluster from *Bifidobacterium longum* DJO10A. *Appl Environ Microbiol, 77*(17), 5879-5887. doi: 10.1128/AEM.00571-11

Liu, W., Zheng, H., Wu, Z. and Wang, Y. (2010). Effects of pH profiles on nisin fermentation coupling with foam separation. *Appl Microbiol Biotechnol, 85*(5), 1401-1407. doi: 10.1007/s00253-009-2217-z

Lucas, R., Grande, M. A., Abriouel, H., Maqueda, M., Ben Omar, N., Valdivia, E., Martinez-Canamero, M. and Galvez, A. (2006). Application of the broad-spectrum bacteriocin enterocin AS-48 to inhibit *Bacillus coagulans* in canned fruit and vegetable foods. *Food Chem Toxicol, 44*(10), 1774-1781. doi: 10.1016/j.fct.2006.05.019

Lv, W., Zhang, X. and Cong, W. (2005). Modelling the production of nisin by *Lactococcus lactis* in fed-batch culture. *Appl Microbiol Biotechnol, 68*(3), 322-326. doi: 10.1007/s00253-005-1892-7

Makarova, K. S. and Koonin, E. V. (2007). Evolutionary genomics of lactic acid bacteria. *J Bacteriol, 189*(4), 1199-1208. doi: 10.1128/JB.01351-06

Mantovani, H. C., Hu, H., Worobo, R. W. and Russell, J. B. (2002). Bovicin HC5, a bacteriocin from *Streptococcus bovis* HC5. *Microbiology, 148*(Pt 11), 3347-3352.

Mantovani, H. C. and Russell, J. B. (2008). Bovicin HC5, a lantibiotic produced by *Streptococcus bovis* HC5, catalyzes the efflux of intracellular potassium but not ATP. *Antimicrob Agents Chemother, 52*(6), 2247-2249. doi: 10.1128/AAC.00109-08

Maqueda, M., Galvez, A., Bueno, M. M., Sanchez-Barrena, M. J., Gonzalez, C., Albert, A., Rico, M. and Valdivia, E. (2004). Peptide AS-48: prototype of a new class of cyclic bacteriocins. *Curr Protein Pept Sci, 5*(5), 399-416.

Martin, N. I., Sprules, T., Carpenter, M. R., Cotter, P. D., Hill, C., Ross, R. P. and Vederas, J. C. (2004). Structural characterization of lacticin 3147, a two-peptide lantibiotic with synergistic activity. *Biochemistry, 43*(11), 3049-3056. doi: 10.1021/bi0362065

Martinez, M. C., Lazdunski, C. and Pattus, F. (1983). Isolation, molecular and functional properties of the C-terminal domain of colicin A. *EMBO J, 2*(9), 1501-1507.

McAuliffe, O., Ryan, M. P., Ross, R. P., Hill, C., Breeuwer, P. and Abee, T. (1998). Lacticin 3147, a broad-spectrum bacteriocin which selectively dissipates the membrane potential. *Appl Environ Microbiol, 64*(2), 439-445.

McCarthy, C., Snyder, M. L. and Parker, R. B. (1965). The indigenous oral flora of man. I. the newborn to the 1-year-old infant. *Arch Oral Biol, 10*, 61-70.

Michel, B. (2005). After 30 years of study, the bacterial SOS response still surprises us. *PLoS Biol, 3*(7), e255. doi: 10.1371/journal.pbio.0030255

Moll, G. N., Roberts, G. C., Konings, W. N. and Driessen, A. J. (1996). Mechanism of lantibiotic-induced pore-formation. *Antonie Van Leeuwenhoek, 69*(2), 185-191.

Moreno, F., Gonzalez-Pastor, J. E., Baquero, M. R. and Bravo, D. (2002). The regulation of microcin B, C and J operons. *Biochimie, 84*(5-6), 521-529.

Morgan, S. M., O'Connor P, M., Cotter, P. D., Ross, R. P. and Hill, C. (2005). Sequential actions of the two component peptides of the lantibiotic lacticin 3147 explain its antimicrobial activity at nanomolar concentrations. *Antimicrob Agents Chemother, 49*(7), 2606-2611. doi: 10.1128/AAC.49.7.2606-2611.2005

Morris, S. L., Walsh, R. C. and Hansen, J. N. (1984). Identification and characterization of some bacterial membrane sulfhydryl groups which are targets of bacteriostatic and antibiotic action. *J Biol Chem, 259*(21), 13590-13594.

Mortvedt, C. I., Nissen-Meyer, J., Sletten, K. and Nes, I. F. (1991). Purification and amino acid sequence of lactocin S, a bacteriocin produced by *Lactobacillus sake* L45. *Appl Environ Microbiol, 57*(6), 1829-1834.

Mota-Meira, M., Lacroix, C., LaPointe, G. and Lavoie, M. C. (1997). Purification and structure of mutacin B-Ny266: a new lantibiotic produced by *Streptococcus mutans*. *FEBS Lett, 410*(2-3), 275-279.

Mulders, J. W., Boerrigter, I. J., Rollema, H. S., Siezen, R. J. and de Vos, W. M. (1991). Identification and characterization of the lantibiotic nisin Z, a natural nisin variant. *Eur J Biochem, 201*(3), 581-584.

Nakayama, K., Takashima, K., Ishihara, H., Shinomiya, T., Kageyama, M., Kanaya, S., Ohnishi, M., Murata, T., *et al.* (2000). The R-type pyocin of *Pseudomonas aeruginosa* is related to P2 phage, and the F-type is related to lambda phage. *Mol Microbiol, 38*(2), 213-231.

Nes, I. F., Diep, D. B. and Holo, H. (2007). Bacteriocin diversity in *Streptococcus* and *Enterococcus*. *J Bacteriol, 189*(4), 1189-1198. doi: 10.1128/JB.01254-06

Ni, J., Teng, K., Liu, G., Qiao, C., Huan, L. and Zhong, J. (2011). Autoregulation of lantibiotic bovicin HJ50 biosynthesis by the BovK-BovR two-component signal transduction system in *Streptococcus bovis* HJ50. *Appl Environ Microbiol, 77*(2), 407-415. doi: 10.1128/AEM.01278-10

Nicolas, G. G., LaPointe, G. and Lavoie, M. C. (2011). Production, purification, sequencing and activity spectra of mutacins D-123.1 and F-59.1. *BMC Microbiol, 11*, 69. doi: 10.1186/1471-2180-11-69

Nissen-Meyer, J., Holo, H., Havarstein, L. S., Sletten, K. and Nes, I. F. (1992). A novel lactococcal bacteriocin whose activity depends on the complementary action of two peptides. *J Bacteriol, 174*(17), 5686-5692.

Orla-Jensen, Sigurd. (1919). *The lactic acid bacteria*. København,: A. F. Høst.

Pajohi, M. R., Tajik, H., Farshid, A. A. and Hadian, M. (2011). Synergistic antibacterial activity of the essential oil of Cuminum cyminum L. seed and nisin in a food model. *J Appl Microbiol*. doi: 10.1111/j.1365-2672.2011.04946.x

Papagianni, M. and Anastasiadou, S. (2009). Pediocins: The bacteriocins of Pediococci. Sources, production, properties and applications. *Microb Cell Fact, 8*, 3. doi: 10.1186/1475-2859-8-3

Perin, L. M., Moraes, P. M., Silva, A., Jr. and Nero, L. A. (2012). Lantibiotics biosynthesis genes and bacteriocinogenic activity of Lactobacillus spp. isolated from raw milk and cheese. *Folia Microbiol (Praha), 57*(3), 183-190. doi: 10.1007/s12223-012-0113-x

Piard, J. C., Kuipers, O. P., Rollema, H. S., Desmazeaud, M. J. and de Vos, W. M. (1993). Structure, organization, and expression of the lct gene for lacticin 481, a novel lantibiotic produced by *Lactococcus lactis*. *J Biol Chem, 268*(22), 16361-16368.

Pilsl, H. and Braun, V. (1995). Strong function-related homology between the pore-forming colicins K and 5. *J Bacteriol, 177*(23), 6973-6977.

Pongtharangkul, T. and Demirci, A. (2006). Effects of fed-batch fermentation and pH profiles on nisin production in suspended-cell and biofilm reactors. *Appl Microbiol Biotechnol, 73*(1), 73-79. doi: 10.1007/s00253-006-0459-6

Qi, F., Chen, P. and Caufield, P. W. (2000). Purification and biochemical characterization of mutacin I from the group I strain of *Streptococcus mutans*, CH43, and genetic analysis of mutacin I biosynthesis genes. *Appl Environ Microbiol, 66*(8), 3221-3229.

Robson, C. L., Wescombe, P. A., Klesse, N. A. and Tagg, J. R. (2007). Isolation and partial characterization of the Streptococcus mutans type AII lantibiotic mutacin K8. *Microbiology, 153*(Pt 5), 1631-1641. doi: 10.1099/mic.0.2006/003756-0

Rodriguez, J. M. and Dodd, H. M. (1996). Genetic determinants for the biosynthesis of nisin, a bacteriocin produced by *Lactococcus lactis*. *Microbiologia, 12*(1), 61-74.

Ross, K. F., Ronson, C. W. and Tagg, J. R. (1993). Isolation and characterization of the lantibiotic salivaricin A and its structural gene salA from *Streptococcus salivarius* 20P3. *Appl Environ Microbiol, 59*(7), 2014-2021.

Sahl, H. G. (1994). Staphylococcin 1580 is identical to the lantibiotic epidermin: implications for the nature of bacteriocins from gram-positive bacteria. *Appl Environ Microbiol, 60*(2), 752-755.

Sahl, H. G., Jack, R. W. and Bierbaum, G. (1995). Biosynthesis and biological activities of lantibiotics with unique post-translational modifications. *Eur J Biochem, 230*(3), 827-853.

Sanders, C. C. and Sanders, W. E., Jr. (1982). Enocin: an antibiotic produced by *Streptococcus salivarius* that may contribute to protection against infections due to group A streptococci. *J Infect Dis, 146*(5), 683-690.

Sass, P., Jansen, A., Szekat, C., Sass, V., Sahl, H. G. and Bierbaum, G. (2008). The lantibiotic mersacidin is a strong inducer of the cell wall stress response of *Staphylococcus aureus*. *BMC Microbiol, 8*, 186. doi: 10.1186/1471-2180-8-186

Schagger, H. (2006). Tricine-SDS-PAGE. *Nat Protoc, 1*(1), 16-22. doi: 10.1038/nprot.2006.4

Schnell, N., Engelke, G., Augustin, J., Rosenstein, R., Ungermann, V., Gotz, F. and Entian, K. D. (1992). Analysis of genes involved in the biosynthesis of lantibiotic epidermin. *Eur J Biochem, 204*(1), 57-68.

Sherman, J. M., Niven, C. F. and Smiley, K. L. (1943). *Streptococcus salivarius* and other non-hemolytic streptococci of the human throat. *J Bacteriol, 45*(3), 249-263.

Siezen, R. J., Rollema, H. S., Kuipers, O. P. and de Vos, W. M. (1995). Homology modelling of the *Lactococcus lactis* leader peptidase NisP and its interaction with the precursor of the lantibiotic nisin. *Protein Eng, 8*(2), 117-125.

Simmonds, R. S., Simpson, W. J. and Tagg, J. R. (1997). Cloning and sequence analysis of zooA, a *Streptococcus zooepidemicus* gene encoding a bacteriocin-like inhibitory substance having a domain structure similar to that of lysostaphin. *Gene, 189*(2), 255-261.

Strauch, E., Kaspar, H., Schaudinn, C., Damasko, C., Konietzny, A., Dersch, P., Skurnik, M. and Appel, B. (2003). Analysis of enterocoliticin, a phage tail-like bacteriocin. *Adv Exp Med Biol, 529*, 249-251. doi: 10.1007/0-306-48416-1_48

Swe, P. M., Cook, G. M., Tagg, J. R. and Jack, R. W. (2009). Mode of action of dysgalacticin: a large heat-labile bacteriocin. *J Antimicrob Chemother, 63*(4), 679-686. doi: 10.1093/jac/dkn552

Tagg, J. R. (2013). Personal communication.

Tagg, J. R. and Bannister, L. V. (1979). "Fingerprinting" beta-haemolytic streptococci by their production of and sensitivity to bacteriocine-like inhibitors. *J Med Microbiol, 12*(4), 397-411.

Tagg, J. R., Pybus, V., Phillips, L. V. and Fiddes, T. M. (1983). Application of inhibitor typing in a study of the transmission and retention in the human mouth of the bacterium *Streptococcus salivarius*. *Arch Oral Biol, 28*(10), 911-915.

Tagg, J. R. and Wannamaker, L. W. (1978). Streptococcin A-FF22: nisin-like antibiotic substance produced by a group A streptococcus. *Antimicrob Agents Chemother, 14*(1), 31-39.

Takada, K., Hayashi, K., Sasaki, K., Sato, T. and Hirasawa, M. (2006). Selectivity of Mitis Salivarius agar and a new selective medium for oral streptococci in dogs. *J Microbiol Methods, 66*(3), 460-465. doi: 10.1016/j.mimet.2006.01.011

Tiwari, S. K. and Srivastava, S. (2008). Purification and characterization of plantaricin LR14: a novel bacteriocin produced by *Lactobacillus plantarum* LR/14. *Appl Microbiol Biotechnol, 79*(5), 759-767. doi: 10.1007/s00253-008-1482-6

Tolonen, M., Saris, P. E. and Siika-Aho, M. (2004). Production of nisin with continuous adsorption to Amberlite XAD-4 resin using *Lactococcus lactis* N8 and L. lactis LAC48. *Appl Microbiol Biotechnol, 63*(6), 659-665. doi: 10.1007/s00253-003-1413-5

Tsang, P., Merritt, J., Nguyen, T., Shi, W. and Qi, F. (2005). Identification of genes associated with mutacin I production in *Streptococcus mutans* using random

insertional mutagenesis. *Microbiology, 151*(Pt 12), 3947-3955. doi: 10.1099/mic.0.28221-0

Turner, D. L., Brennan, L., Meyer, H. E., Lohaus, C., Siethoff, C., Costa, H. S., Gonzalez, B., Santos, H., *et al.* (1999). Solution structure of plantaricin C, a novel lantibiotic. *Eur J Biochem, 264*(3), 833-839.

Udompijitkul, P., Paredes-Sabja, D. and Sarker, M. R. (2012). Inhibitory effects of nisin against Clostridium perfringens food poisoning and nonfood-borne isolates. *J Food Sci, 77*(1), M51-56. doi: 10.1111/j.1750-3841.2011.02475.x

Upton, M., Tagg, J. R., Wescombe, P. and Jenkinson, H. F. (2001). Intra- and interspecies signaling between *Streptococcus salivarius* and *Streptococcus pyogenes* mediated by SalA and SalA1 lantibiotic peptides. *J Bacteriol, 183*(13), 3931-3938. doi: 10.1128/JB.183.13.3931-3938.2001

Van der Meer, J. R., Polman, J., Beerthuyzen, M. M., Siezen, R. J., Kuipers, O. P. and De Vos, W. M. (1993). Characterization of the *Lactococcus lactis* nisin A operon genes nisP, encoding a subtilisin-like serine protease involved in precursor processing, and nisR, encoding a regulatory protein involved in nisin biosynthesis. *J Bacteriol, 175*(9), 2578-2588.

Van Heusden, H. E., de Kruijff, B. and Breukink, E. (2002). Lipid II induces a transmembrane orientation of the pore-forming peptide lantibiotic nisin. *Biochemistry, 41*(40), 12171-12178.

Vaughan, E. E., van den Bogaard, P. T., Catzeddu, P., Kuipers, O. P. and de Vos, W. M. (2001). Activation of silent gal genes in the lac-gal regulon of *Streptococcus thermophilus*. *J Bacteriol, 183*(4), 1184-1194. doi: 10.1128/JB.183.4.1184-1194.2001

Wan, X., Li, R., Saris, P. E. and Takala, T. M. (2012). Genetic characterisation and heterologous expression of leucocin C, a class IIa bacteriocin from *Leuconostoc carnosum* 4010. *Appl Microbiol Biotechnol*. doi: 10.1007/s00253-012-4406-4

Wescombe, P. A., Burton, J. P., Cadieux, P. A., Klesse, N. A., Hyink, O., Heng, N. C., Chilcott, C. N., Reid, G., *et al.* (2006). Megaplasmids encode differing combinations of lantibiotics in *Streptococcus salivarius*. *Antonie Van Leeuwenhoek, 90*(3), 269-280. doi: 10.1007/s10482-006-9081-y

Wescombe, P. A., Dyet, K. H., Dierksen, K. P., Power, D. A., Jack, R. W., Burton, J. P., Inglis, M. A., Wescombe, A. L., *et al.* (2012). Salivaricin G32, a homolog of the prototype *Streptococcus pyogenes* nisin-like lantibiotic SA-FF22, produced by the commensal species *Streptococcus salivarius*. *Int J Microbiol, 2012*, 738503. doi: 10.1155/2012/738503

Wescombe, P. A., Hale, J. D., Heng, N. C. and Tagg, J. R. (2012). Developing oral probiotics from *Streptococcus salivarius*. *Future Microbiol, 7*(12), 1355-1371. doi: 10.2217/fmb.12.113

Wescombe, P. A. and Tagg, J. R. (2003). Purification and characterization of streptin, a type A1 lantibiotic produced by *Streptococcus pyogenes*. *Appl Environ Microbiol, 69*(5), 2737-2747.

Wescombe, P. A., Upton, M., Dierksen, K. P., Ragland, N. L., Sivabalan, S., Wirawan, R. E., Inglis, M. A., Moore, C. J., *et al.* (2006). Production of the lantibiotic salivaricin A and its variants by oral streptococci and use of a specific induction assay to detect their presence in human saliva. *Appl Environ Microbiol, 72*(2), 1459-1466. doi: 10.1128/AEM.72.2.1459-1466.2006

Wescombe, P. A., Upton, M., Renault, P., Wirawan, R. E., Power, D., Burton, J. P., Chilcott, C. N. and Tagg, J. R. (2011). Salivaricin 9, a new lantibiotic produced by *Streptococcus salivarius*. *Microbiology, 157*(Pt 5), 1290-1299. doi: 10.1099/mic.0.044719-0

Wiedemann, I., Breukink, E., van Kraaij, C., Kuipers, O. P., Bierbaum, G., de Kruijff, B. and Sahl, H. G. (2001). Specific binding of nisin to the peptidoglycan precursor lipid II combines pore formation and inhibition of cell wall biosynthesis for potent antibiotic activity. *J Biol Chem, 276*(3), 1772-1779. doi: 10.1074/jbc.M006770200

Willey, J. M. and van der Donk, W. A. (2007). Lantibiotics: peptides of diverse structure and function. *Annu Rev Microbiol, 61*, 477-501. doi: 10.1146/annurev.micro.61.080706.093501

Wilson-Stanford, S. and Smith, L. (2011). Commercial development and application of type A lantibiotics. *Recent Pat Antiinfect Drug Discov, 6*(2), 175-185.

Wirawan, R. E., Klesse, N. A., Jack, R. W. and Tagg, J. R. (2006). Molecular and genetic characterization of a novel nisin variant produced by *Streptococcus uberis*. *Appl Environ Microbiol, 72*(2), 1148-1156. doi: 10.1128/AEM.72.2.1148-1156.2006

Wirawan, R. E., Swanson, K. M., Kleffmann, T., Jack, R. W. and Tagg, J. R. (2007). Uberolysin: a novel cyclic bacteriocin produced by *Streptococcus uberis*. *Microbiology, 153*(Pt 5), 1619-1630. doi: 10.1099/mic.0.2006/005967-0

Xiao, H., Chen, X., Chen, M., Tang, S., Zhao, X. and Huan, L. (2004). Bovicin HJ50, a novel lantibiotic produced by *Streptococcus bovis* HJ50. *Microbiology, 150*(Pt 1), 103-108.

Xie, L. and van der Donk, W. A. (2004). Post-translational modifications during lantibiotic biosynthesis. *Curr Opin Chem Biol, 8*(5), 498-507. doi: 10.1016/j.cbpa.2004.08.005

Ye, S. Y., Koponen, O., Qiao, M., Immonen, T. and Saris, P. E. (1995). NisP is related to nisin precursor processing and possibly to immunity in *Lactococcus lactis*. *J Tongji Med Univ, 15*(4), 193-197.

Yonezawa, H. and Kuramitsu, H. K. (2005). Genetic analysis of a unique bacteriocin, Smb, produced by *Streptococcus mutans* GS5. *Antimicrob Agents Chemother, 49*(2), 541-548. doi: 10.1128/AAC.49.2.541-548.2005

Yuzenkova, J., Delgado, M., Nechaev, S., Savalia, D., Epshtein, V., Artsimovitch, I., Mooney, R. A., Landick, R., *et al.* (2002). Mutations of bacterial RNA polymerase leading to resistance to microcin j25. *J Biol Chem, 277*(52), 50867-50875. doi: 10.1074/jbc.M209425200

Zendo, T., Fukao, M., Ueda, K., Higuchi, T., Nakayama, J. and Sonomoto, K. (2003). Identification of the lantibiotic nisin Q, a new natural nisin variant produced by *Lactococcus lactis* 61-14 isolated from a river in Japan. *Biosci Biotechnol Biochem, 67*(7), 1616-1619.

Zendo, T., Yoneyama, F. and Sonomoto, K. (2010). Lactococcal membrane-permeabilizing antimicrobial peptides. *Appl Microbiol Biotechnol, 88*(1), 1-9. doi: 10.1007/s00253-010-2764-3

Zhao, M. (2011). Lantibiotics as probes for phosphatidylethanolamine. *Amino Acids, 41*(5), 1071-1079. doi: 10.1007/s00726-009-0386-9