ISOLATION, PURIFICATION AND MODE OF ACTION OF ANTIMICROBIAL PEPTIDES PRODUCED BY LACTIC ACID BACTERIA OF DAIRY ORIGIN

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ABSTRACT

Lactic acid bacteria (LAB) are found in fermented food products. They help in improving shelf-life and enhance the flavour of food. They also produce bacteriocins to prevent the growth of undesirable bacteria. The goal of this study was to search for microbial strains with potent antimicrobial activity that can combat emerging food-borne pathogens and spoilage bacteria. In this study, LAB namely *Lactococcus lactis* A1, *Weissella confusa* A3 and *Enterococcus faecium* C1 were isolated from fermented cow milk and found to produce antimicrobial compounds. PCR amplification of genes encoding known bacteriocins proved that *L. lactis* A1 harboured Nis Z gene. As nisin has been well documented, it was not chosen for further investigations. *E. faecium* C1 did not harbour genes for enterocin A, B and P production. Therefore further tests were done to identify and characterise the bacteriocin from *E. faecium* C1. No gene encoding bacteriocin production was available for *W. confusa*. Both bacteriocins were purified through four steps namely ammonium sulphate precipitation, hydrophobic interaction, centrifugal filter, size separation concentrator and finally reverse phase HPLC. Both bacteriocins were active towards *Bacillus cereus, Escherichia coli, Pseudomonas aeruginosa* and *Micrococcus luteus.* The purified bacteriocins were named BacA3 and BacC1 for bacteriocin purified from *Weissella confusa* A3 and *Enterococcus faecium* C1 respectively. SDS-PAGE showed that the molecular weight of BacA3 was around 2.5 kDa. MALDI-TOF analysis suggested that BacA3 might be approximately 2.7 kDa. The molecular weight of BacC1 estimated by SDS-PAGE was around 10 kDa. The trypsin digested BacC1 showed unique molecular weight which did not match with any known proteins from UniProt database. BacA3 exhibited thermostability when exposed to temperature of 100 °C but BacC1 showed reduced activity after heating to 80 °C. Both BacA3 and BacC1 retained their activity at pH ranging from 2 to 6. When treated with proteinase and peptidase, both bacteriocins showed reduction in activity. Hence, confirmed the antimicrobial substances were of proteinaceous nature. The membrane permeability test using SYTOX® green nucleic acid stain showed that both bacteriocins caused significant disruptions to the test bacterial membrane and this was confirmed by transmission electron microscopy. The N-terminal sequence of BacA3 was VAPGEIVESL and BacC1 was GPXGPXGP. The search for genes related to virulence, superantigens and diseases by Rapid Annotation using Subsystem Technology (RAST) showed that both strains did not harbour the genes. The antibiotic resistance genes as listed in the pathogenicity island database were also absent in *E. faecium* C1. The virulence genes detected from the virulence factor database showed that 6 genes (*Asm*, *SagA*, *EfaAfm*, *CdsA*, *UppS*, *BopD*) present in C1 were also present in probiotic strain T110. The genomes of A3 and C1 showed the presence of several probiotic function genes. *In vitro* testing of viable *W. confusa* A3 and *E. faecium* C1 and their bacteriocins on milk also showed significant reduction of total bacterial count. Strains A3 and C1 were non-haemolytic and not antibiotic resistant. They therefore have high potential for application in the food industry as antimicrobial agents to extend the shelf-life of food products.

ABSTRAK

Bakteria asid laktik (LAB) didapati dalam produk makanan yang ditapai dan mereka membantu meningkatkan janka penimpan dan meningkatkan rasa makanan. Mereka juga menghasilkan bakteriosin untuk menghalang pertumbuhan bakteria yang tidak diingini atau bakteria patogenic. Tujuan kajian ini adalah untuk mencari strain mikrob dengan aktiviti antimikrob terhadap patogen makanan dan bakteria yang oleh membawa kerosakan kepada makanan. Dalam karya ini, tiga LAB bernama *Lactococcus lactis* A1, *Weissella confusa* A3 dan *Enterococcus faecium* C1 telah diasingkan daripada susu lembu yang ditapai dan dapat menghasilkan sebatian antimikrob. Amplifikasi PCR dengan gen yang berkaitan dengan bakteriosin dari *L. lactis* A1 dan *E. faecium* C1 membuktikan bahawa *L. lactis* A1 menaruh gen Nis Z. Oleh kerana nisin telah didokumenkan dengan baik, ia tidak dipilih untuk ujian selanjutnya. *E. faecium* C1 tidak menaruh gen pengeluaran enterocin A, B dan P. Oleh itu ujian lanjut telah dijalankan untuk mengenal pasti bakteriosin tersebut. Tiada pengekodan gen bakteriosin pengeluaran boleh didapati dari *W. confusa*. Bakteriosins tersebut telah ditulenkan melalui empat langkah iaitu ammonium sulfat, interaksi hidrofobik, serangkaian langkah sentrifugal vivaspin dan fasa-terbalik kromatografi cecair berprestasi tinggi (RP-HPLC). Bakteriosins tersebut telah terbukti mempunyai aktiviti perencatan terhadap *Bacillus cereus*, *Escherichia coli*, *Pseudomonas aeruginosa* dan *Micrococcus luteus*. Bacteriocins yang tulen telah dinamakan sebagai BacA3 dan BacC1 untuk bacteriocin yang ditulenkan daripada *Weissella confusa* A3 and *Enterococcus faecium* C1 masing-masing. SDS-PAGE menunjukkan berat molekul BacA3 adalah sekitar 2.5 kDa. Analisis MALDI-TOF mencadangkan BacA3 kira-kira 2.7 kDa. Berat molekul BacC1 dianggarkan dengan SDS-PAGE sekitar 10 kDa. Pencernaan trypsin BacC1 tersebut menunjukkan berat pecahan molekul tidak sepadan dengan mana-mana protein daripada pangkalan data UniProt mencadangkan bahawa itu mungkin protein novel. BacA3 menamerkan kestabilan thermo apabila terdedah kepada suhu 100 ° C tetapi BacC1 menunjukkan pengurangkan aktiviti selepas pemanasan hingga 80 °C. Kedua-dua BacA3 dan BacC1 mengekalkan aktiviti pada pH antara 2 hingga 6. Apabila dirawat dengan proteinase dan peptidase, kedua-dua bacteriocins menunjukkan pengurangan yang ketara dalam aktiviti. Ini mengesahkan bahan antimikrob itu bersifat protin. Ujian kebolehtelapan membran dengan menggunakan SYTOX® hijau menunjukkan bahawa kedua-dua bakteriosin menyebabkan gangguan besar kepada membran bakteria ujian dan juga dibuktikan oleh imej dari mikroskop elektron transmisi. Urutan terminal N daripada BacA3 adalah VAPGEIVESL dan penjujukan N-terminal mendedahkan urutan sebahagian BacC1 sebagai GPXGPXGP. Pencarian gen virulensi, superantigen, penyakit dengan Anotasi Pantas menggunakan Teknologi Subsistem (RAST) menunjukkan bahawa kedua strain tidak melabuh gen-gen tersebut. Gen rintangan antibiotik yang terdapat di pangkalan data pathogenicity pulau juga tidak hadir dalam *E. faecium* C1. Gen virulensi yang dikesan daripada pangkalan data faktor virulensi menunjukkan bahawa 6 gen (*Asm*, *SagA*, *EfaAfm*, *CdsA*, *UppS*, *BopD*) hadir dalam C1 turut hadir di T110 strain probiotik. Kedua-dua A3 dan C1 genom menunjukkan gen yang boleh membantu bakteria ini untuk mendapatkan potensi probiotik. Dalam pengujian *in vitro W. confusa* A3 dan *E. faecium* C1 dan bacteriocins dalam susu juga menunjukkan pengurangan yang ketara daripada nombor bakteria kerosakan. Strain bakteria adalah bukan hemolitik dan resistansi kepada antibiotik. Oleh itu mereka mempunyai potensi yang tinggi untuk aplikasi dalam industri makanan sebagai agen antimikrob untuk melanjutkan hayat simpanan produk makanan.

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Lastly, I offer my regards and blessing to all those who supported and advised me as I overcome all obstacles to complete this project.

DEDICATION

I dedicate this PhD to my father, mother and family for their endless support and love

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LIST OF SYMBOLS AND ABBREVIATIONS

%	:	Percentage
°C	:	Degree Celsius
AMP	:	Antimicrobial peptide
BLAST	:	Basic local alignment search tool
bp	:	Base pair
CFU	:	Colony forming unit
cm	:	Centimetre
CO_2	:	Carbon dioxide
Da	:	Dalton
DNA	:	Deoxyribonucleic acid
ed.	:	Editor
e.g.	:	Exempli gratis (example)
et al.	:	et alia (and others)
g	:	Gram
G+C	:	Guanine and cytosine
h	:	Hour
H_2S	:	Hydrogen sulfide
kb	:	Kilobase
kDa	:	Kilodalton
L	:	Litre
LAB	:	Lactic acid bacteria
min	:	Minute
ml	:	Millilitre
mm	:	Millimetre

µl	:	Microlitre
MALDI-TOF	:	Matrix-assisted laser desorption/ ionization time of flight
MS	:	Mass spectrometry
MWCO	:	Molecular weight cut-off
NaCl	:	Sodium chloride
NCBI	:	National Center for Biotechnology Information
nm	:	Nanometre
No.	:	Number
O.D.	:	Optical density
PAGE	:	Polyacylamide gel electrophoresis
PAIs	:	Pathogenicity islands
PCR	:	Polymerase chain reaction
pH	:	Hydrogen ion concentration
RAST	:	Rapid Annotation using Subsystem Technology
REIs	:	Antimicrobial Resistance Islands
RP-HPLC	:	Reversed-Phase High-performance liquid chromatography
SDS	:	Sodium dodecyl sulphate
SEM	:	Scanning Electron Microscopy
TEM	:	Transmission Electron Microscopy
UniProt	:	Universal Protein Resource
UV	:	Ultraviolet
v	:	Volume

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

During the past decade, there has been a dramatic increase in bacterial resistance towards conventional antibiotics. The overwhelming increase in antibiotic resistance is now recognised as a global crisis and as such requires the immediate attention of the pharmaceutical industry, academia and government institutions. Antimicrobial peptides (AMPs) have emerged as a promising new group to be evaluated in therapeutic intervention of infectious diseases. The development of resistance towards AMPs has occurred to a much lesser degree compared to conventional antibiotics as the mechanism of killing bacteria by AMPs usually involves attacking multiple hydrophobic and/or polyanionic targets (Fjell *et al*., 2012). AMPs have several advantages over antibiotics which include a broad spectrum of antimicrobial activity and selective cytotoxicity for hosts which are human endothelial cells and do not easily induce resistance (Matsuzaki, 2009). Thus, they are promising candidates for the development of antibiotics.

Apart from combatting multidrug-resistant microbes, antimicrobial peptides are also used to control harmful microflora in the food industry. The demand for natural, non-chemical and healthy products that comply with biosafety standards is increasing. One of the limitations of using nisin in food products is the inability of nisin to inhibit Gram-negative bacteria. Therefore there is need to search for bacteriocins which can exhibit antimicrobial activity against Gram-negative bacteria. Whether deliberately added or produced *in situ* by lactic acid bacteria, bacteriocins can play a beneficial role to control undesirable microbes and in the establishment of a desirable microbial population in selected food products.

This study investigates novel peptides from selected lactic acid bacteria that have significant antimicrobial effects against food spoilage bacteria and to develop an

alternative to current antibiotics in view of the increasing global antibiotic resistance. Lactic acid bacteria (LAB) are a heterogeneous group of bacteria used in various industrial applications, ranging from food and beverage fermentation, bulk and fine chemicals production such as lactic acid and B vitamins to the production of pharmaceuticals.

The project focused on the isolation of novel bacteriocins, a class of antimicrobial peptides of bacteria origin, from an indigenous source of cow milk supplied from Malaysian dairy retailers and the modes of action of the isolated peptides from these sources. The specific objectives and approaches used in this study included:

1. Isolation of lactic acid bacteria with antimicrobial activity from fermented raw cow milk.
2. Identification and characterisation of the bacteriocin producers.
3. Purification and characterisation of the bacteriocins.
4. To evaluate binding mechanism and the effect of the bacteriocin on targeted bacterial membranes
5. Genomes sequencing of the bacteriocin producers and basic genome analysis.

The above mentioned objectives were achieved by isolation of lactic acid bacteria with good inhibitory activity against selected food pathogens and a series of purification steps that were carried out in order to identify the bacteriocins. Further study was then carried out to evaluate the mode of action of these bacteriocins on the target pathogens. The lactic acid bacteria isolated and its bacteriocins were tested *in vitro* using a milk matrix to elucidate their effect on milk to reveal vital information for large scale industry application.

In the past when there was lack of chemical preservatives, most foods were preserved by drying, salting, smoking or with added concentrated sugar and also

through biopreservation using fermentation by lactic acid bacteria. The lactic acid bacteria not only produced acid to lower the pH of the food to prevent the growth of other bacteria but also antimicrobial peptides which kill pathogenic bacteria.

General food preservatives currently used to preserve food products are mostly derived from synthetic chemical processes. Such chemicals may have deleterious effect on the health of consumers. Therefore safer and more natural sources of food preservatives are investigated as alternatives to synthetic preservatives. The production of bacteriocins by LAB is not only advantageous to the bacterial propagation but can also be used in the food industry as natural preservatives to enhance the shelf life of certain foods likely more acceptable to consumers. Nisin which was isolated from *Lactococcus lactis* is an example of a bacteriocin isolated from lactic acid bacteria that was widely used as a biopreservative in the food industry.

CHAPTER 2: LITERATURE REVIEW

2.1 Lactic acid bacteria

Lactic acid bacteria designated as LAB have long been used for food preservation. The documented history started from 1857 when Louis Pasteur studied lactic acid fermentation and discovered the role of lactic acid in spoilage of wine (Wibowo *et al*., 1985). After 10 years of the discovery, a pure lactic acid bacteria was isolated from milk by Joseph Lister, student of Louis Pasteur. Then the bacteria was named as "*Bacterium lactis*" (Josephsen *et al*., 2006; Newsom, 2003). LAB are Gram positive, non-spore formers, cocci or rod-shaped and usually non-motile bacteria. They also do not have cytochromes and are unable to synthesize porphyrins and do not have catalase and oxidase enzymes. They are called lactic acid bacteria because lactic acid is the major end product during their fermentation of different carbohydrates (Stiles & Holzapfel, 1997).

Most of the LAB are aerotolerant anaerobes. They do not require oxygen for their growth, but they can grow in the presence of oxygen. LAB are found in a large variety of nutrient rich environments especially when the carbohydrates and proteins are abundant. These include milk or dairy products, vegetables and plants, cereals, meat and meat products (Wiley & Sons, 2010). The biosynthetic ability to generate amino acids from inorganic nitrogen sources by LAB is very limited. Therefore, they must be cultivated in complex media with all the required amino acids and vitamins to fulfil their nutritional needs (Hugenholtz & Kleerebezem, 1999).

LAB have DNA base composition of less than 53 mol % G+C content. According to taxonomic revisions, LAB consists of 17 genera namely *Aerococcus*, *Alloiococcus*, *Carnobacterium*, *Dolosigranulum*, *Globicatella*, *Enterococcus*,

Lactobacillus, *Lactococcus*, *Lactosphaera*, *Leuconostoc*, *Mlissococcus, Streptococcus*, *Oenococcus*, *Pediococcus*, *Tetragenococcus*, *Vagococcus,* and *Weissella* (Holzapfel *et al*., 2001; Ruas-Madiedo *et al*., 2012). The main pylogenetic groups of LAB and related Gram positive bacteria is shown in Figure 2.1. Based on biochemical pathways, LAB comprises of both the homofermenters and heterofermenters. In homofermentative LAB, the end product of metabolism is mainly lactic acid. On the other hand, heterofermenters can produce lactic acid as well as other fermentation end products such as ethanol, acetic acid, formic acid and carbon dioxide (Kleerebezem & Hugenholtz, 2003). Carbon dioxide production is a distinct product of heterofermentation. Some of the *Lactobacilli* and most species of *Enterococci*, *Tetragenococci*, *Pediococci*, *Lactococci*, *Streptococci*, and *Vagococci*, which ferment hexoses by the Embden-Meyerhof pathway (EMP) are in the homofermetative LAB group. The heterofermentative LAB comprise of some *Lactobacilli*, *Leuconostoc*, *Oenococci*, and *Weissella* species. They use the pentose phosphate pathway or alternately called the phosphoketolase pathway (PK) to break down sugars. The significant difference in these two types of fermentation is their enzyme action which includes the production of the important cleavage enzymes, fructose 1,6-diphosphate of the EMP and phosphoketolase of the PK pathway (Conway, 1992; König & Fröhlich, 2009). Figure 2.2 summarises the metabolic pathway of lactic acid bacteria.

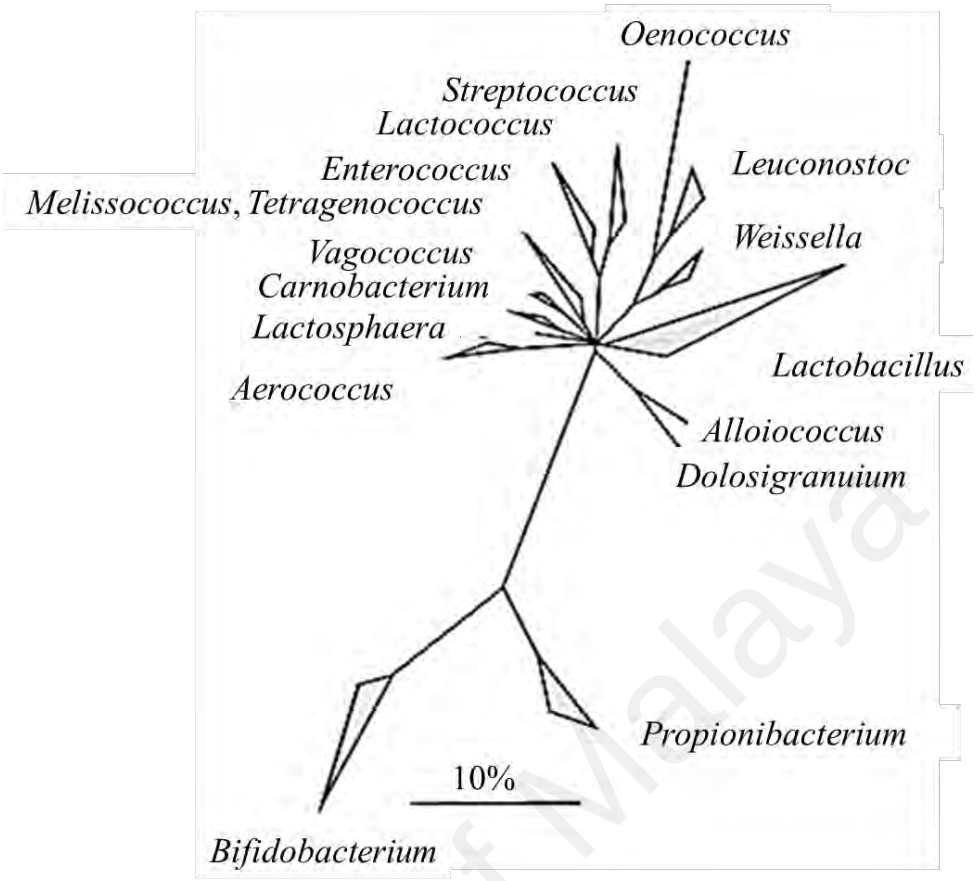


Figure 2.1: Major pylogenetic groups of LAB and related Gram positive bacteria with low and high mol % G+C in DNA. Adapted from Stiles & Holzapfel (1997).

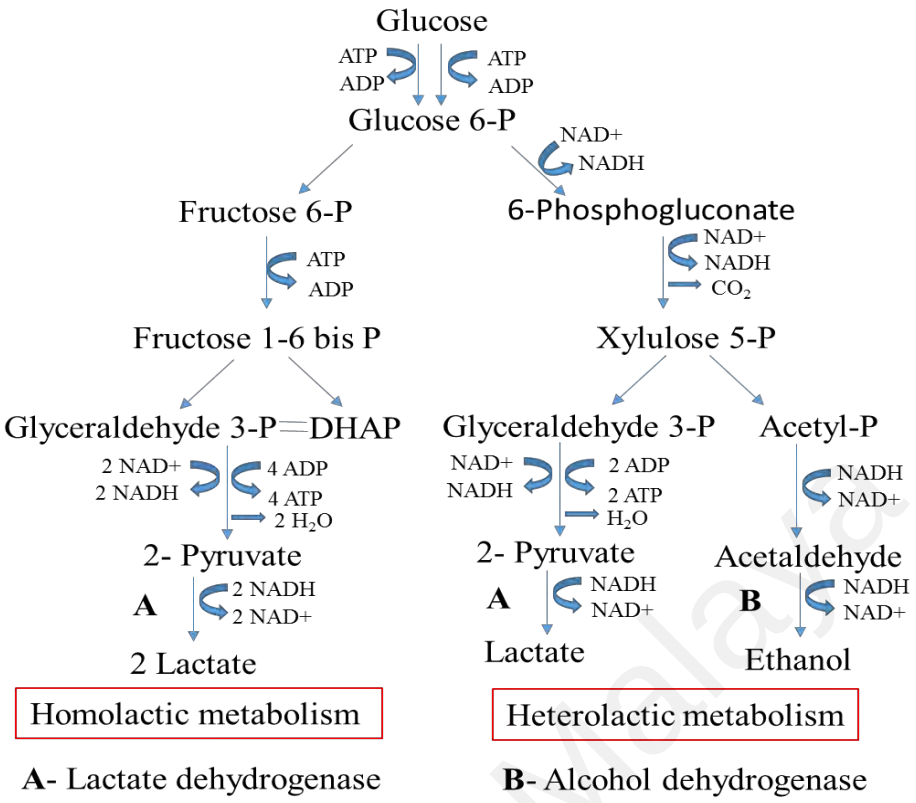


Figure 2.2: The metabolic pathway of lactic acid bacteria. Adapted from Reddy *et al*. (2008).

Most of the LAB are beneficial bacteria that are harmless to humans except for a few species such as *Streptococcus pneumoniae* and *Streptococcus pyogenes*. They are considered as "Generally Recognised as Safe" (GRAS) microorganisms (Hardie & Whiley, 1997). They have been used as starter cultures for thousands of years in the production of fermented foods and beverages (Stiles & Holzapfel, 1997). Some of them are also known as probiotics. Probiotics are defined by the Food and Agricultural Organization (FAO) as live microorganisms that when administered in adequate amounts will confer a health benefit on the host. The health-promoting effects of these beneficial bacteria include inhibition of carcinogenesis, anticholesteraemic compounds, increased calcium resorption, decrease of lactose intolerance, synthesis of vitamins, prevention of genital and urinary tract infections and immunostimulatory effects (Masood *et al*., 2011; Tannock, 1997).

2.2 Bacteriocins as antimicrobial peptides produced by LAB

AMPs are small biological molecules with less than 10 kDa that direct antimicrobial activity. They have an overall positive charge which is generally +2 to +9 and a substantial proportion of more than 30 % of hydrophobic residues. These properties allow the peptides to fold into three dimensional amphiphilic structures. The AMPs discovered so far are divided into several groups according to their length, secondary and tertiary structure and presence or absence of disulfide bridges (Reddy *et al*., 2004; Xiao *et al*., 2013). There are four broad structural groups for folded peptides which are: (1) β-sheet peptides stabilised by two to four disulfide bridges (human α- and β-defensins or plectasinorprotegrins). (2) α-helical peptides (LL-37, cecropins or magainins). (3) extended structures rich in glycine, proline, tryptophan, arginine and/or histidine (for example indolicidin). (4) Loop peptides with one disulfide bridge (for example bactenecin). They have been isolated from different organisms including bacteriocins from bacteria, fungal peptide antibiotics, insect defensins and cecropins, plant thionins and defensins, amphibian magainins and temporins, as well as defensins and cathelicidins from higher vertebrates (Bommarius *et al*., 2010; McPhee & Hancock, 2005; Sang & Blecha, 2008). In bacteria antimicrobial compounds are referred to as bacteriocins (Joerger, 2003). The term bacteriocin was introduced by Jacob and co-workers in 1953 (Jacob *et al*., 1953). Bacteriocin has been defined as an antagonistic compound of proteinaceous nature with bactericidal activity against a limited range of closely related organisms (Tagg *et al*., 1976). Bacteriocins are also defined as proteinaceous compounds produced by bacteria that exhibit a bactericidal mode of action against related as well as unrelated organisms (Ogunbanwo *et al*., 2003).

LAB is one of the prominent groups of bacteria inhabiting the gastrointestinal tract, and the importance of these non-pathogenic bacteria in producing beneficial

antimicrobial compounds has recently been more noticed. The antimicrobial compounds produced may act as bacteriostatic or bactericidal agents that inhibit or kill other bacteria. The antimicrobial activities are due to the production of antimicrobial metabolites such as bacteriocins, hydrogen peroxide and organic acids (Wood, 1992). The basic structure of bacteriocin consists of a heterogenous group of small peptides or high molecular weight proteins or protein complexes (Dillon, 1998; Papagianni,2003). The synthesis of bacteriocins is widespread among different bacterial species and it is proposed that virtually all bacterial species synthesise bacteriocins (O'Connor *et al*., 2015; Majeed *et al*., 2013). The bacteriocin production is made possible by relatively simple biosynthetic machineries that are often linked with elements like plasmids and conjugative transposons (Yamashita *et al*., 2011; Phelan *et al*., 2013). This process is further simplified by the fact that bacteriocin associated genes are often clustered on plasmids, chromosomes or transposable elements (Abriouel *et al*., 2006; Cavera *et al*., 2015).

The nomenclature of bacteriocin is straight forward. Just like "ase" is used in enzyme nomenclature, the suffix "cin" is used to denote bacteriocinogenic activity. The "cin" suffix is appended to either the genus name or (more correctly) to the species name. For example, colicins were isolated from *Escherichia coli*, subtilin is produced by *Bacillus subtilis*, salivaricins were originally isolated from *Streptococcus salivarius*, and so on. Sequential letters assigned in the order of discovery are used after the bacteriocin name to differentiate unique bacteriocins produced by different strains of the same bacterial species. For example lacticin F was the sixth bacteriocin reported for a *Lactobacilli* species (de Lima & Filho, 2005).

Most of the AMPs kill bacteria by membrane-targeting pore-forming mechanisms which disrupt the membrane integrity. Therefore they are thought to be less

likely to cause resistance and are being widely evaluated as novel antimicrobial drugs. Unlike conventional antibiotics which are generally active against bacteria or fungi, AMPs are more powerful and active against a wider spectrum of microorganisms including bacteria, fungi, parasites, enveloped viruses and even some cancer cells (Reddy *et al*., 2004; Sang & Blecha, 2008).

Lactic acid bacteria are known to inhibit some psychrotrophs in milk and ground beef (Ammor *et al*., 2006; Wong & Chen,1998). Viable cultures or components of lactic acid bacteria are useful in the treatment of displaced endogenous intestinal microflora which is the characteristic of many intestinal disorders and they will enhance the gut permeability of the host (Collado *et al*., 2009; Quigley, 2010). They are able to survive the low pH gastric condition and colonise the intestine at least temporarily by adhering to the epithelium (Marteau *et al*., 1997). Pigs and calves fed with these beneficial bacteria showed significant decrease in the occurrence of diarrhoea. Enterotoxins from *Escherichia coli* which are pathogenic to pigs are believed to be neutralised by lactic acid bacteria as reviewed by Teo & Tan (2006).

In recent years, the increased consumption of foods containing additives such as chemical preservatives and the increase in consumer concerns have created a high demand for natural and minimally processed foods. LAB can be considered as a substitute for chemical food preservatives because they have antagonistic effects towards other bacteria and do not produce other adverse effects (Pingitore *et al*., 2007). LAB can be naturally found in fermented foods, so the use of antimicrobial compounds produced by LAB is safe and is a natural way of food preservation (Tiwari *et al*., 2009; Topisirovic *et al*., 2006). However, currently only nisin is licensed for use as food additives and other bacteriocins are present in foods mostly through production by starter cultures. Antibiotic is a major tool to combat bacterial infections as reviewed by

Balciunas *et al*. (2013). However, bacteria are highly adaptable microbes and are capable of developing resistance to antibiotics. Currently, several bacteria have become superbugs resistant to many types of antibiotics available in the market. The emergence of these antibiotic resistant bacteria may be overcome by using naturally occurring LAB which produces bacteriocins or antimicrobial peptides (AMPs) as secondary metabolites (Corr *et al*., 2007; Hassan *et al*., 2012).

2.3 Production and purification of bacteriocins

The discovery of bacteriocins from the LAB culture involves several steps. The first step starts from screening the bacteriocin-producing LAB from a large number of isolates. Then the selected potential producer is grown on the most suitable medium to produce large amount of bacteriocin which is then purified through a series of steps leading to identification and characterisation (De Vuyst & Leroy, 2007; Parente & Hill, 1992). The initial screening usually is done by direct detection method by growing the potential producer cells and indicator strains on agar surface. The tests normally used for initial identification of the antagonistic activity are based on the diffusion of the antimicrobial compounds in the culture media to inhibit a sensitive target microorganism (Miao *et al*., 2014; Parente *et al*., 1995). During incubation both producer and indicator strains are cultured simultaneously and antimicrobial activity indicated by the presence of inhibition zones around the producer cells after incubation. Other commonly used methods for initial detection include spot on lawn and agar well diffusion assay (Fleming *et al*., 1975; Tagg & McGiven, 1971).

The next step of purification involves growing the bacteria in an appropriate medium under optimum conditions to yield high production of the bacteriocin. The bacteriocin which is extracellularly secreted into the culture broth is then purified and separated from the contents of the medium through different purification steps. Table

2.1 shows different purification methods used to purify bacteriocins produced by different producers.

Table 2.1: Purification method of bacteriocins produced by LAB

Producer strain	Bacteriocin	Purification method	Reference
E. faecalis MR99	Enterocin MR99	AS, HIC	Sparo *et al*., 2006
E. faecium CT492	Enterocin A	AS, CEX, HIC, RP-HPLC	Aymerich *et al*., 1996
E. faecium T136	Enterocin B	XAD 16, CEX, HIC, RP-HPLC	Casaus *et al*., 1997
E. faecium P13	Enterocin P	AS, GF, CEX, HIC, RP-HPLC	Cintas *et al*., 1997
E. avium XA83	Avicin A	AS, CEX, RP-HPLC	Birri *et al*., 2010
L. plantarum C-11	Plantaricin A	AS, CEX, HIC, RP-HPLC	Nissen-Meyer *et al*., 1993
L. plantarum C19	Plantaricin C19	pH mediated adsorption – desorption, RP-HPLC	Atrih *et al*., 2001
L. plantarum 423	Plantaricin 423	AS, Dialysis, chloroform-methanol extraction, CEX	Van Reenen *et al*., 2003
L. plantarum A-1	Plantaricin ASM-1	AS, CEX, HIC, RP-HPLC	Hata *et al*., 2010
L. sake C2	Sakacin C2	Cold ethanol precipitation, GF	Gao *et al*., 2010
L. acidophilus DSM20079	Acidocin D20079	AS, Dialysis, CEX, HIC	Deraz *et al*., 2005
L. curvatus CWBI-B28	Curvalicin 28a, b & c	AS, HIC, RP-HPLC	Ghalfi *et al*., 2010)
L. divergens	Divergicin M35	CEX, C-18 Sep-Pak, RP-HPLC	Tahiri *et al*., 2004
L. rhamnosus 68	Rhamnosin A	Lyophilization, Ethanol precipitation, RP-HPLC	Dimitrijević *et al*., 2009
L. lactis spp. *Lactis* CNRZ 481	Lacticin 481	AS, GF, RP-HPLC	Piard *et al*., 1992
L. lactis spp. *Lactis* IPLA 972	Lactococcin 972	Acetone precipitation, CEX	Martínez *et al*., 1996
L. lactis spp. *lactis* 61-14	Nisin Q	XAD-16, CEX, RP-HPLC	Zendo *et al*., 2003
P. pentosaceus SA132	Pediocin SA131	Ethanol precipitation, IEX, Ultrafiltration	Lee *et al*., 2010
S. thermophilus SFi13	Thermophilin 13 (A and B)	Trichloroacetic acid precipitation, HIC, RP-HPLC	Marciset *et al*., 1997
S. salivarius 20P3	Salivaricin A	XAD-2, CEX, GF, RP-HPLC	Ross *et al*., 1993

Table 2.1 continued

L. mesenteroides	Leucocin C	CEX, RP-HPLC	Fimland *et al*., 2002
L. pseudomesenteroides	Leucocin Q	XAD-16, CEX, RP-HPLC	Sawa *et al*., 2010

Abbreviation: AS: Ammonium sulphate precipitation; GF: Gel filtration; IEX: Ion-Exchange; CEX: Cation Exchange; RP-HPLC: Reverse-phase high performance liquid chromatography; HIC: Hydrophobic interaction chromatography.

Since bacteriocins are produced and secreted into the culture medium during bacterial growth and considering the relatively low specific production of these peptides, therefore a first necessary step is the concentration of the cell-free culture supernatant. The most commonly used method is by ammonium sulphate precipitation which reduces initial volume and thereby concentrates the bacteriocins. Although most bacteriocins display a reduced activity at high salt concentrations, ammonium sulfate as concentrated as 80 % saturation does not interfere with the antimicrobial activity (Ivanova *et al*., 1998; Rashid *et al*., 2013). In ammonium sulphate precipitation, the proteins and peptides of the growth medium are also concentrated along with the bacteriocins. Therefore, further purification steps are needed to separate the bacteriocins from the contaminants and purify the bacteriocin to homogeneity. Some characteristics of bacteriocins including low molecular weight, hydrophobicity and cationic nature are usually exploited to further purify the bacteriocin (Altuntas *et al*., 2014; Héchard & Sahl, 2002; Parada *et al*., 2007).

The purification scheme described above is most commonly used and contributed to purification and identification of many novel bacteriocins. However, there are limitations. The scheme involves a number of steps and some steps are time consuming and laborious (Nigutová *et al*., 2008). For example, the ammonium sulphate precipitation requires stirring overnight at 4 °C. Moreover, there are losses at each step and hence the greater number of steps can mean greater loss in bacteriocin activity. The

bacteriocins are produced in minute quantities; therefore, the entire bacteriocin activity may be lost after final purification (Yang *et al*., 1992).

An interestingly shorter and inexpensive alternative to concentrate LAB bacteriocins was described in 1992 in which bacteriocins such as sakacin A, nisin, pediocin AcH, and leuconocin Lcm1 were adsorbed onto producer bacteria at pH 5–7 and then desorbed later by lowering the pH of the medium (Yang *et al*., 1992). The rationale of this method is based on the fact that most bacteriocins have a specific range of pH where the bacteriocins are completely adsorbed onto the bacterial cell surfaces. Therefore, after an overnight incubation, cultures are heated at about 70 °C to kill the bacterial cells. The pH of the medium is adjusted to assure complete adsorption of bacteriocins to the heat-killed cells followed by a number of washes in order to remove contaminants from the culture medium. Finally, the peptides are released by lowering the pH with strong acids and using 50 mM sodium dodecyl sulfate (SDS). The same method is reported to be effective in purifying other bacteriocins. For instance, nisin, piscicolin 126, brevicin 286 and pediocin PO2 were extracted from fermentated culture broth by adsorption onto Micro-Cel which is a food-grade diatomite calcium silicate (anti-caking agent) and subsequent desorption with 1 % sodium deoxycholate and 1 % SDS (Coventry *et al*., 1996). However, it is not suitable for all bacteriocins; for instance, the yield of two component bacteriocins is often low and inappropriate for large-scale purification (Anderssen *et al*., 1998).

Since the concentration steps only reduce the working volume and do not provide a high degree of purity, several subsequent chromatographic steps are still required. Reverse-phase high performance liquid chromatography is commonly used in the final step of the purification scheme to separate the bacteriocin from any other remaining compounds (Saavedra & Sesma, 2011). The purified bacteriocin is then run

on a SDS-PAGE gel to confirm its purity and estimate the molecular weight of the bacteriocin. The presence of a single band in the gel confirms that the bacteriocin has been purified to homogeneity. Finally the structure of the bacteriocin is determined by N-terminus sequencing and mass spectrometry techniques (Farías *et al*., 1996; Himeno *et al*., 2012; Saraiva *et al*., 2012).

2.4 Classification of bacteriocins

Some bacteriocins are small and comprise of 19 to 37 amino acids while some are of larger molecular weight of 90 kDa (Joerger, 2003). Post translational modifications may occur resulting in unusual amino acid residues such as class I bacteriocins. Different classifications of bacteriocin were developed by different researchers. In 1993, Klaenhammer proposed to classify the bacteriocin into 4 which is class I, II, III and IV (Klaenhammer, 1993). The class I bacteriocin are post-translationally modified lantibiotics containing unusual amino acids such as lanthionine or methyl lanthionine residues. Class II bacteriocins are heat-stable, unmodified peptides and do not contain the unusual amino acid and hence they are known as non-lantibiotics. They are small peptide molecules which are smaller than 10 kDa. The class II bacteriocins are further split into 3 sub-classes, namely IIa, IIb, and IIc. Pediocin-like bacteriocins, two-component bacteriocins and thiol-activated bacteriocins are organised under the IIa, IIb, and IIc sub-classes respectively. Class III bacteriocins are heat-labile and larger (>10 kDa) protein molecules and class IV bacteriocins are complex proteins that contain lipid or carbohydrate moieties. In 2005 and 2013, Paul D. Cotter suggested to group bacteriocin into three main classes (Cotter *et al*., 2005; Cotter *et al*., 2012). The classification scheme of Cotter *et al*., (2005) is further discussed in the following sub-section.

2.4.1 Class I bacteriocins

Class I bacteriocins are called lantibiotics. Lantibiotics are gene-encoded peptides that have intra-molecular ring structures by formation of the thioether bridges between dehydrated serine or cysteines and threonine that confer lanthionine and methyl-lanthionine residues, respectively. They are small (<5 kDa), heat stable and undergo post-translational modification. A special characteristic of class I bacteriocin is the presence of unusual amino acids such as lanthionine, methyl-lanthionine, dehydrobutyrine and dehydroalanine (Cleveland *et al*., 2001). Class I bacteriocins are further divided into two sub-classes. The class Ia bacteriocins are relatively elongated, flexible with cationic and hydrophobic peptides (Deegan *et al*., 2006). Class Ib bacteriocins have either no charge or are negatively charged and they are globular peptides with rigid structures. The typical example of class Ia bacteriocin is nisin and class Ib is mersacidin. Besides, class I bacteriocins also have been sub-classified into 12 sub-groups based on their distinctive features (Cotter *et al*., 2005). The different groups are shown in Table 2.2.

Table 2.2: Class I bacteriocins and their examples (Cotter *et al*., 2005)

Group	Distinctive feature	Examples
MccC7-C51-type bacteriocins	Is covalently attached to a carboxy-terminal aspartic acid	MccC7-C51
Lasso peptides	Have a lasso structure	MccJ25
Linear azole- or azoline-containing peptides	Possess heterocycles but not other modifications	MccB17
Lantibiotics	Possess lanthionine bridges	Nisin, planosporicin, mersacidin, actagardine, mutacin 1140
Linaridins	Have a linear structure and contain dehydrated amino acids	Cypemycin
Proteusins	Contain multiple hydroxylations, epimerizations and methylations	Polytheonamide A
Sactibiotics	Contain sulphur–α-carbon linkages	Subtilosin A, thuricin CD
Patellamide-like cyanobactins	Possess heterocycles and undergo macrocyclization	Patellamide A
Anacyclamide-like cyanobactins	Cyclic peptides consisting of proteinogenic amino acids with prenyl attachments	Anacyclamide A10
Thiopeptides	Contain a central pyridine, dihydropyridine or piperidine ring as well as heterocycles	Thiostrepton, nocathiacin I, GE2270 A, philipimycin
Bottromycins	Contain macrocyclic amidine, a decarboxylated carboxy-terminal thiazole and carbon-methylated amino acids	Bottromycin A2
Glycocins	Contain S-linked glycopeptides	Sublancin 168

The primary translation product of lantibiotics is a pre-peptide consisting of a leader peptide at the N-terminus. The length of the leader peptide may vary from 23 to 59 amino acids. Although there are no amino acid modifications occurring in the leader peptide region, extensive modifications take place in the pro-peptide region while the leader peptide is still attached to the pro-peptide. Post-translational modifications only involve three amino acids which are serine, threonine and cysteine. Occasionally lysine, alanine and isoleucine may also be post-translationally modified as reviewed by Sahl & Bierbaum (1998).

2.4.2 Class II bacteriocins

Class II bacteriocins do not have lanthionine and do not undergo post-translational modification. They are less than 10 kDa and heat stable peptides. Currently it was divided into 5 sub-groups. Class IIa bacteriocins are pediocin-like, active against *Listeria* and have amino acid sequence of YGNGV 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; Johnsen *et al*., 2005; Uteng *et al*., 2003). Class IIb bacteriocins are two peptides bacteriocin. Sometimes, individual peptide present will not cause antimicrobial activity. Two peptides need to be present in order to give antimicrobial activity. The two peptides are encoded by genes adjacent to each other and even though they consist of two peptides, only one immunity gene is required for them to protect themselves from their own bacteriocin action (Cleveland *et al*., 2001). The class IIc bacteriocins are circular peptide because their N and C terminals are covalently bonded to each other (Kawai *et al*., 2004; Maqueda *et al*., 2008). On the other hand, class IId bacteriocins are linear and unmodified bacteriocins which do not belong to any of the class discussed above. Class IIe are peptides that have a serine-rich C- terminal and they undergo non-ribosomal siderophore-type modification (Lagos *et al*., 2009).

2.4.3 Class III bacteriocins

Although the bacteriocins characterised from Gram-positive bacteria are predominantly small (<10 kDa) peptides, some large antimicrobial proteins have been described at both the biochemical and genetic levels. The class III bacteriocins are large and heat sensitive proteins. One apparent exception is propionicin SM1 which is a heat-stable antimicrobial agent produced by *Propionibacterium jensenii* DF1 isolated from Swiss raw milk (Miescher *et al*., 2000). Bacteriocins in this class are sub-grouped into

two, namely bacteriolysin (bacteriolytic enzyme) and the non-lytic antimicrobial protein. So far, the number of class III LAB bacteriocins identified is very limited (Bali *et al*., 2016; Bastos *et al*., 2010). Class III bacteriocins have domain-type structure for translocation, receptor binding and lethal activity. In the past few years, much progress has been made in the characterisation of bacteriolysins produced by LAB, mainly from members of genera *Streptococcus* and *Enterococcus*. The prototype streptococcal bacteriolytic enzyme is zoocin A, which is specified by a chromosomally located gene (*zooA*) in *Streptococcus equi* subsp. *zooepidemicus*. Zoocin A contains 262 amino acids organised in distinct domains with different functions (Simmonds *et al*., 1996; Simmonds *et al*., 1997). Several large bacteriocins have been shown to kill target cells by non-lytic mean. This could involve dissipation of the proton motive force, leading to ATP starvation and ultimately cell death. Helveticin J, a 37 kDa bacteriocin produced by *Lactobacillus helveticus* is one of the non-lytic bacteriocin (Joerger & Klaenhammer, 1986). The list of bacteriocins from class II and class III is shown in Table 2.3.

Table 2.3: Classification of class II and class III bacteriocins

Group		Distinctive feature	Examples
Class II	IIa	Possess a conserved YGNGV motif (in which N represents any amino acid)	Pediocin PA-1, Enterocin CRL35, Carnobacteriocin BM1
	IIb	Two unmodified peptides are required for activity	ABP118, Lactacin F
	IIc	Cyclic peptides	Enterocin AS-48
	IId	Unmodified, linear, non-pediocin-like, single-peptide bacteriocins	Microcin V, Microcin S, Epidermicin NI01, Lactococcin A
	IIe	Contain a serine-rich carboxy-terminal region with a non-ribosomal siderophore-type modification	Microcin E492, Microcin M
Class III		Large, heat-labile proteins	Lysostaphin, Helveticin J, Enterolysin A, Zoocin

Adapted and modified from Cotter *et al*. (2013).

2.4.4 Universal scheme of bacteriocin classification

Considering the two proposed schemes, a universal bacteriocin classification was established as shown in Figure 2.3 (Heng & Tagg, 2006; Ramu *et al*., 2015). The universal scheme is built based on the Klaemhammer scheme of classification as a foundation and at the same time incorporate elements of Cotter *et al*. (2005) classification. In this universal scheme of classification, Class I lantibiotics are subdivided into Ia (linear), Ib (globular) and Ic (multi-component). The class II cyclic

peptides from Cotter *et al*. (2005) classification is restructured into an individual class which is class IV in the universal classification scheme.

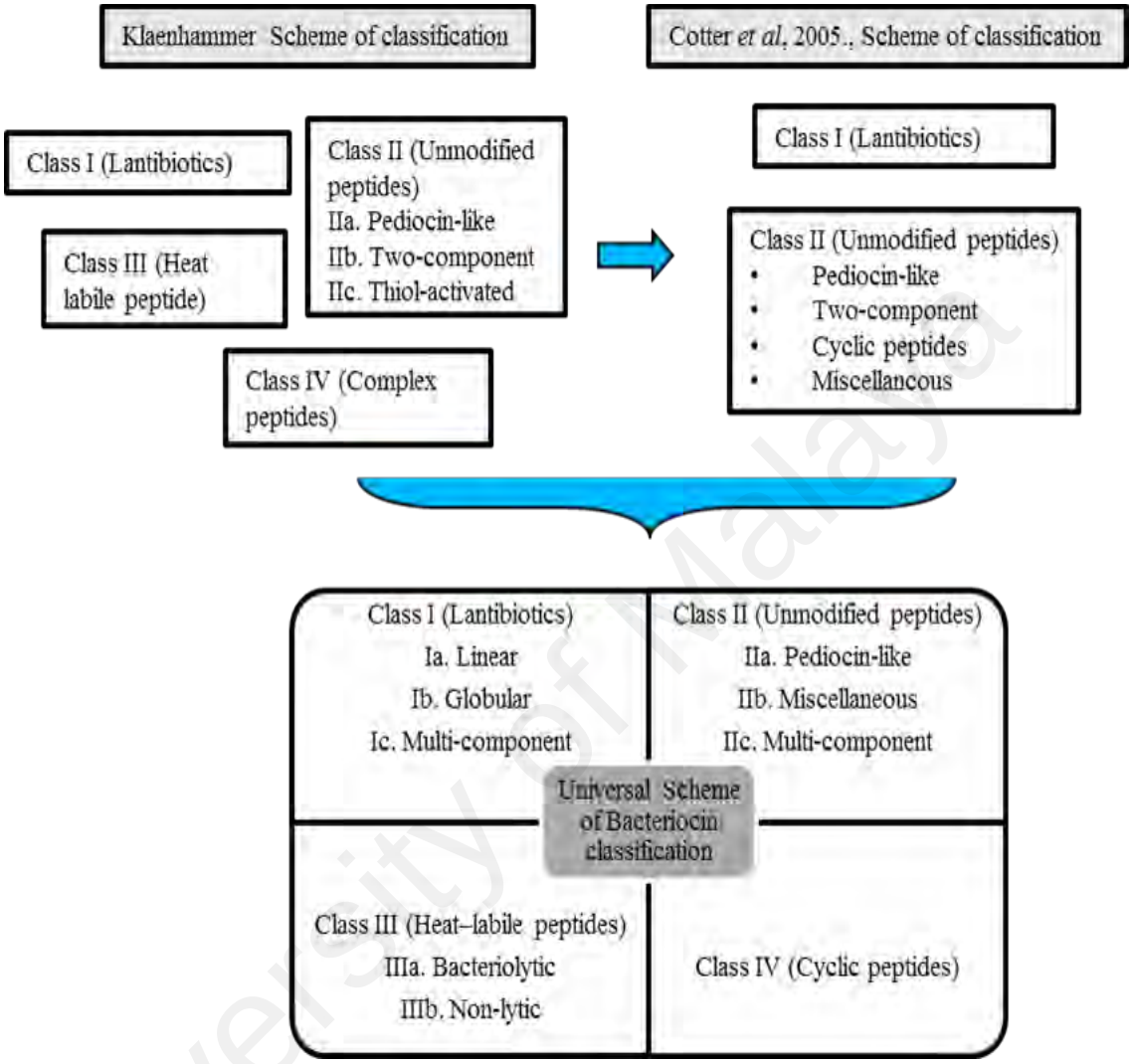


Figure 2.3: Classification of bacteriocin. Adapted from Heng & Tagg (2006).

2.5 Genetics of bacteriocin production

In recent years, the DNA sequences encoded for the production of bacteriocins has been determined. Studies have revealed that the genes required for biosynthetic machinery of lantibiotics are complex and are often organised in operons (Eijsink *et al*., 2002). The whole operon usually consists of the structural genes, extracellular translocation of the bacteriocins, genes encoding modification enzymes as well as immunity genes to protect the producer for self-destruction are needed (Nes *et al*., 1996). Besides, studies have shown that the production of some lantibiotics is also regulated by a two-component regulatory system (Kleerebezem & Quadri, 2001) and alternative regulatory systems have also been identified in some lantibiotics as seen for lactocin S (Rawlinson *et al*., 2005). These entire genes are either organised on transposon, plasmid or chromosome. The modifications in lactibiotics are introduced either by one biosynthetic enzyme (LanM) or by a combination of dehydratase (LanB) and a cyclase (LanC) (Chatterjee *et al*., 2006; Li & van der Donk, 2007; Rink *et al*., 2007; You & van der Donk, 2007). For the synthesis of Class II bacteriocins, the structured genes encoding the bacteriocin production are usually present along with a dedicated immunity gene, a transporter and its accessory gene. As seen with some lantibiotics, the class II bacteriocins can also be regulated by a two- or three-component regulatory system, and under such circumstances, extra three genes are needed: a gene that encodes a peptide pheromone, and genes for a sensor (histidine protein kinase) and also a DNA-binding protein that activates gene expression (response regulator) (Nes *et al*., 1996; Nes & Eijsink, 1999). Furthermore, several bacteriocins are clustered together and share both the transport and regulatory systems as seen in plantaricin (Diep *et al*., 2009). In Figure 2.4, comparison of the gene clusters indicate the presence of a number of conserved genes proposed to encode similar functions.

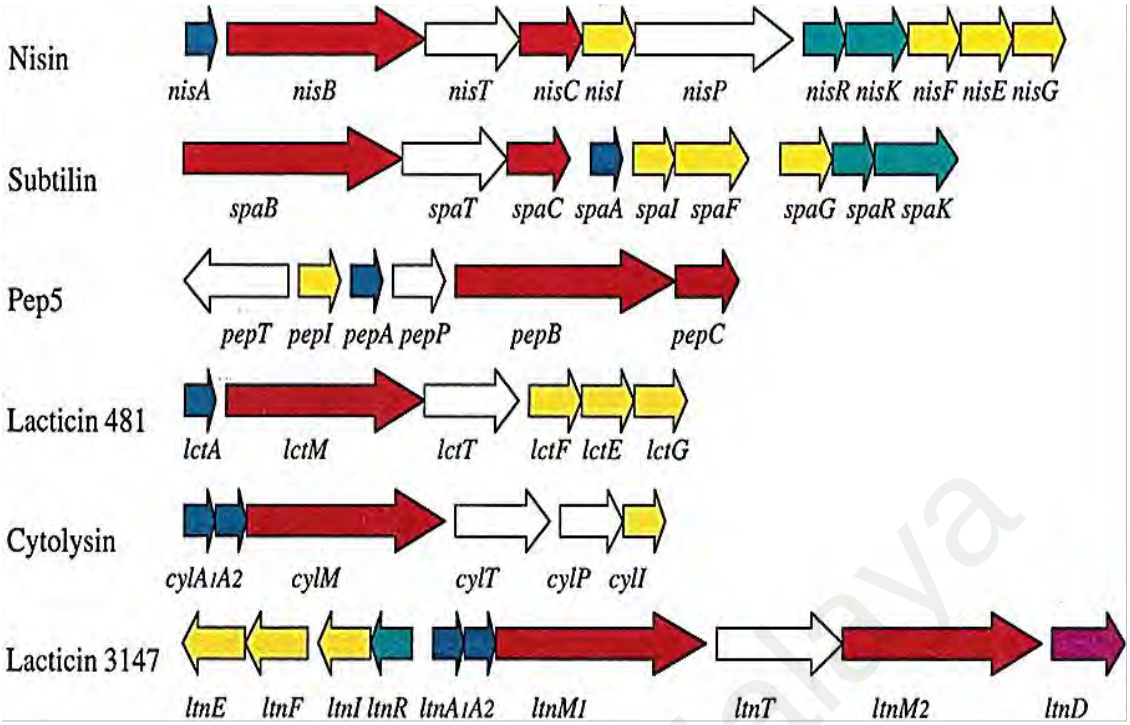


Figure 2.4: Gene clusters of characterised lantibiotics. Structural genes are highlighted in blue; genes with similar proposed functions are highlighted in the same colour (yellow for immunity, white for transport/processing, green for regulatory red for modification, and blue for unknown function). Copied with permission from McAuliffe *et al*. (2001).

2.6 Mode of action of AMPs or bacteriocins

The exact mode of action of AMPs remains a matter of controversy. However there is a consensus that these peptides selectively disrupt the bacterial membranes and the amphipathic structural arrangement of the peptides is believed to play a significant role in this mechanism. Peptides with a net positive charge will interact with the bacterial membranes which are more negatively charged than mammalian cells and initiate the inhibitory activity towards bacterial membrane. It is well documented that biophysical characteristics such as secondary structure, overall charge and hydrophobicity affect the interaction of AMPs with biological cells and model membranes (Chen *et al*., 2007; Shai, 2002; Reddy *et al*., 2004). Initial binding to the cell surface is driven by charge and based on the electrostatic attraction of the peptide to

the membrane. Thereby, the cationic peptides interact with acidic polymers, such as the cell wall associated teichoic acids, as well as with acidic phospholipids e.g. cardiolipin and phosphatidylglycerol (Matsuzaki, 1999). This binding induces a conformational change that results in a rearrangement of hydrophilic and hydrophobic amino acids into an amphipathic helix and, thus in formation of a 3-dimensional structure that allows membrane insertion.

The mode of action of bacteriocins can be generally grouped into two classes. In the first class mode of action, the bacteriocins act by membrane disruptive following barrel stave, toroidal, micellar or carpet aggregate mechanisms (Figure 2.5). Second is the non-membrane disruptive class which targets the intracellular components (Giuliani *et al*., 2007). The membrane disruptive mechanism will cause the efflux of intracellular contents by disrupting the cytoplasmic membrane. In the barrel-stave model, a number of channel-forming peptides are arranged in a barrel-like ring around an aqueous pore. The peptide which is most likely in monomeric structure will associate with the surface of the membrane prior to insertion. Then the hydrophobic region of the bound peptides is inserted into the targeted membrane to a depth that varies depend on the hydrophobicity of the membrane outer leaflet. When the bound peptide reaches a certain threshold concentration, the peptide monomers then self-aggregate and are entered deeper into the hydrophobic membrane core. The hydrophobic regions of the peptides then align and face the hydrophobic lipid core region whereas their hydrophilic faces create the interior region of a water-filled pore (Mihajlovic & Lazaridis, 2010; Ramamoorthy *et al.*, 2010).

In carpet mechanism, the antimicrobial peptides are accumulate on the surface membrane where they are electrostatically bound to the anionic phospholipid head groups and carpeting the membrane surface at several sites. When the concentration of

the peptides reach a threshold, membrane disruption happens in a detergent-like pattern that does not involve pore formation. The peptides do not necessarily insert into the hydrophobic core (Mihajlovic & Lazaridis, 2010). This model explains how cecropin P1 disrupt membranes (Gazit *et al.*, 1996).

In toroidal model, antimicrobial peptides bind to the phospholipid head group of the membrane bilayer and induce a high-curvature fold in the lipid bilayer, enabling the two leaflets of the bilayer to communicate directly at a torus lined by the leaflets (Park *et al.*, 2006). This is different from the barrel-stave model where the antimicrobial peptides are collaborated onto the phospholipid head groups even when they are perpendicularly inserted into the lipid bilayer.

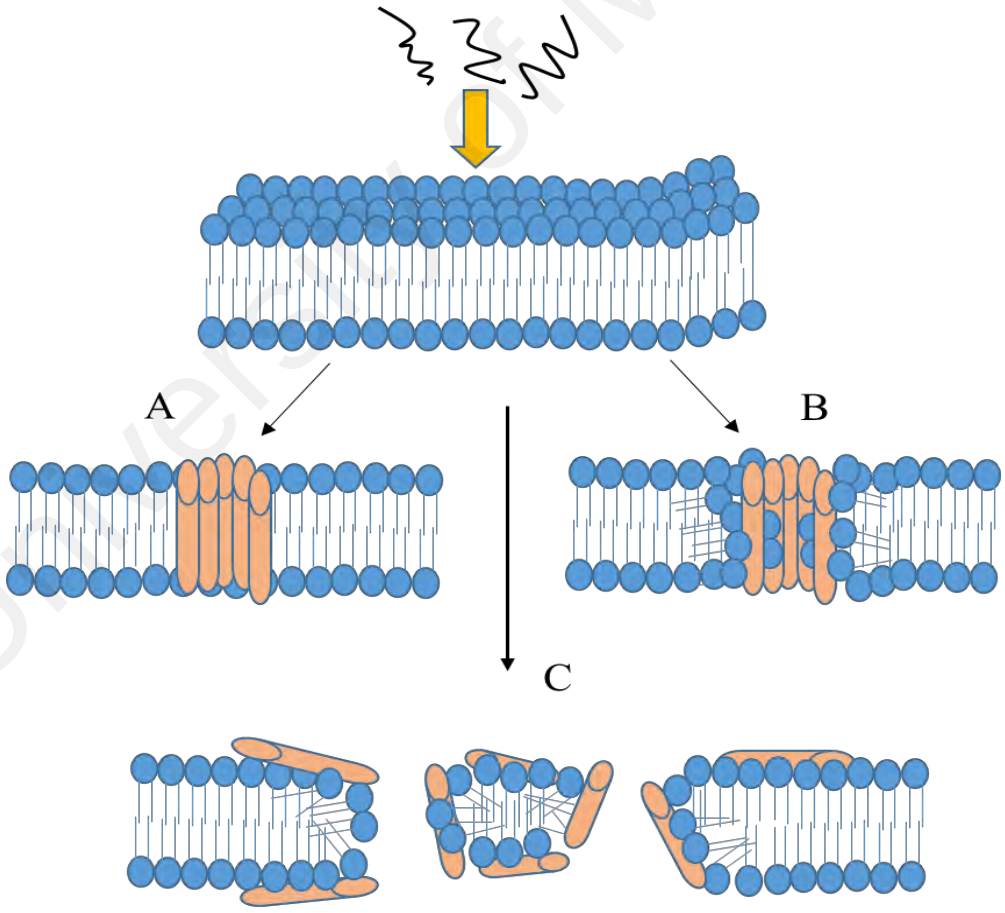


Figure 2.5: Three typical modes of action of antimicrobial peptides against cytoplasmic membranes. (A) barrel-stave model; (B) toroidal pore model; (C) carpet model. Adapted from Park *et al*. (2001).

The range of killing other bacteria by bacteriocins can vary significantly, from relatively narrow as lactococcins A, B, and M that are found to kill only *Lactococcus* to extraordinarily broad (Martínez-Cuesta *et al*., 2006). For example, some lantibiotics like nisin A and mutacin B-Ny266 have been shown to kill a wide range of genera including *Bacillus*, *Clostridium*, *Corynebacterium*, *Gardnerella*, *Mycobacterium*, *Listeria*, *Micrococcus*, *Propionibacterium*, *Staphylococcus* and *Actinomyces*. They also kill other lactic acid bacteria such as *Enterococcus*, *Lactococcus* and *Streptococcus* (Mota-Meira *et al*., 2005). Most of the bacteriocins in class IIa have relatively narrow killing spectra compared to those in class I where most only inhibit closely related Gram-positive bacteria (Heng *et al*., 2007).

Nisin and some other bacteriocins target on the lipid II which is an important component for cell wall synthesis. In this mode of action, the bacteriocin bind to the lipid II and hence prevent the docking molecule forming a nisin and lipid II stoichiometry and subsequently leading to pore formation (Christ *et al*., 2007; de Kruijff *et al*., 2008). Figure 2.6 shows the amino acid sequences of nisin and its different modes of action namely inhibition of cell wall biosynthesis, pore formation in bacterial membranes, inhibition of spore outgrowth, lipid II dependent pore formation and activation of autolytic enzyme.

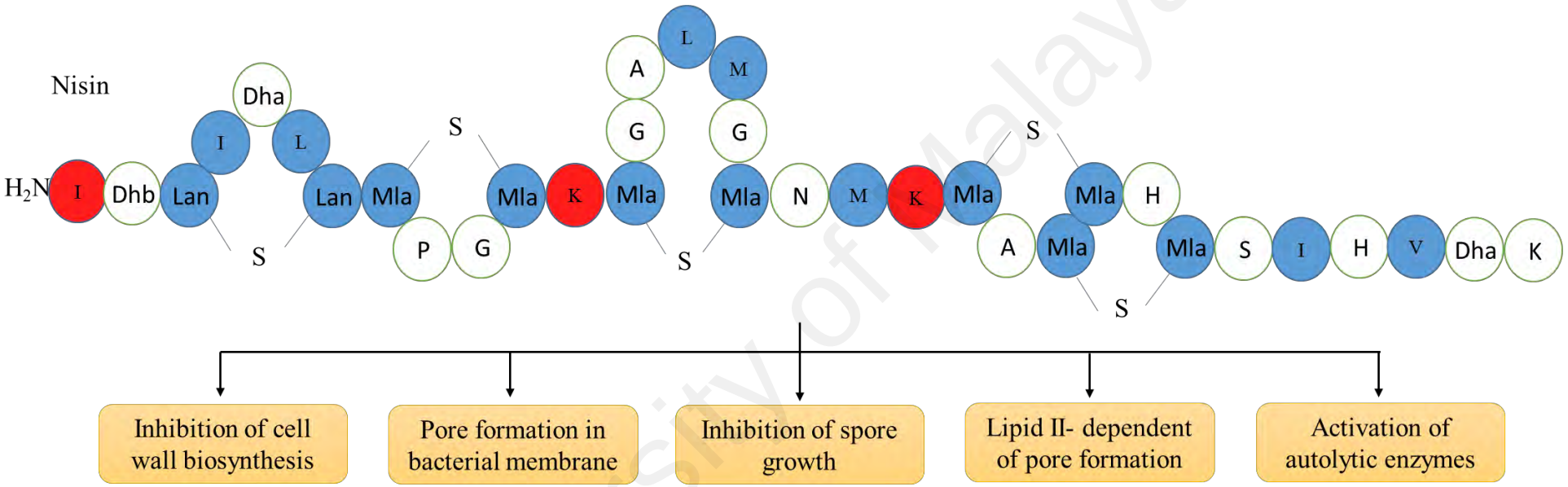


Figure 2.6: Nisin and its variable modes of action: residues showed in red have a positive net charge; those in blue are hydrophobic; amino terminus is indicated with NH_2. Dha, dehydroalanine; Dhb, dehydrobutyrine; Lan, lanthionine; Mla, methyllanthionine; S, thioether bridge (Peschel & Sahl, 2006).

Figure 2.7 summarises the basis of mode of action by different classes of bacteriocins.

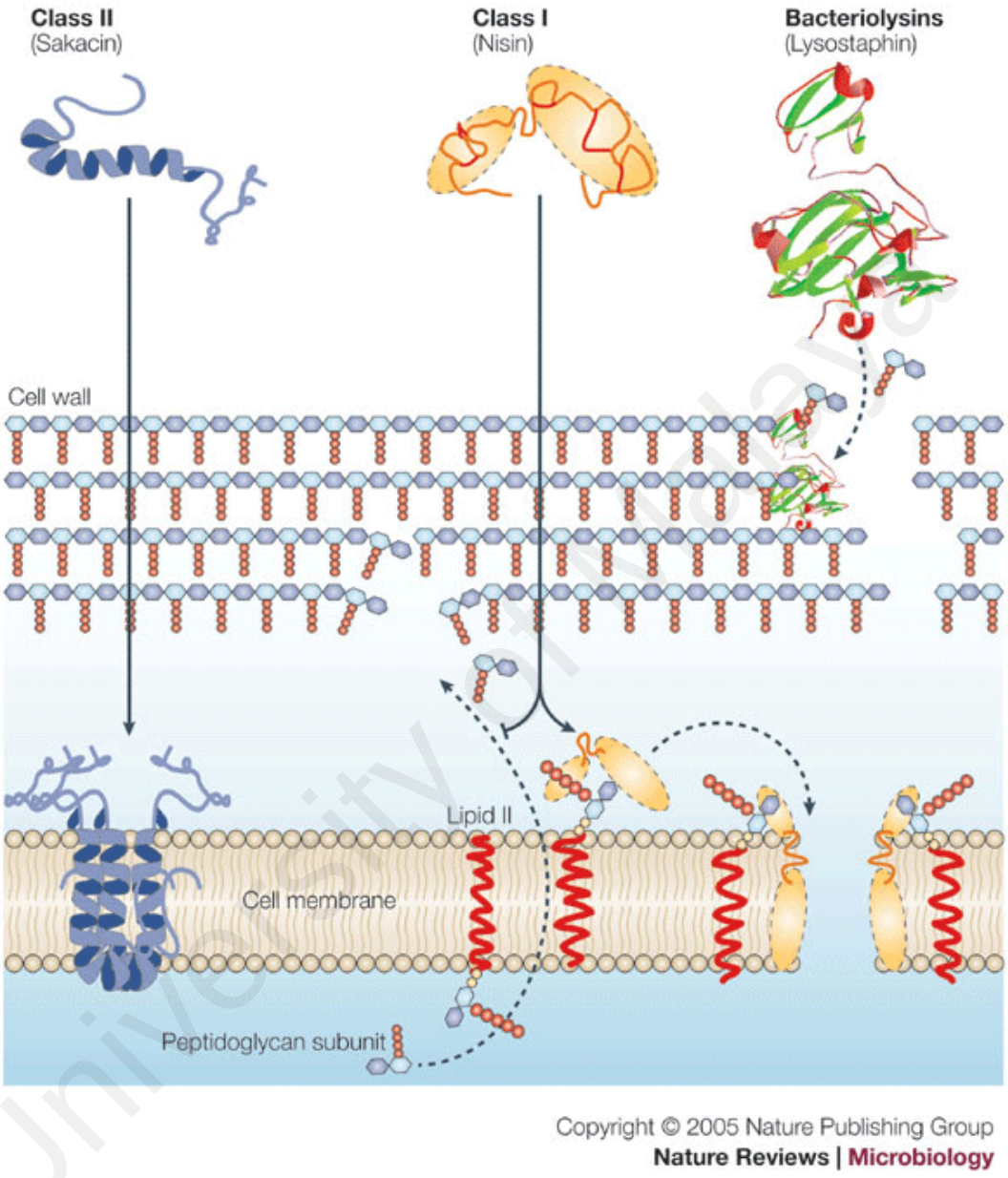


Figure 2.7: The basis of mode of action of different classes of bacteriocins. Copied with permission from Cotter *et al*. (2005).

Some of the class I (or lantibiotic) bacteriocins, for example nisin, was shown to have dual modes of action. Nisin can bind to lipid II which is the main transporter of peptidoglycan subunits from the cytoplasm to the cell wall, and subsequently inhibit

correct cell wall synthesis, leading to cell death. In another mode of action, they can use lipid II as a docking molecule to initiate membrane insertion on the target and pore formation which can lead to rapid cell death. The dual modes of actions are assigned to specific regions of the active peptide. The N-terminal rings A and B form a binding pocket called pyrophosphate cage that allows specific interaction and binding of nisin to the pyrophosphate moiety of lipid I or lipid II. As shown in NMR spectra, the binding of lipid II depends on five hydrogen bonds presence between the nisin's amides backbone and the pyrophosphate linkage group of lipid II (Hsu *et al*., 2004). The two-peptide lantibiotics have a dual mode of action like in nisin case, but here it depends on two synergistically acting peptides (Morgan *et al*., 2005). For example, a two peptides bacteriocin lacticin 3147 have these dual activities distributed across two peptides. It was found that the lipid II binding will result in a conformational change within the α-peptide leading to enhance complex formation with the second peptide (Wiedemann *et al*., 2006). Besides, proline residue of the β-peptide motif is apparently involved in complex formation because mutations in this site will strongly affect the two peptide synergism as reviewed by Lawton *et al*. (2007). The formation of the complex itself strengthens the binding of the α-peptide to lipid II and leads to a deeper insertion of the complex into the membrane. At the same time this complex arrangement also allows the β-peptide to adopt a trans-bilayer orientation and results in pore formation. However, these pores were found to be significantly smaller (0.6 nm) than those formed by nisin (Bonelli *et al*., 2006). On the other hand, in the case of mersacidin, it is able to bind to lipid II but does not form any pores.

In general, the amphiphilic helical structure of the class II bacteriocins helps them to get inserted into the membrane of the target cell and subsequently lead to depolarisation and finally cell death (Brashears *et al*., 1998; Drider *et al*., 2006). The initial interaction takes place when the heads of the hydrophilic N-terminus of the

peptides bind to anionic phospholipids on the membranes. The C-terminus of the peptide which is more hydrophobic than the N-terminal is thought to be involved in hydrophobic interactions with the membrane (Alonso *et al*., 2000).

Large bacteriolytic proteins (bacteriolysins), such as lysostaphin can act directly on the cell wall of Gram-positive bacteria, causing death and lysis of the target cell (Cotter *et al*., 2005; Cotter *et al*., 2012). Interestingly, for some lantibiotics (e.g. subtilin) an inhibiting effect on the outgrowth of endospores is reported (Oman & van der Donk, 2009). A recent publication dealing with the molecular basis of the antisporal effect of nisin demonstrated that this effect also depends on the membrane and lipid II binding activity of bacteriocin. By preventing the establishment of a membrane potential and cell wall synthesis, the spore outgrowth is inhibited at the stage of spore germination (Gut *et al*., 2011).

The mechanisms within the targeted bacterial cell include inhibition of gene expression and protein production. The antimicrobial peptides bound to the component involved in DNA synthesis after entering the bacterial cell and prevent DNA replication of the bacteria and eventually lead to death of the target bacteria. For example, microcin B17 target DNA gyrase, an enzyme that is needed in the ATP-dependent negative super-coiling of double stranded DNA (Reece *et al*., 1991). Hence, the DNA replication of the bacteria will be affected. Cellular structures and enzymes are primarily made of proteins. Protein synthesis is an essential and important process necessary for all bacteria to multiply and survive. Therefore, several types of antibacterial substances target the bacterial protein synthesis. They act by binding to either the 30S or 50S subunits of the intracellular ribosomes. The binding to the ribosomal subunits then results in the disruption of the normal cellular metabolism of the bacteria and consequently leads to the death of the organism or the inhibition of its growth and

multiplication. For example thiazomycin, ocathiacins, thiostrepton and some other thiopeptides act by targeting the bacterial ribosome and binding the 23s rRNA region of the 50S ribosomal subunit (Bagley *et al*., 2005). The mode of action of bottromycins is by blocking aminoacyl-tRNA binding to the 50S ribosome (Kobayashi *et al*., 2010). Other thiopeptides such as GE2270 A acts by binding to the bacterial chaperone elongation factor Tu (EF-Tu) to inhibit protein synthesis (Bagley *et al*., 2005).

2.7 Cow milk as a growth medium of LAB

Cow milk is sterile while it is produced in a cow's udder. However, during milking, the cow milk is contaminated with natural microflora present on the cow. The three main sources that usually contaminate the milk are within the udder, the exterior of the udder, the surfaces of the milk processing and storage equipment (Godefay & Molla, 2000). This is because milk contains highly nutritional components which can support the growth of different kinds of bacteria. Some of these may cause spoilage and diseases to human while some are harmless and may give beneficial effect to human. The good bacteria present in the milk are mainly lactic acid bacteria and its presence can be easily detected after fermentation of milk (Furet *et al*., 2004).

Past research publications have shown that milk consists of a wide range of bioactive compounds. These compounds originally from the mother are transferred to the young in order to give nutrients and immunity to the young. Besides, there are also a lot of bioactive peptides present in the milk and they are believed to possess very important biological functions including antimicrobial, antihypertensive, antioxidant, anticytotoxic, immune modulatory, opioid, and mineral-carrying activities. Most of the bioactive peptides are only released during milk fermentation and hence the lactic acid bacteria in the milk are presumed to generate these bioactive compounds (Armand & Flouris, 2013; Hajirostamloo, 2010; Nagpal *et al*., 2011)

Pasteurisation is a term derived from the name of Louis Pasteur. He discovered that heating liquids especially wines at low temperatures (60°C) will improve the storage period. This low temperature heat treatment destroys wine spoilage organisms, while retaining its original characteristics (Holsinger *et al*., 1997). Pasteurisation is defined as a heating process with temperatures of not less than 142°F (61.1°C) for 30 min in approved equipment (Westhoff, 1978). The earliest pasteurisation condition is known as flash pasteurisation. The milk is heated to 155 to 178°F (68.3 to 81°C) for 16 seconds followed by cooling (Annand, 1967). Pasteurisation conditions were adjusted to 143°F (61.7°C) for 30 minutes or 160°F (71.1°C) for 15 seconds to inactivate the growth of *Mycobacterium bovis* which caused tuberculosis (Westhoff, 1978). In 1957, these pasteurisation conditions were proven to be insufficient for to kill *Coxiella burnetii* which causes Q fever in humans (Enright *et al*., 1957). New pasteurisation conditions of 145°F (62.8°C) for 30 minutes for a batch process or 161°F (71.7°C) for 15 sec for a continuous process are then adopted in order to inactivate *Coxiella burnetii*. These conditions are still in use today. However, the pasteurisation process will not kill the heat resistant endospores produced by *Bacillus cereus* although the vegetative cells are rapidly killed. *B. cereus* can be considered as a major spoilage organism in the dairy industry and caused losses to the industry (Holsinger *et al*., 1997).

2.8 Choice of test microorganisms

The test microorganisms used in this study are Gram-positive and Gram-negative bacteria namely *Bacillus cereus* ATCC14579, *Escherichia coli* UT181, *Micrococcus luteus* ATCC10240, *Pseudomonas aeruginosa* PA7 *and Staphylococcus aureus* RF122. According to the European Food Safety Authority (2005), *B. cereus* causes two kinds of foodborne diseases namely an emetic intoxication (vomiting) due to the ingestion of a toxin (cereulide) pre-formed in the food and a diarrhoeal infection due

to the ingestion of bacterial cells or spores which produce enterotoxins in the small intestine. The occurrence of *B. cereus* in milk product was frequently reported due to its psychotropic and spore-forming characteristics (Bartoszewicz *et al*., 2008; Holsinger *et al*., 1997; Larsen & Jørgensen, 1997). *B. cereus* can survive even after pasteurisation affecting the shelf-life of pasteurised milk. Consumption of milk contaminated with *B. cereus* can cause food poisoning (Andersson *et al*., 1995; Christiansson *et al*., 1999). *E.coli* is a normal microflora in the gastrointestinal tract. However, some strains of *E.coli* are able to produce a toxin that causes serious infections. The symptoms may range from mild to severe bloody diarrhoea. Surveillance data indicates resistance that occurred in *E. coli* is consistently highest for antibiotics that have been in use the longest time in human and veterinary medicine (Tadesse *et al*., 2012). The past two decades have witnessed major increases in the emergence and spread of multidrug-resistant bacteria. Besides, reports on resistance to newer compounds such as fluoroquinolones and certain cephalosporins also increase (Levy & Marshall, 2004). In parts of Southeast Asia and China, 60 to 70 % isolates of *E. coli* are found resistant to fluoroquinolones (Wang *et al*., 2001). This report is worrisome because the trend jeopardizes the value of this drug family. *Micrococcus luteus* was a Gram- positive, coccal shaped bacteria and was known to caused food spoilage and food poisoning (Agaoglu *et al*., 2007). *P. aeruginosa* causes persistent and life-threatening infections in patients with cystic fibrosis despite highly aggressive antimicrobial therapy. The presence of it in food can cause food spoilage and reduce the shelf-life of food (Gram *et al*., 2002; Tajkarimi *et al*., 2010). *S. aureus* can produce enterotoxins that cause food poisoning if ingested and affect human health (Stewart & Hocking, 2003).

2.9 Applications of bacteriocins in food

Microbes and their natural products have been used in food preservation for a very long time. Their main advantage over chemical preservatives is they are able to preserve without changing the sensory qualities of the food while adhering to the demand for natural preservatives. Application of bacteriocins in food industry may be beneficial in several ways (Galvez *et al*., 2007): (1) decrease of risks in food poisoning, (2) reduce cross-contamination in the food chain, (3) enhance the shelf-life of food products, (4) protect food during temperature-abuse episodes, (5) reduce economic losses due to food spoilage, (6) reduce the usage of added chemical preservatives, (7) reduce the usage of physical treatments and thereby achieving a better preservation of the food nutritional value and possibly reduce the processing cost, (8) has potential to provide alternative preservation barriers for "novel" foods which are less souring, with a lower salt content, and with a higher water content and (9) possibly satisfy the demands of consumers for foods that are lightly preserved, fresh-tasting, and ready to eat. There may also be a potential market for bacteriocins as natural substitutes for chemical preservatives and also in the preservation of functional foods and nutraceuticals (Robertson *et al*., 2003).

Bacteriocins can be introduced into food by either in peptide form or together with the producer as a probiotic. In solely peptide form without the producer, the bacteriocin is obtained by growing the producer strain in a suitable growth medium followed by extraction of the bacteriocin and final step with lyophilisation or spray-drying to preserve the bacteriocin in dry powder form. Commercial preparations such as Nisaplin™, Alta™ products or Microgard™ are some examples. Other bacteriocins such as variacin from *Kokuria varians*, lacticin 3147 or enterocin AS-48 have also been obtained in powder form (Ananou *et al*., 2010; Morgan *et al*., 1999; O'mahony *et al*.,

2001). The second method is to add bacteriocin producer strains as starter cultures. When the bacteria propagate in the food, the bacteriocin will be produced as a by-product. In non-fermented food products, they can also be applied as bioprotective cultures provided that they do not have adverse effects on the foods. Table 2.4 summarises the primary application of LAB.

Table 2.4: Primary application of LAB

Strain name	**Primary applications**	**References**
Enterococcus faecium M3K31	Antilisterial	Arbulu *et al*., 2015
Enterococcus faecium Y31	traditional Chinese fermented foods	Liu *et al*., 2015
Lactobacillus acidophilus NCFM	Probiotics	Altermann *et al*., 2005
Lactobacillus casei BL23	Probiotics, milk fermentation, and flavour development of cheese	Alcantara *et al*., 2011
Lactobacillus delbrueckii ssp. *bulgaricus* ATCC 11842	Yogurt fermentation	Chervaux *et al*., 2000
Lactobacillus fermentum IFO 3956	Probiotics	Morita *et al*., 2008
Lactobacillus gasseri ATCC 33323	Probiotics	Azcarate-Peril *et al*., 2008
Lactobacillus helveticus DPC 4571	Cheese manufacture	Callanan *et al*., 2008
Lactobacillus johnsonii NCC 533	Probiotics	Pridmore *et al*., 2004
Lactobacillus plantarum WCFS1	Food preservation, feed raw materials such as milk, meat, vegetables, probiotics	Kleerebezem *et al*., 2003
Lactobacillus plantarum ZJ316	Kimchi fermentation, anti-Salmonella	Jiang *et al*., 2016
Lactobacillus reuteri F275	Probiotics	Morita *et al*., 2008

Table 2.4 continued

Lactobacillus reuteri JCM 1112	Probiotics	Morita *et al*., 2008
Lactobacillus sakei ssp. *sakei* 23K	Food preservation, meat fermentation	Chaillou *et al*., 2005
Lactobacillus salivarius ssp. *salivarius* UCC118	Probiotics	Claesson *et al*., 2006
Lactococcus lactis ssp. *cremoris* MG1363	Dairy food fermentation, model strain	Wegmann *et al*., 2007
Lactococcus lactis ssp. cremoris SK11	Dairy food fermentation, model strain	Siezen *et al*., 2005
Lactococcus lactis ssp. l*actis* IL1403	Dairy food fermentation, model strain	Bolotin *et al*., 2001
Leuconostoc citreum KM20	Kimchi fermentation	Kim *et al*., 2008
Oenococcus oeni PSU-1	Wine making	Mills *et al*., 2005
Streptococcus salivarius K12	Probiotic targeting the oral cavity	Burton *et al*., 2006
Streptococcus thermophilus CNRZ1066	Yogurt and cheese fermentation	Bolotin *et al*., 2004
Streptococcus thermophilus LMG 18311	Yogurt and cheese fermentation	Bolotin *et al*., 2004

Adapted and modified from Zhu *et al*. (2009).

There is intriguing evidence to show that bacteriocins may function in diffent mechanisms within the gastrointestinal tract. Bacteriocins may act as colonising peptides and facilitate the introduction of a producer into an established microbial ecological niche. They may function as killing peptides and directly inhibit the invasion of competing strains or pathogens, or they may function as signalling peptides by modulating the composition of the microbiota and thereby influence the host immune system (Dobson *et al*., 2012). Figure 2.8 summarises the mechanism of action of bacteriocin as a probiotic.

Bacteriocin aids in the GI tract for colonizing bacteria as shown by evidence of *E.coli* producing colicin colonizing in the large intestine of streptomycin-treated mice for an extended time compared to *E.coli* that did not produce colicin (Gillor *et al*., 2009). Besides, bacteriocin produced by *L. salivarius* UCC118 acted as a killing peptide and protected mice against *L. monocytogenes*. The finding showed that *L. salivarius* UCC118 produced two- peptide bacteriocin Abp118 that prevented infection in mice while non-bacteriocin producing strains failed to protect the mice from infection (Millette *et al*., 2008). On the other hand, bacterial communication via extracellular diffusible signaling molecules (quorum sensing) allows populations of bacteria to synchronize group behavior and thereby facilitate coordinated multicellular functionality (Gillor & Ghazaryan, 2007).

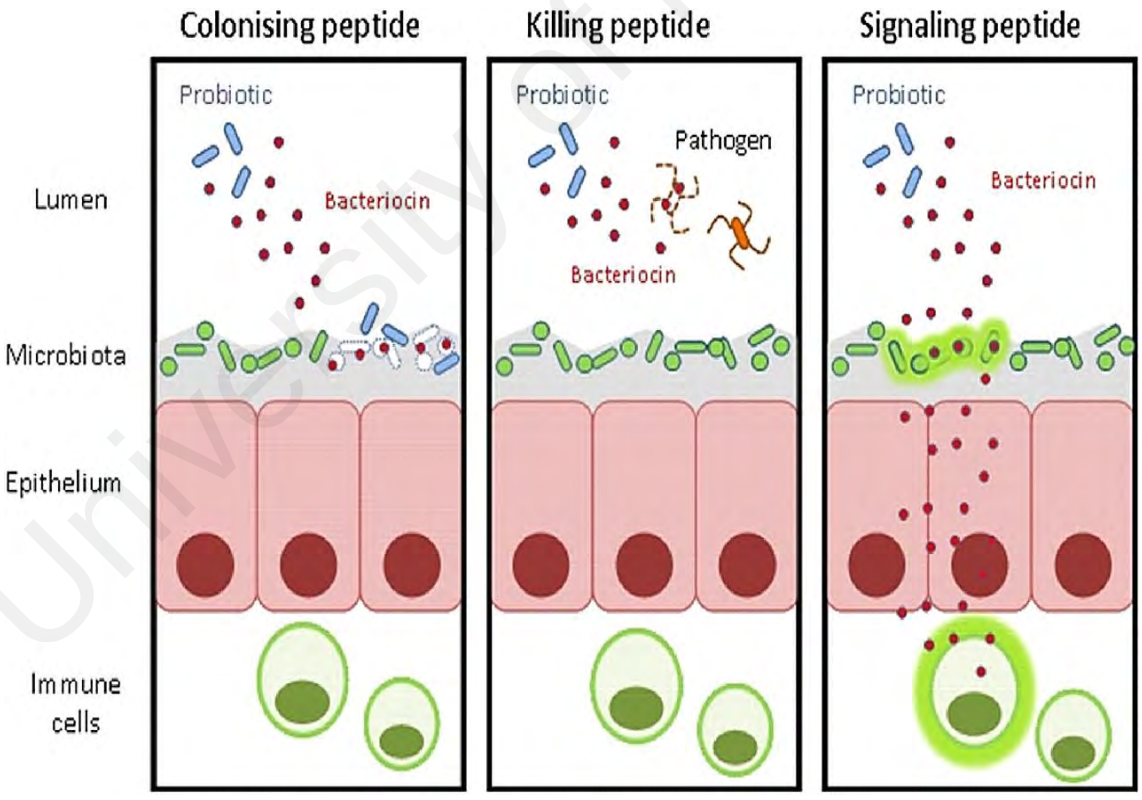


Figure 2.8: Mechanisms via which bacteriocin production could contribute to probiotic functionality. Copied with permission from Dobson *et al*. (2012).

2.10 Background of lactic acid bacteria

2.10.1 *Lactococcus lactis*

Lactococcus lactis can be considered the first in the LAB group that has been used in food preservation. It is well known as a producer of nisin which can inhibit a wide range of Gram-positive bacteria including endospore-former namely *Bacillus* and *Clostridium* (Thomas *et al*., 2002). For the LAB bacteriocins identified to date, nisin which is a lantibiotic (class I bacteriocin) has been the most extensively investigated (Saraiva *et al*., 2014). Class I lantibiotic also includes lactococcal bacteriocins, lacticin 481 and lacticin 3147. Nisin is the only bacteriocin approved for use in food preservation by the European Union and the Food and Drug Administration (FDA). It is a 34 amino acids peptide with a wide inhibitory spectrum against Gram-positive bacteria in favourable conditions (Guinane *et al*., 2005). Nisin has been used in a variety of applications because of its broad spectrum of activity. Nisin is frequently utilised to control spoilage and pathogenic bacteria. It is easily broken down by gut proteases and does not cause harm to the gut microflora (Mozzi *et al*., 2010). There are six variants of nisin known until now (Khan & Oh, 2016). Nisin A is the originally isolated form of nisin and five other natural variants include nisin Z, Q, F, U and U2 with up to a difference by 10 amino acids from a total of 34 amino acids in nisin A. Nisins A, Z, F and Q are produced by *Lactococcus lactis* while nisins U and U2 are produced by *Streptococcus* sp (Perin & Nero, 2014; Piper *et al*., 2011). Nisin was first marketed in England in 1953 and since then has been approved for use in over 48 countries. In 1969, it was assessed to be safe to be used as a food additive by the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additives (Ross *et al*., 2002). In 1988, nisin has attained GRAS (generally recognised as safe) status and is approved as a food additive in the United States (Food

& Administration, 1988). The chronological development of nisin is shown in Figure 2.9.

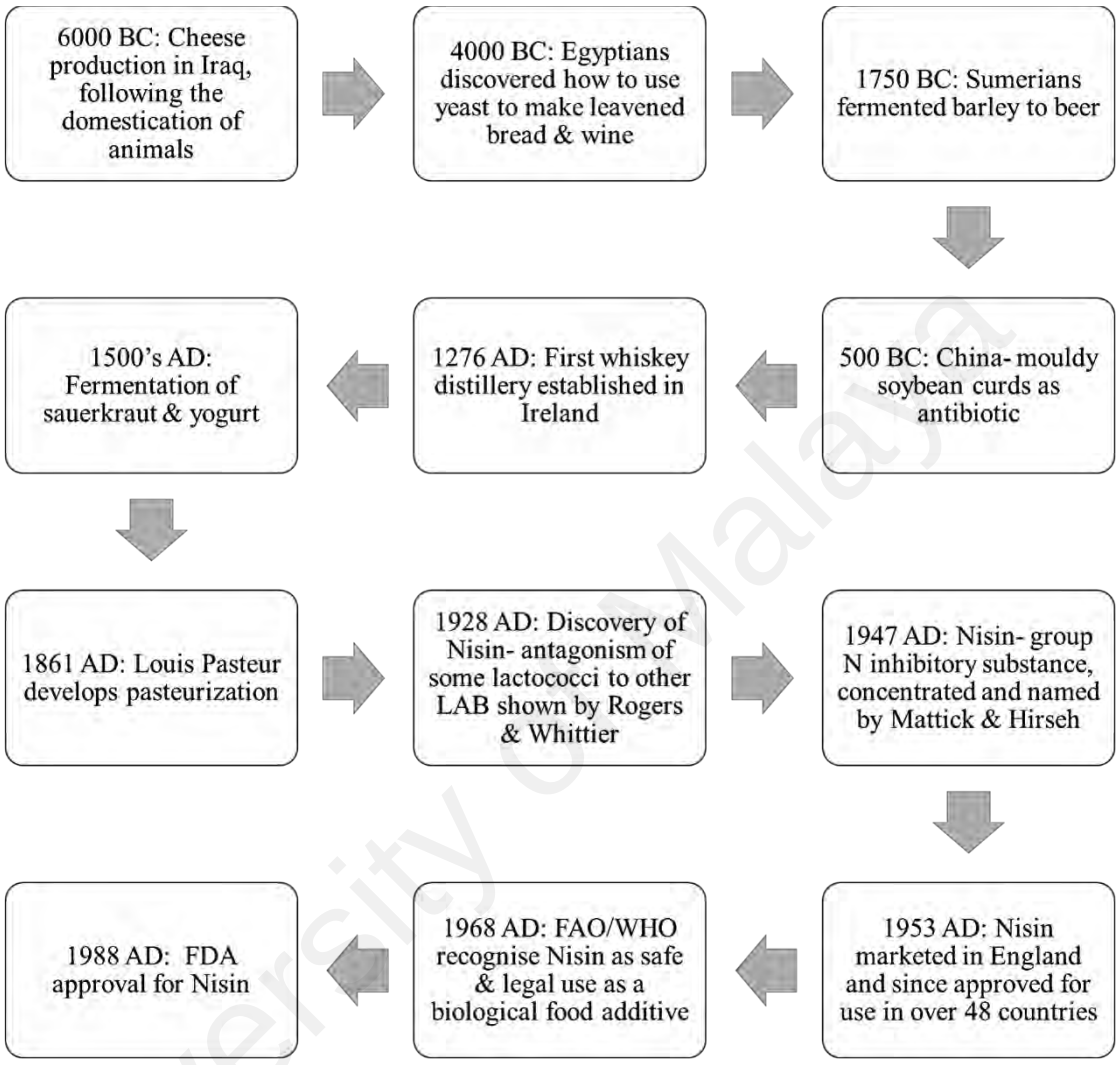


Figure 2.9: Schematic diagram of fermentation development and use of nisin in food industry. Adapted from Ross *et al*. (2002).

2.10.2 *Weissella confusa*

Weisella is phylogenetically related to the genera *Leuconostoc* and *Oenococcus* and the phylogentic status of *Weissella* was clarified in 1990 by using data from 16S and 23S rRNA gene sequencing (Collins *et al*., 1991). The data showed that *L. paramesenteroides* is phylogenetically different from *L. mesenteroides* and forms a natural grouping with certain heterofermentative *Lactobacillus* species which include *L. confusus*, *L. kandleri, L. minor* and *L. viridescens*. In a study of *Leuconostoc*-like organisms isolated from fermented sausages produced in Greece, the phylogenetic status of this group of microorganisms was further assessed (Collins *et al*., 1993). An in-depth study based on phenotypic, biochemical and 16S rRNA gene analysis allowed the differentiation of the new genus *Weissella* and the re-assignment of the species previously grouped in the genus *Lactobacillus*. The genus *Weissella* was named after the German microbiologist Norbert Weiss, who is well known by his contributions in the field of LAB research (Collins *et al*., 1993). Currently, there are only 19 validated species in the genus *Weissella* and they include *W. confusa*, *W. halotolerans*, *W. hellenica*, *W. kandleri*, *W. minor*, *W. thailandensis*, *W. paramesenteroides*, *W. viridescens*, *W. cibaria* and several others with 93.9 to 99.1 % of 16S sequence similarity (Björkroth & Holzapfel, 2006). The phylogenetic tree based on 16S rRNA sequence of genus *Weissella* is shown in Figure 2.10. *Weissella confusa* has been isolated from different sources including sugar cane, carrot juice, milk, fermented foods, fermented beverages and also human and animal samples. Apart from lactic acid production, *Weissella confusa* also produces exopolysaccharide from sucrose. This exopolysaccharide has been reported to produce antifungal activity and highlight the use of *Weissella confusa* in biotechnology applications (Amari *et al*., 2012). The application of exopolysaccharides is raising interest due to their prebiotic potential as they may decrease the risk of infections and diarrhea, increase bowel function and metabolism

through the gastrointestinal tract and stimulate the growth of resilient beneficial bacteria, mainly the *Bifidobacteria* (Rastall & Gibson, 2015).

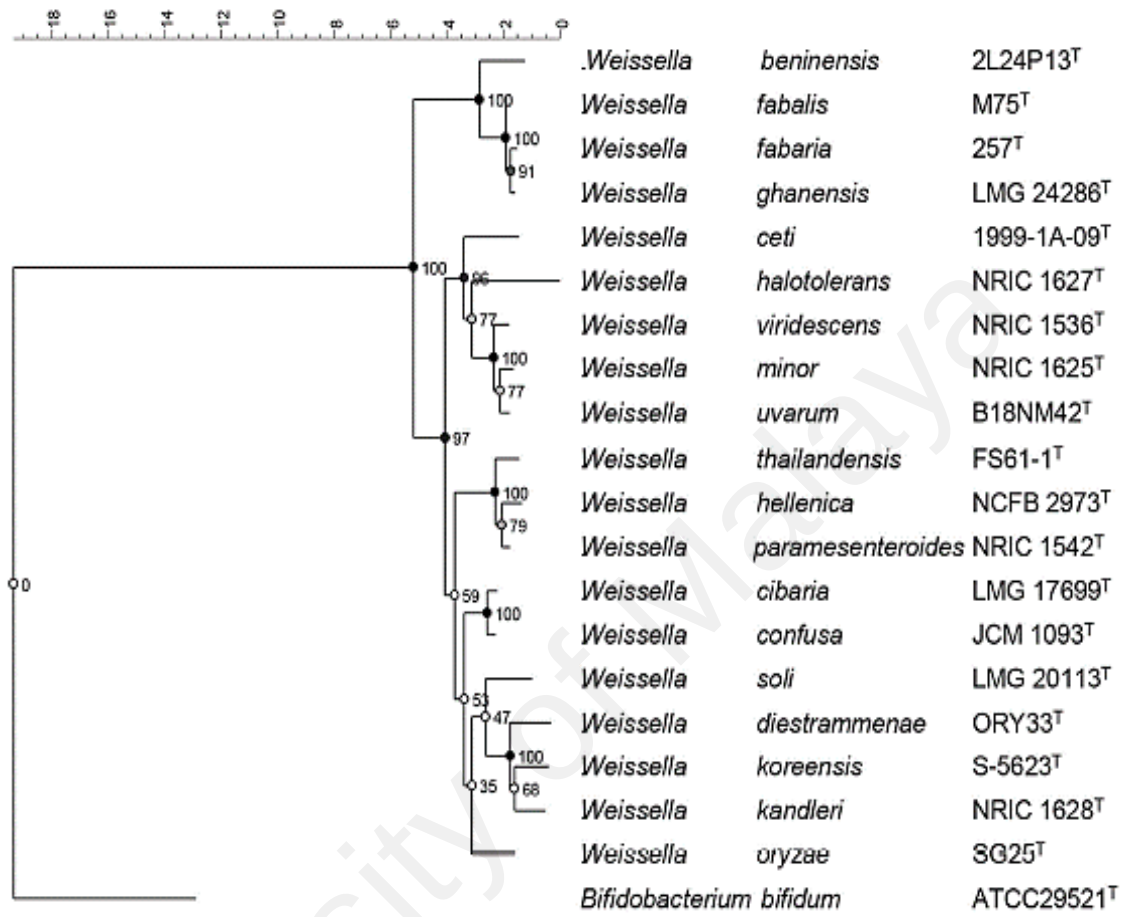


Figure 2.10: Phylogenetic tree based on 16S rRNA sequence with *Bifidobacterium bifidum* used as an outgroup sequence. Adapted from Fusco *et al*. (2015).

In recent studies, *Weissella* strains were screened for their antimicrobial activity and six bacteriocins (Table 2.5) have been reported from *W. cibaria*, *W. paramesenteroides* and *W. hellenica*. Among these bacteriocin, weissellin A which is a listericidal bacteriocin was further investigated for its technological application in fermented sausages (Papagianni, 2012; Papagianni & Papamichael, 2012; Papagianni & Sergelidis, 2013), while the bacteriocinogenic *W. hellenica* strain D1501was successfully used to enhance the shelf-life of tofu (Chen, *et al*., 2014).

Table 2.5: Bacteriocin produced by *Weissella* strains

Name	Class	Producer organisms	References
Weissellicin 110	Unclassified	*W. cibaria* 110	Srionnual *et al*., 2007
Weissellin A	Class IIA	*W. paramesenteroides* DX	Papagianni & Papamichael, 2011
Weissellicin L	Unclassified	*W. hellenica* 4-7	Leong *et al*., 2013
Weissellicin D	Unclassified	*W. hellenica* D1501	Chen, *et al*., 2014
Weissellicin M **Weissellicin Y**	Unclassified Unclassified	*W. hellenica* QU 13	Masuda *et al*., 2012

2.10.3 *Enterococcus faecium*

Enterococci are found in many food products such as dairy products, fermented sausages and olives (Omar *et al*., 2004). *Enterococci* were originally classified as *Streptococci*. However, with the development of genetic studies based on DNA–DNA and DNA–RNA hybridization, the new generic name *Enterococcus* was given as reviewed by Schleifer & Kilpper-Bälz (1987). *Enterococci* are now being used as probiotics but at the same time they are among the most common nosocomial pathogens associated with many human infections. They are known to cause endocarditis, bacteremia, and infections of the central nervous system, urinary tract, intra-abdominal and pelvic infections, as well as cause multiple antibiotic resistances. Furthermore, the number of vancomycin-resistant *Enterococci* (VRE) is increasing in numbers. However the *Enterococci* that possess virulence genes are only isolated from patients (Foulquié Moreno *et al*., 2006; Franz *et al*., 2003).

A study with 139 strains of *E. faecium* and *E. faecalis* from canine faeces, boiler meat samples, swine faeces, wild waterfowl faeces, and human faeces was carried out to check for their capabilities to produce enterocins and 51 % of the isolates were able to produce enterocins and 46 % of them were able to produce more than one enterocin (Theppangna *et al*., 2007). The contribution of *Enterococci* to the organoleptic sensory properties of fermented food products and their common ability to produce bacteriocins especially enterocins may be of interest in food technology. *Enterococci* play a fundamental role in the ripening of dairy products, probably through lipolysis, proteolysis, exopolysaccharide production and citrate breakdown, thus conferring a unique taste and flavour to dairy foods (Manolopoulou *et al.* 2003; Yerlikaya & Akbulut, 2011). Most the enterocin produced by *E. faecium* are found within class IIa and class IIb bacteriocins. Class II bacteriocins are heat stable, small, cationic and hydrophobic peptides which do not undergo post-translation modification (Rodrıguez *et al*., 2003). Examples of enterocin are listed in the Table 2.6.

Table 2.6: Enterocins produced by *Enterococcus faecium*

Class IIa	**Producer strain**	**M.W (Da)**	**Amino acid sequence of the purified enterocin**	**pI**	**Reference**
Enterocin A	*E. faecium* CTC492	4833	TTHSGKYYGNGVYCTK NKCTVDWAKATTCIAG MSIGGFLGGAIPGKC	9	Aymerich *et al*., 1996
Enterocin CRL 35	*E. faecium* CRL 35	4290	KYYGNGVSCN KKGCSV DWGK AIGIIGNNSA AN LATGGAAG WKS	9.5	Farías *et al*., 1996
Enterocin P	*E. faecium* P13	4630	ATRSYGNGVYCNNSKC WVNWGEAKENIAGIVIS GWASGLAGMGH	8.3	Cintas *et al*., 1997
Bacteriocin RC714	*E. faecium* RC714	4937	ATYYGNGLYCNKEKCW VDWNQAKGEIGKIIVNG WVNHGPWAPR	9.3	Campo *et al*., 2001
Class IIb	**Producer strain**	**M.W (Da)**	**Amino acid sequence of the purified enterocin**	**pI**	**Reference**
Enterocin W beta	*E. faecalis NKR-4-1*	2855	VTTSIPCTVMVSAAVCP TLVCSNKCGGRG	8.34	Sawa *et al*., 2012
Class IIc	**Producer strain**	**M.W (Da)**	**Amino acid sequence of the purified enterocin**	**pI**	**Reference**
Enterocin B	*E. faecium* T136	5465	ENDHRMPNELNRPNNLS KGGAKCGAAIAGGLFGI PKGPLAWAAGLANVYS KCN	9.6	Casaus *et al*., 1997
Enterocin L50A	*E. faecium* L50	5190	MGAIAKLVAKFGWPIVK KYYKQIMQFIGEGWAIN KIIEWIKKHI	10.4	Cintas *et al*., 1998
Enterocin L50B	*E. faecium* L50	5178	MGAIAKLVTKFGWPLIK KFYKQIMQFIGQGWTID QIEKWLKRH	10.7	Cintas *et al*., 1998
Enterocin Q	*E. faecium* L50	3952	MNFLKNGIAKWMTGAE LQAYKKKYGCLPWEKIS C	9.7	Cintas *et al*., 2000

* Data adapted and modified from Moreno *et al*. (2006).

CHAPTER 3: METHODOLOGY

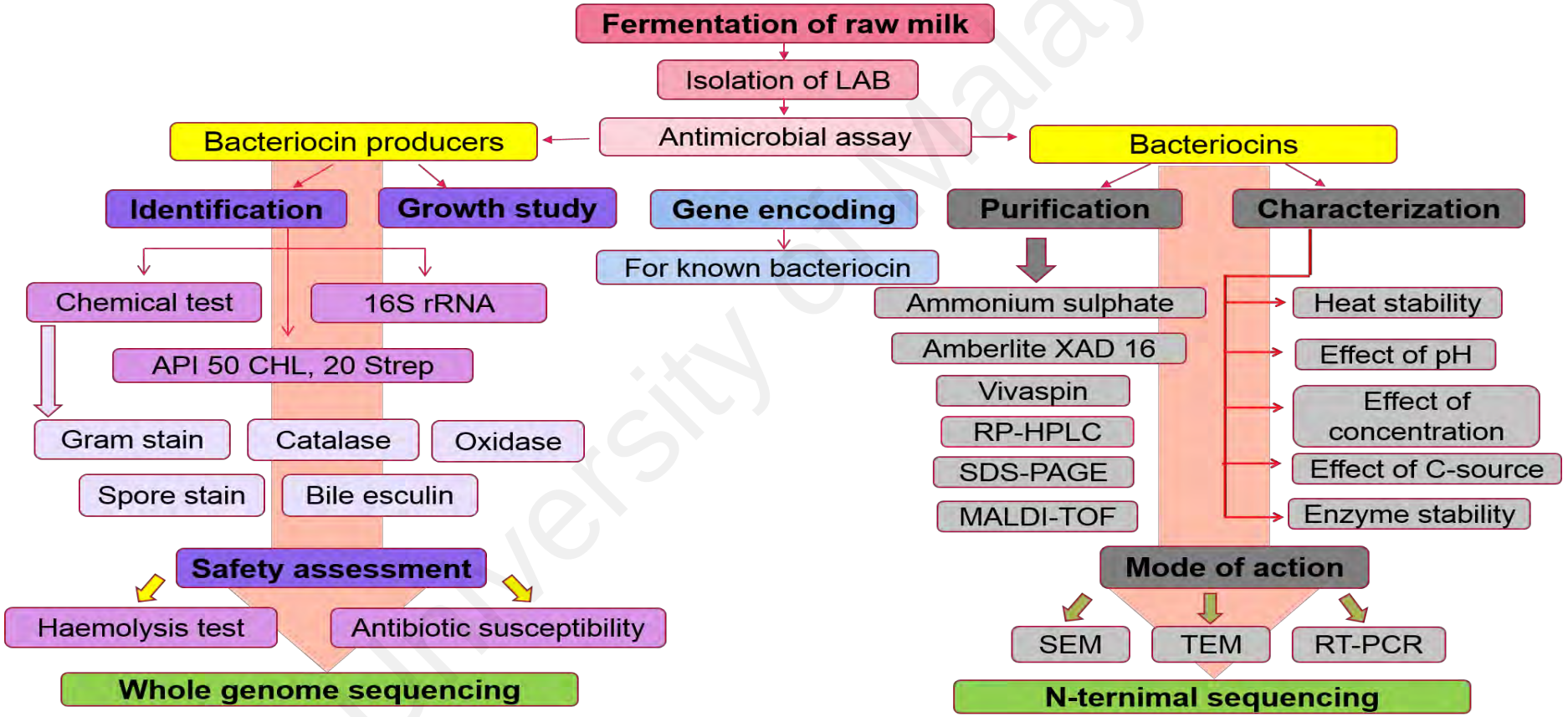


Figure 3.1: Schematic diagram of methodology.

Figure 3.1 outlines the methods used in this study. The study started with fermentation of raw cow milk. The lactic acid bacteria were then isolated from fermented milk. MRS media were used as a selective media to isolate the lactic acid bacteria. Then antimicrobial assay was done to select lactic acid bacteria with inhibition against the test bacteria. The experiment was then divided into two parts, the bacteriocin producers and the bacteriocins. The bacteriocin producers were identified through chemical test, API strips and 16S rRNA gene sequencing. Safety assessments were carried out to check for haemolysis and antibiotic supcetibility. The genes encoding known bacteriocin production in the bacteria were also screened. The bacteriocins were purified through several steps and the molecular weights determined by SDS-PAGE and MALDI-TOF. The bacteriocins were characterised based on their heat stability, pH stability, effect of different concentrations of target bacteria, effect of carbon source in the producer culture medium used to produce bacteriocins and enzyme stability test. The mode of action of the bacteriocins were then evaluated by SEM, TEM and RT-PCR with SYTOX green dye. Finally, whole genome sequencing for the bacteriocin producers and N-terminal sequencing for the bacteriocins were performed. All experiments were conducted in triplicates while the RT-PCR was done in quadruplicate.

3.1 Media preparation and sterilization

All media used in this study were prepared by autoclaving at 121 ºC for 15 minutes and cooled to 30 ºC prior to use. Solid media was prepared by adding 1 % (w/v) of agar powder to the media. Agar plates were prepared by pouring 20 ml of the autoclaved media into sterile petri dishes with size of 90 mm X 15 mm. After the agar had solidified, the plates were placed under the UV light in laminar flow for 15 minutes then transferred to incubator and the plates were incubated in inverted position overnight at 37 ºC to ensure no contamination before bacterial inoculation. Both liquid and agar media were stored at 4 ºC for subsequent use. De Man, Rogosa and Sharpe (MRS) media was used to culture lactic acid bacteria (de Man *et al.*, 1960), LAPTg media was used to grow *Enterococcus faecium* (Raibaud *et al.*, 1963) and Mueller-Hinton media to culture target bacteria (Baker *et al.*, 1985).

MRS media: 52.2 g of MRS media
1 L of distilled water

LAPTg media : 15 g of peptone
10 g of tryptone
10 g of yeast extract
10 g of glucose
1 ml of Tween- 80
1 L of distilled water

Mueller-Hinton media: 21 g of Mueller- Hinton media
1 L of distilled water

3.2 Indicator strains and culture conditions

The indicator bacteria used are *Bacillus cereus* ATCC14579, *Escherichia coli* UT181, *Listeria monocytogenes* NCTC10890, *Micrococcus luteus* ATCC10240, *Pseudomonas aeruginosa* PA7 and *Staphylococcus aureus* RF122. All strains were grown on Mueller-Hinton agar or broth (Merck, Germany) at 37 ºC for at least 18 hours. These cultures were obtained originally from ATCC and maintained in the Fermentation Laboratory, Microbiology Division, Institute of Biological Sciences, University of Malaya, Kuala Lumpur, Malaysia.

3.3 Antimicrobial assays by using well diffusion or spot on lawn methods

The test bacteria were grown on Mueller-Hinton broth at 37 ºC for 18 hours. The turbidity of the test bacteria was fixed at 0.1 value of optical density at 600 nm wavelength using GENESYS™ 20 visible spectrophotometer (Thermo Scientific). The test bacteria were first lawn grown on the Muller-Hinton agar (Merck, Germany) and allowed to dry. The thickness of the plate were standardised by 15 ml of media per plate. Wells were made by using a cork borer to make 5 mm diameter wells. Another way of doing antimicrobial assay was by spotting the sample on bacterial lawn. All antimicrobial tests were carried out in triplicate. The plate was incubated for 18 hours at 37 °C and the inhibition zone was measured (Lewus & Montville, 1991). The steps of antimicrobial assay are shown in Figure 3.2.

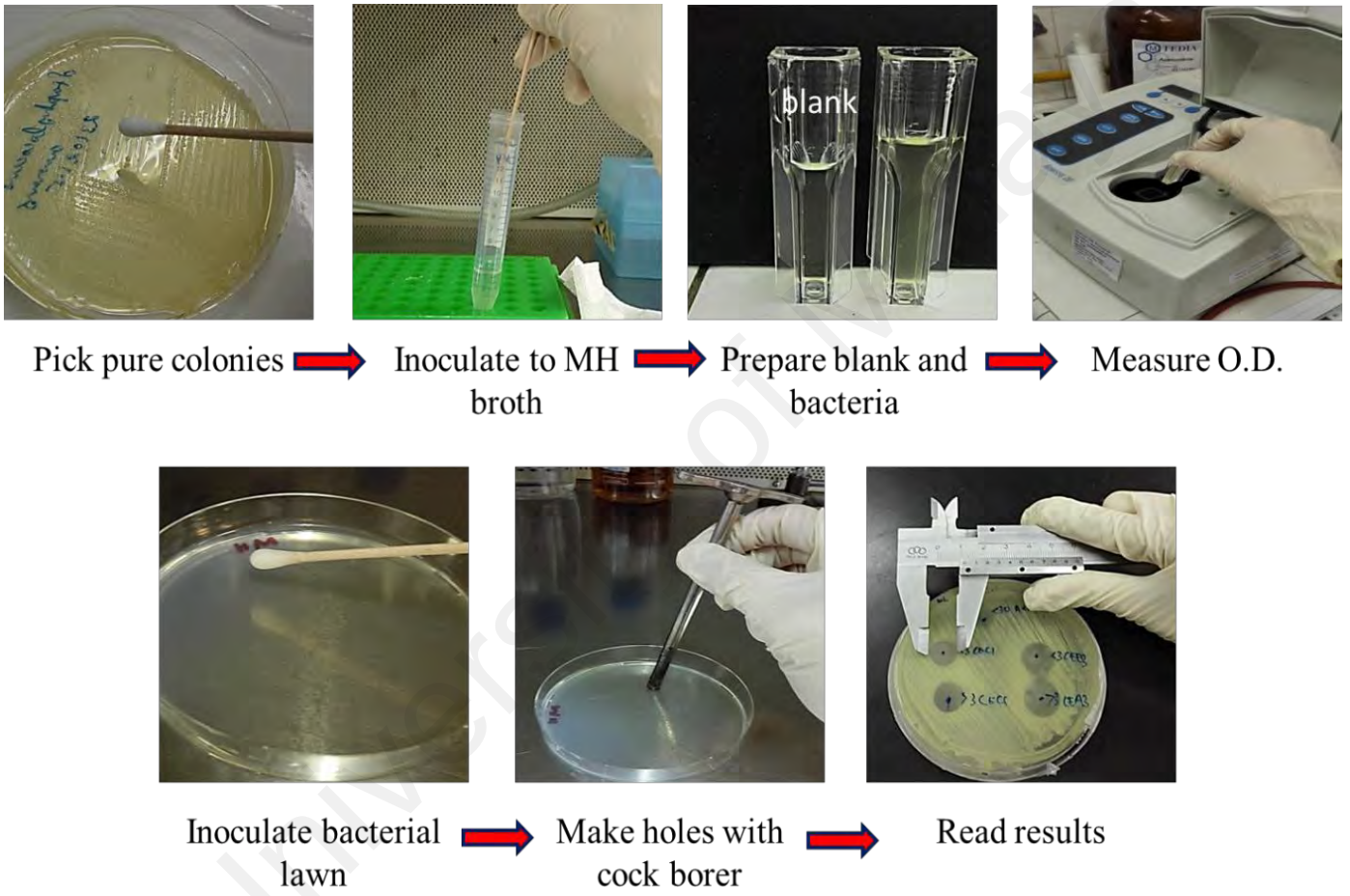


Figure 3.2: Antimicrobial assay protocol.

3.4 Sampling

Sampling was done from raw cow milk randomly collected from two different food retail outlets located in Petaling Jaya, Selangor, Malaysia. 150 ml of the raw milk samples (3) were transferred into sterile conical flasks and pasteurised at 63 ºC for 30 minutes in order to kill the harmful bacteria present in the raw milk. The milk was left to ferment in the sterile flask under room temperature for two days.

3.5 Isolation of lactic acid bacteria from fermented milk

Serial dilution was performed by transferring 1 ml of the fermented milk into 9 ml of sterile distilled water. Then 0.1 ml was taken from dilutions of 10^{-3} and 10^{-4} and spread onto a fresh de-Mann, Rogosa and Sharpe (MRS) agar (Merck, Germany). These plates were placed in an inverted position in an incubator and then incubated for 24 hours at 37 °C. Single colonies were picked from the plate and subcultured twice to get a pure colony. The isolates were grown in MRS broth for 24 hours and centrifuged at 10,000 x g for 20 minutes. The supernatant was used to test for preliminary antimicrobial properties (de Man *et al.*, 1960).

3.6 Identification of lactic acid bacteria

For identification of bacteria five tests were performed including Gram stain, spore stain, catalase test, oxidase test and growth ability on bile esculin azide agar (Merck, Germany). Cell morphology and Gram- staining reaction were examined under light microscope with final magnification of 1000X. Schaeffer and Fulton's spore staining method (Ashby, 1938) was followed with some modifications. Smears of bacteria were done and then gently heat fixed on glass slide. Each slide was placed over a beaker of boiling water, resting it across the rim with the bacterial smear on the upper slide surface. The smear was flooded with malachite green for 1 minute and washed

away. Then it was stained with safranin for 30 seconds and washed away. All slides of bacteria were examined under the oil immersion lens. Bacterial spore was stained green whereas bacterial cell was stained red. Catalase test was used to detect the presence of catalase enzyme in the bacteria which broke down the hydrogen peroxide into water and oxygen. This was done by adding one drop of 3 % hydrogen peroxide into a fresh culture of bacteria after which formation of bubbles were observed to indicate catalase positive while a lack of bubbles indicated catalase negative. On the other hand oxidase test was used to identify whether the bacteria produce enzyme cytochrome oxidase. The test was carried out by adding one drop of oxidase reagent (1 % N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride from Sigma, USA) directly onto the agar plate with the culture of bacteria and a blue colour appeared within one minute to indicate positive result while no colour indicated negative result. Bile esculin azide agar tested the ability of the bacteria to hydrolyse esculin in the presence of bile which was a characteristic of *Enterococci* and group D *Streptococci*. Bacteria that is positive for esculin hydrolysis will hydrolyse the glycoside esculin to esculetin and dextrose. The esculetin reacts with the ferric citrate to form a dark brown or black complex. The bile was used to inhibit Gram-positive bacteria other than *Enterococci* and the sodium azide inhibits Gram-negative bacteria (Facklam & Moody, 1970).

3.7 Molecular assay to identify bacteria

The bacteria that showed antimicrobial activity were then further identified using 16S rRNA gene sequencing. Previously developed PCR method was followed with some modifications (Weisburg *et al.*, 1991). The total DNA of the bacteria was extracted using i-genomic BYF DNA Extraction Mini Kit (iNtRON Biotechnology, Korea). Polymerase chain reaction (PCR) was done by using forward universal bacterial primer, 27f (5'-AGAGTTTGATC(A/C)TGGCTCAG-3') and reversed universal bacterial primer, 1492r (5'-ACGG(C/T)TACCTTGTTACGACTT-3'). PCR reaction mixture (25 µl total) contains 2.5 µl PCR buffer, 2 µl of dNTP mix (2.5 mM each), 1 µl of each primer (20 pmol), 100 ng of DNA template and 0.5 µl of i-Taq^{TM} DNA polymerase 5 U/µl (iNtRON Biotechnology, Korea). The PCR condition was set for initial denaturation at 94 ºC for 5 minutes; denaturation at 94 ºC for 1 minute; annealing at 52 ºC for 1 minute; extension at 72 ºC for 1.5 minutes for a total of 30 cycles run with a final extension at 72 ºC for 10 minutes. The PCR product was cleaned up using MEGA quick-spin PCR & Agarose Gel DNA Extraction Kit (iNtRON Biotechnology, Korea). Gel electrophoresis was carried out for total DNA, PCR products and PCR product after clean up by using 1 % (w/v) agarose (Bio-rad, USA) gel, 1 µL of 1 Kb DNA ladder (Fermentas, Lithuania). The gels were run in Tris-acetate-EDTA, TAE buffer (0.04 M Tris, 0.02 M acetic acid and 1 mM EDTA at pH 8.0) at 100 voltage. The gel was stained by using Gel Red (Biotium, USA). The cleaned up PCR product was sequenced by Genomics BioSci & Tech. Ltd, Taiwan. The sequencing results were then compared with the database in National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST) http://blast.ncbi.nlm.nih.gov/Blast.cgi.

3.8 API 50 CHL and API 20 strep bacteria identification

The bacteriocin producers were also identified by using API 50 CHL (bioMérieux, France) to study the ability of the bacteria to metabolize 49 kinds of carbohydrates and API 20 strep to identify *Enterococcus* spp. The API identification system was performed according to the manufacturer's instructions. Pure colonies of the bacteria were transferred into the test medium and loaded onto the test strip. A drop of mineral oil was loaded in each well of the strip. The test strip was incubated for 48 hours at 37˚C. The result obtained after 48 hours was analysed using APIWEB^{TM} identification software by comparing the bacterial similarity to the database. The identification of a microorganism is accompanied by the following information: (i) The percentage of identification (% id) is an estimate of how closely the profile corresponds to the taxon relative to all the other taxa in the database. (ii) The T-index represents an estimate of how closely the profile corresponds to the most typical set of reactions for each taxon. Its value varies between 0 and 1, and is inversely proportional to the number of atypical tests. (iii) Comments on the quality of identification derived from the % id and the T-index of the selected taxon (excellent identification % id > 99.9 and T> 0.75).

3.9 Growth study

The growth of the bacteriocin producers was measured by using Miles and Misra's method (Miles *et al*., 1938). The growth of the bacteria was also monitored by measuring the optical density of the fermented broth at 600 nm wavelength. Ten percent of the starter cultures of the bacteriocin producers with O.D. of 0.1 was added to 1 litre of sterile MRS broth. The colony forming units and the O.D. of the bacteria within 72 hours was monitored. To determine the colony forming units, 20 µl of the fermented broth with different dilutions was spotted on MRS agar in triplicates and allowed to dry. Then the plates were incubated at 37 °C for 18 hours. The colony forming unit was calculated as below:

CFU per ml= Average number of colonies for a dilution × 50 × dilution factor

3.10 PCR amplification to detect genes encoding known bacteriocins

Lactococcus lactis A1 and *Enterococcus faecium* C1 isolated in this study were subjected to PCR amplification to detect the structural genes of nisin, lacticin 481 and lactococcin A for *L. lactis* A1 and enterocin A, B and P for *E. faecium* C1 by using the primers listed in Table 3.1.

Table 3.1: List of primers used to detect structural genes from *L. lactis* A1 and *E. faecium* C1

Primer	Specificity	5'-sequence-3'	Size (bp)	Reference
P1 P2	Nisin	AAGAATCTCTCATGAGT CCATGTCTGAACTAACA	898	Rodriguez *et al.*, 1995
P3 P4	Lactococcin A	CAATCAGTAGAGTTATTAACATT TG GATTTAAAAAGACATTCGATAA TTAT	771	Martinez *et al.*, 1998
P5 P6	Lacticin 481	TCTGCACTCACTTCATTAGTTA AAGGTAATTACACCTCTTTTAT	366	Martinez *et al.*, 1998
E1 E2	Enterocin A	AAATATTATGGAAATGGAGTGT AT GCACTTCCCTGGAATTGCTC	NA*	du Toit *et al.*, 2000
E3 E4	Enterocin B	GAAAATGATCACAGAATGCCTA GTTGCATTTAGAGTATACATTTG	NA*	
E5 E6	Enterocin P	TATGGTAATGGTGTTTATTGTAA T ATGTCCCATACCTGCCAAAC	NA*	

* No data available

The template DNA was extracted using the same protocol used for extraction of total DNA in 16S as descript previously. The PCR conditions consisted of 30 cycles of initial denaturation 94 °C for 4 min, 94 °C for 40 sec, 50 °C for 1 min, 72 °C for 1 min and final extension of 72 °C for 10 min for structural genes of nisin. For the Lactococcin A, Lacticin 481 and Enterocin A, B and P genes, similar PCR conditions were employed except that annealing temperatures of 50 °C and 55 °C (both) were used. Amplified fragments were visualised on 1 % (w/v) agarose gels by staining with Gel Red (Biotium, USA). 2 µl of 1 Kb ladder (Fermentas, Lithuania) was loaded into a well

as the molecular weight standard. PCR products obtained with the primers were purified with the MEGA quick-spin PCR & Agarose Gel DNA Extraction Kit (iNtRON Biotechnology, Korea). The product was sequenced by Genomics, Taiwan. The sequences were compared with the BLAST programs of the National Center for Biotechnology Information (NCBI).

3.11 Antibiotic susceptibility and haemolytic tests of the bacteria

In order to determine any potential pathogenic activity, the producer strains were tested for haemolytic activity. The producer strain was grown overnight on MRS agar (Merck, Germany) at 37 °C and subcultured again on blood agar base (Difco, France) containing 7 % (v/v) sheep blood. The plates were incubated at 37 °C overnight. The haemolytic activity was recorded as α-haemolysis (agar under the colony was dark and greenish), β-haemolysis (clear zone around the colonies) and γ-haemolysis (unchanged). To indicate any potential for antibiotic resistance, antibiotic susceptibility test of *E. faecium* C1 was done in triplicate according to Clinical and Laboratory Standard Institute (CLSI) 2007, Volume 27. Bacteria suspension equivalent to 0.5 McFarland standard was lawned on Mueller-Hinton agar and incubated at 37 °C for 18 hours and 24 hours for vancomycin. If the growth of the bacteria was inhibited by the antibiotic disc, a zone of inhibition will appear. The diameter of the zone of inhibition is reported in millimetres. Results are reported as S (sensitive), I (intermediate) or R (resistant) according to CLSI breakpoints. The antibiotic susceptibility test for *W. confusa* A3 was carried out in the same way as *E. faecium* C1 but the media used was MRS agar supplemented with 0.05 % (w/v) of cysteine hydrochloride (Sigma-aldrich, United State). The antibiotic disc (Oxoid, United Kingdom) used were amoxicillin (10 µg), ampicillin (10 µg), chloramphenicol (30 µg), clindamycin (2 µg), gentamicin (10 µg), novobiocin (5 µg), ofloxacin (5µg), penicillin (1 unit), penicillin V (10 µg),

streptomycin (10 µg) and vancomycin (30 µg). All antibiotic susceptibility tests were performed in triplicates (CLSI, 2007)

3.12 Bacteriocin production in different media

To examine and compare the effectiveness of different culture media on the production of bacteriocin, the bacteriocin producers were grown in 10 types of broth and tested against *B. cereus*. The media used were de Man, Rogosa and Sharpe (MRS), M17 supplemented with 1.5 % glucose, M17 (Difco, France) supplemented with 1.5 % sucrose, M17 supplemented with 1.5 % lactose, Brain-Heart Infusion (BHI) (Merck, Germany), LAPTg (Pingitore *et al*., 2007), tryptic soy broth (TSB) (Merck, Germany) with 1 % tween 80, Brucella broth (Merck, Germany), nutrient broth (NB) (Merck, Germany) with 1.5 % glucose and Miller's LB broth (Merck, Germany). After 18 hours of incubation, the fermented broth was centrifuged at 10,000 x g for 20 minutes and the supernatant was precipitated with 80 % ammonium sulphate overnight at 4 °C. Then the precipitated supernatant was centrifuged and the pellet dissolved in minimum amount of sterile water and tested for inhibitory activity by well diffusion assay.

3.13 Time plot of bacteriocin production

The time plot of bacteriocin production by *W. confusa* A3 (in MRS broth) and *E. faecium* C1 (in LAPTg broth) was investigated. The production of bacteriocin was monitored during log to stationary phases by measuring the inhibition zones of the crude bacteriocin. 20 ml of the bacteria culture was centrifuged at 10,000 x g for 15 minutes and the supernatant was subjected to 80 % ammonium sulphate precipitation. The dissolved precipitate from each interval was tested against *B. cereus* and the inhibition zones were measured.

3.14 Effect of carbon sources

The MRS broth (200 ml) was supplemented with 2 % of different carbon sources namely glucose, lactose and sucrose and inoculated with 2 ml of the bacteria of OD_{600} value of 0.5. After 18 hours of incubation period, the broth was centrifuged to remove the cell pellet. The supernatant was subjected to 80 % ammonium sulphate precipitation and the resulting precipitate after centrifugation was dissolved in minimal amount of sterile double distilled water. Then the inhibition zones produced from the different carbon sources used were compared.

3.15 Purification of bacteriocins

3.15.1 Ammonium sulphate precipitation

The bacteria were grown until late log phase in MRS broth or LAPTg broth depend on the suitability. The fermented broth was centrifuged under 10,000 x g for 20 minutes. After centrifugation, the bacterial pellet was discarded and the cell free supernatant was filtered using 0.2 µm membrane filter (Sartorius, Germany). The supernatant was added slowly with ammonium sulphate (Merck, Germany) until the solution reached 80 % saturation and then stirred overnight at 4 °C. The product was then centrifuged under 10,000 x g for 15 minutes and the supernatant was discarded. The precipitate was dissolved in minimum amount of sterile double distilled water. The product was lyophilised and kept in 4 °C for further use. 0.025 g of the lyophilised powder was dissolved in 50 µl sterile distilled water and used for antimicrobial assay as described above (Ivanova *et al*., 2007).

3.15.2 Amberlite XAD 16 hydrophobic interaction column

Amberlite XAD 16 (Sigma-Aldrich, Germany) is a nonionic macro-reticular resin with cross-linked polymers provided as small white beads that adsorb and release ionic species through hydrophobic and polar interactions. A published method was followed with some modifications (Casaus, *et al*., 2007). Before packing the column, 20 g of the Amberlite XAD 16 resin was first soaked in 100 ml of 100 % acetonitrile in a beaker for one hour to activate the resin. The column used to pack the resin was washed thoroughly with sterile distilled water prior to use. Then the activated resin was poured into the cleaned column together with the 100 % of acetonitrile. The column was prepared by removing the 100 % acetonitrile in the resin and washed with 1 L of sterile distilled water. The lyophilised sample was dissolved in 100 ml of sterile distilled water and loaded into the column. Then the column was washed with 1 L of sterile distilled water to elute out unwanted products and then the fractions were eluted out by gradually increasing the concentration of acetonitrile from 10 %, 50 % to 90 % with 1 L, 200 ml and 100 ml respectively. Figure 3.3 shows the setup of Amberlite XAD 16 hydrophobic interaction column.

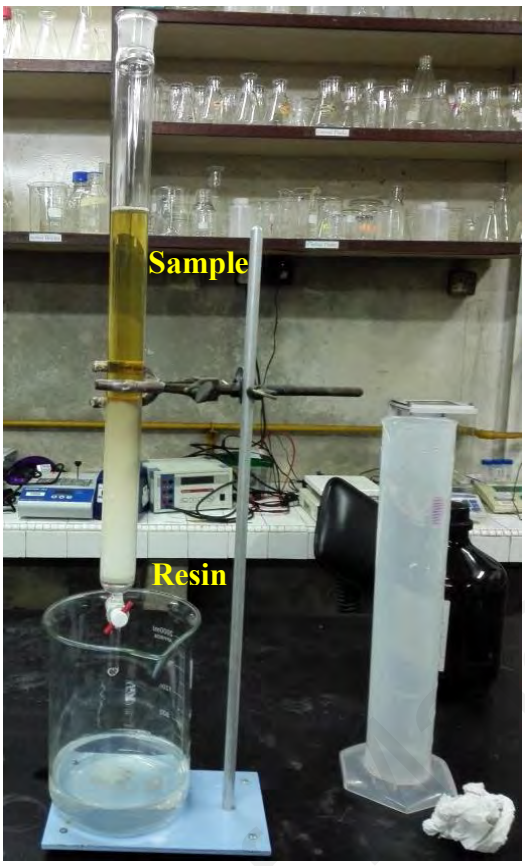


Figure 3.3: Amberlite XAD 16 column setup.

3.15.3 Vivaspin centrifugal filter and size separation concentrator

The active fraction obtained from Amberlite XAD 16 column was further fractionated by size using Vivaspin centrifugal concentrator column (Sartorius, Germany) with different molecular weights cut off (MWCO) of 0.2 µm, 1,000,000 MWCO, 50,000 MWCO, 30,000 MWCO, 10,000 MWCO, 5,000 MWCO and 2,000 MWCO. The centrifuge speeds used for 50,000 MWCO and below was 8,000 × g, and 0.2 µm and 1,000,000 MWCO was 6,000 × g. After centrifugation, antimicrobial test was performed by spotting 20 µl of the fraction on Mueller-Hinton agar seeded with *B cereus*.

3.16 Reverse-phase high-performance liquid chromatography (RP-HPLC)

The fraction collected from Vivaspin centrifugal filter and size separation concentrator was loaded in an HPLC system (Waters, USA) equipped with Chromolith SemiPrep RP-18e column (Merck, Germany). EMPOWER software (Waters, USA) was used for data acquisition and processing. Figure 3.4 shows the HPLC gradient profile used in this study. The chromatographic separation was carried out by gradient separation using two solvents which constituted the mobile phase: A which is 95 % Mili-Q water (Millipore, USA) and 5 % acetonitrile (Merck, Germany) and B which is 100 % acetonitrile (ACN) (Merck, Germany). Solvent A with 95 % water was used to prevent damage to the column. All the solvents used were filtered through with nylon filter with pore size 0.45 µm. The flow rate of the mobile phase was set at 1 ml min^{-1}. The following gradient was used starting with 0 % of solution B, 40 % of solution B after 3 minutes and 100 % of solution B after 45 min. Upon completion of the separation, the system remained unchanged for the next 5 min and then restored to 100 % solution A (column rinsing). Elution was monitored at wavelength 214 nm and fractions were collected manually at 3 minutes intervals. Fractions that exhibit antimicrobial activity were collected at the same retention time during different HPLC runs and then pooled and lyophilised. The purified bacteriocins were named as BacA3 (bacteriocin produced by *W. confusa* A3) and BacC1 (bacteriocin produced by *E. faecium* C1).

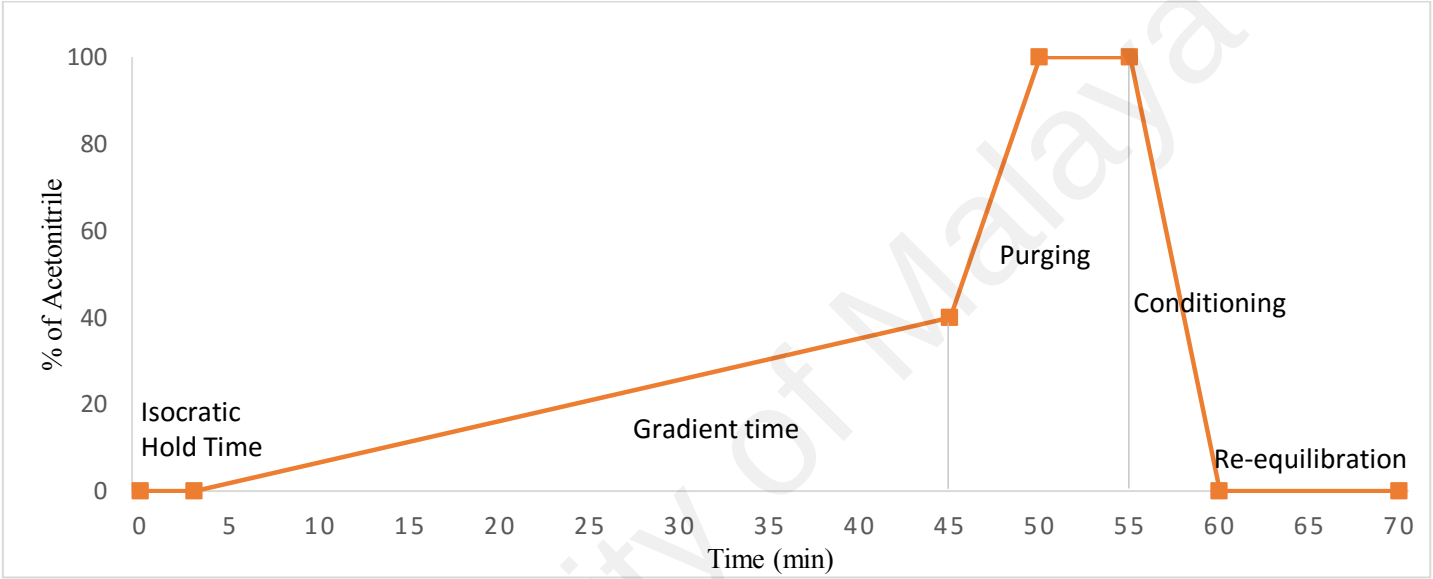


Figure 3.4: Gradient HPLC profile. Fractions were collected every 3 minutes until 60 minute of elution time.

3.17 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC of antimicrobial substances was done based on a standardised method (Wiegand *et al*., 2008). The indicator strains were inoculated into 5 ml of Mueller-Hinton broth and incubated at 18 h at 37 °C on a shaker. Cultures were diluted with Mueller- Hinton broth to give 7×10^5 CFU/ml. Two-fold serial dilutions were performed from the stock bacteriocin with Bradford concentration of 1.48 (BacA3) and 1.57 (BacC1) mg/ml in 0.2 % BSA and 0.01 % acetic acid in Eppendorf tubes (polypropylene). 11 µl of each concentration was added to a well in a 96-well poplypropylene plate. Then 100 µl of the diluted indicator strains was added into each corresponding well containing each concentration of the bacteriocin. The final concentrations of the bacteriocin were progressively halved to 74, 37, 18.5 until 1.16 µg/ml (BacA3) and 78.5, 39.25, 19.63 until attaining a concentration 1.23 µg/ml (BacC1). The 96-well plate was incubated for 18 h at 37 °C. MIC value was taken for the lowest concentration of the bacteriocin that reduced the growth by more than 50 % compared to that of the control. 10 µl of the content of the well with higher MIC value was plated onto Mueller-Hinton agar and incubated at 37 ºC overnight. The lowest concentration of bacteriocin that prevents any viable cell growth was defined as the minimum bactericidal concentration (MBC). Agar dilution method was also performed to check the MIC of the sample purified from Amberlite XAD 16 because the colour of the sample. The bacteriocin was two-fold diluted and 50 µl of each dilution was transferred to wells made with sterile cock borer on Mueller-Hinton plate seeded with *B. cereus*. After incubation at 37 °C overnight, activity was determined by the diameter of the inhibition zones around the wells.

3.18 Effect of temperature, pH and enzyme on activity of bacteriocin

The HPLC purified bacteriocins were used in the following experiments.

3.18.1 Heat stability test

To determine the effect of heat on bacteriocin activity, the bacteriocins were exposed to temperatures of 40, 60, 80 and 100 °C for 20 minutes. The heat exposed bacteriocins were cooled to room temperature and tested for inhibition on *B. cereus*. The residue activity was then determined using the following equation:

$$\text{Residue activity} = \frac{(\text{Inhibition zone} - 5)}{(\text{Inhibition zone of Untreated} - 5)} \times 100$$

The inhibition zones were subtracted with 5 mm which is the initial diameter of the wells on agar plates.

3.18.2 pH stability test

To evaluate the effect of pH on bacteriocin activity, the bacteriocins were adjusted to a pH value ranging from 2 to 10 by using concentrated NaOH (Merck, Germany) and HCl (Merck, Germany). The samples were incubated for 2 hours at 25˚C before determining the antimicrobial activity by using agar well diffusion assay.

3.18.3 Effect of enzymes

The vulnerability of the bacteriocins to breakdown by different enzymes namely proteinase K, lysozyme, lipase, catalase, lyticase and peptidase were also tested. All the enzymes used were supplied by Sigma-Aldrich Corporation (St Loius, MO, USA). 500 µl of the bacteriocin was treated with enzymes with 1 mg/ml final concentration and a control without treatment was prepared. All preparations were incubated at 37 °C for 1

hour and tested against *B. cereus*. The residue activity was then determined using the following equation:

$$\text{Residue activity} = \frac{(\text{Inhibition zone} - 5)}{(\text{Inhibition zone of Untreated} - 5)} \times 100$$

The inhibition zones were reduced by 5 mm which is the initial diameter of the wells on agar plates.

3.19 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out according to the method described in Laemmli SDS-PAGE (Schagger, 2006). The molecular weights of the bacteriocins were estimated using SDS-PAGE. The gel used for the separation was 16.5 % tris- tricine SDS-PAGE gel. Precision Plus Protein™ Dual Xtra Standards (Bio-Rad, USA) was used as a molecular weight marker to determine the size of the samples. The gel was run at voltage of 100 V in tris- tricine buffer for one and a half hour until the dye front reached the bottom of the gel. Then the gel cassette was removed and the gel was first rinsed with sterile water and placed on a belly shaker for 5 minutes. This step was repeated three times to remove the buffer residues and other chemical from the gel. Then it was stained with 20 ml of SimplyBlue™ SafeStain (Invitrogen, UK) which was sensitive and non- hazardous Coomassie® G-250 stain. The gel was stained on a rotory shaker for 30 minutes and de-staining was continued until the protein bands could easily be distinguished from the blue background.

3.20 MALDI-TOF analysis

Mass spectrometry analysis was done by using matrix-assisted laser desorption ionization- time of flight (MALDI-TOF) mass spectrometry (MS) at the Medical Biotechnology Laboratory, Faculty of Medicine, University of Malaya.

3.20.1 Sample in liquid form, without digestion

The bacteriocin purified from HPLC was subjected to matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis. The targeted plate was spotted with aliquots consisting 4 µl of matrix (α-cyano-4-hydroxycinnamic acid in acetonitrile: 0.1 % TFA in Milli Q water, 1:3) and 4 µl of sample cleaned up with zip-tip C18 (Millipore, Bedford, USA). The sample was air-dried before analysis using MALDI TOF/TOF (ABI 4800 Plus).

3.20.2 Sample in gel form, with digestion

The band from SDS-PAGE was cut and digested with trypsin by shaking the gel plugs in 50 µl of 50 % acetonitrile in 50 mM ammonium bicarbonate for three times until the gel became clear. Then the gel plug was incubated in 150 µl of 10 mM dithiothreitol in 100 mM ammonium bicarbonate for 30 minutes at 60 °C. The gel plug was cooled to room temperature and continued with alkylation and dehydration step. The gel was then digested with trypsin. The sample was extracted and desalted with C18 Zip- tip before subjecting to MALDI TOF/TOF (ABI 4800 Plus). Protein identification was performed by sending trypsin digested peptide masses to SWISS-PROT (protein sequence database) using the MASCOT (Matrix Science) Peptide Mass Finger printing programme. The mono-isotopic masses were used and significance threshold $p < 0.05$ was used.

3.21 Membrane permeabilization test using Real-Time PCR

Membrane permeabilization assay was carried out using SYTOX® green fluorescent dye (Invitrogen, USA) (Bourbon *et al*., 2008). This molecular probe can only permeate depolarised membranes and is a high-affinity DNA stain which cannot enter intact bacterial membranes. Real-time PCR was used to monitor the quantity of DNA present when different concentrations of bacteriocins were added to the target bacteria. If the bacteriocins shear the membrane of the target bacteria, DNA of the bacteria is released and incorporated with the florescence dye. Hence, the higher fluorescence indicated the bacteriocins to be more powerful. The target *B. cereus* was grown in Mueller-Hinton broth for 18 hours at 37 °C. The bacterial pellets were washed twice with 10 mM sodium phosphate buffer pH 7.2 before re-suspending in the same buffer to attain an optical density of 0.6 at 600 nm wavelength. Then 5 µl of SYTOX® green stock solution was added to 5ml *B. cereus* to give a final concentration of 5 µM of SYTOX® green dye. Ninety µl of the bacteria added with SYTOX® dye was mixed with 10 µl of bacteriocin. The positive control used was 1 M sodium hydroxide and negative control was bacterial cells without bacteriocin. Tetracycline (1.5 µg/ml) was also tested as a known antimicrobial having a different mode of action. The experiment was performed in quadruplicate. The wells were sealed by using adhesive cover and the plate was placed immediately in the Step One Plus real-time PCR system (Applied Biosystem, USA). The system was programmed with SYBR Green filter selected and 60 cycles of 1 min duration at 37 ◦C read at the end of each cycle. The raw data was analysed using Microsoft Excel software and the fluorescence uptake of each treatment was plotted.

3.22 Effect of bacteriocin on bacteria examined by electron microscopy (EM)

The effect of bacteriocins on the target bacteria was examined using scanning and transmission electron microscopes available in the University of Malaya. Electron microscope has the ability to visualise the structure of bacteria at high resolution.

3.22.1 Scanning electron microscope (SEM)

The test bacteria *B. cereus* was grown in Mueller- Hinton broth for 18 hours till it reached the exponential phase. The cells were then centrifuged at 2,000 x g for 15 minutes. The resulting supernatant was discarded and bacterial pellet was dissolved in 1 ml of purified bacteriocin and incubated for 3 hours at 37 °C. Bacteria without addition of bacteriocin was prepared and used as a negative control. Then the bacteriocin was washed away thrice by using sodium phosphate buffer by centrifuging at 2,000 x g for 15 minutes. Then the cells were fixed with 2.5 % 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 % osmium tetroxide (OsO_4) mixed with 1:1 (v/v) water and then left overnight. After washing the samples 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 sample preparation before critical point drying (CPD). Finally, the sample was coated with gold and then observed under a scanning electron microscope (model JEOL JSM-7001F, Japan).

3.22.2 Transmission electron microscope (TEM)

The test bacteria strain was prepared following the same method used for SEM. The cells were fixed with 4 % glutaraldehyde overnight at 4 °C. The suspensions were

washed thrice with cacodylate buffer and post-fixed for 2 hours with osmium tetroxide and cacodylate buffer in 1:1 ratio. Then the samples were washed three times in cacodylate buffer and incubate in cacodylate buffer overnight. Samples were then washed thrice with water, once with uranyl acetate and thrice with water. The samples were dehydrated in a graded ethanol series and embedded in Epon which is an embedding resin. Ultrathin sections (0.1 μm) were prepared and coated on copper grids and stained with uranyl acetate and lead citrate. The grids were examined using LEO-Libra 120 transmission electron microscope (Carl Zeiss, Germany).

3.23 Amino-terminal sequence analysis

The N-terminal amino acid sequences of the purified bacteriocin BacA3 and BacC1 were performed by Edman degradation using Procise 494 sequencer (Applied Biosystems, USA) equipped with PTH-C18 column (Applied Biosystems, Roissy, France) for the quantitative determination of phenylthiohydantoin (PTH) amino acids. The Procise employed Edman degradation in liquid-pulse mode to sequentially cleave and identify amino acids starting at the amino terminus (N-terminus) of the protein. It can detect all 20 common amino acids, as well as several modified forms of cysteine. The partial sequences obtained from Edman degradation were then searched and compared with known proteins in UniProt database (http://www.uniprot.org/blast/).

3.24 Genome sequencing and analysis

DNA with required quality and quantity was isolated with DNeasy Blood & Tissue Kits (Qiagen, Valencia) following manufacturer's instructions. DNA concentration of each sample was measured with Qubit (Invitrogen, USA) and Nanodrop spectrophotometer (Thermofisher, USA). The DNA was then fragmented using the Covaris S220 system (Applied Biosystems, USA) to fragment it into a size ranging 600-900 bp following optimised conditions. DNA library (using 800 ng sonic

cleaned DNA) was prepared using Nextflex DNA Sample Prep Kit (Bio scientific, Texas, USA) as per the manufacturer's instructions. Around 2.5 µg of genomic DNA was sonicated using Bioruptor® sonication system to obtain 300 to 600 bp fragment size. The size distribution was checked by running an aliquot of the sample on Agilent HS DNA Chip. The resulting fragmented DNA was cleaned up using HighPrep™ PCR beads (Magbio). Library QC was performed on Bioanalyzer (Agilent technologies) to check the fragment size distribution. The QC passed library was sequenced on MiSeq sequencer (Illumina) with MiSeq Reagent Kits v3 (300 cycle, paired-end). Fluorescent images were analysed using the MiSeq Control Software, and FASTQ-formatted sequence data was created using MiSeq Reporter Analysis.

3.24.1 De-novo whole genome sequencing for *W. confusa* A3

The Illumina MiSeq paired end raw reads were quality checked using Genotypic Technology Pvt. Ltd., proprietary tool SeqQC. Illumina raw reads were processed by in-house perl script for adapters and low quality bases trimming towards 3'-end. De-novo assembly of Illumina MiSeq data was performed using SPAdes assembler (Bankevich *et al*., 2012). SPAdes assembler is intended for de-novo assembly after error-correction of sequenced reads. Thereafter, Scaffolding was carried out using SSPACE scaffolder. SSPACE scaffolds pre-assembled contigs using paired-ended data. By using the distance information of paired-end data, SSPACE is able to assess the order, distance and orientation of contigs and combine them into larger scaffolds. Genes were predicted for bacterial scaffolds using Prodigal.v2_60 (Hyatt *et al*., 2010). Predicted genes were annotated with proteins of bacterial sequences taken form Uniprot database using NCBI BLAST 2.2.29 (Altschul *et al*., 1990). All annotations along with statistics were provided. The scaffolds were subjected to evaluate Simple Sequence Repeats using MIcroSAtellite identification tool (MISA tool).

3.24.2 Re-sequencing for *E. faecium* C1

The DNA sequences were aligned with Bowtie 2-2.0.5. The reference strain used was *Enterococcus faecium* CRL1879. Variant detection was performed with SAMtools – 0.1.18 and SnpEFF 3.4. SNPs or single nucleotide polymorphisms which are the heritable single base changes in a genome versus a reference sequence were detected.

3.24.3 Analysis of the genome data

Basic analysis was performed with Rapid Annotation using Subsystem Technology (RAST) server (http://rast.nmpdr.org/). The analysis for *W. confusa* A3 and *E. faecium* C1 focussed on the search of genes encoding bacteriocin production, antibiotic resistance genes and virulence factors. Both strains were searched for genes related for probiotic function by comparing the genome obtained with sequence from NCBI database. For *E. faecium* C1, virulence genes listed in virulence factor database (VFDB) (http://www.mgc.ac.cn/VFs/) was searched with the genome sequence obtained. Five categories of virulence factors were covered including adherence, antiphagocytosis, biofilm formation, exoenzyme (gelatinase, hyaluronidase and serine protease) and toxin (cytolysin) production. To further detect the pathogenicity islands (PAIs) and antimicrobial resistance islands (REIs) in *E. faecium* C1, the genes involved in the same species listed in pathogenicity island database (PAI DB) http://www.paidb.re.kr/about_paidb.php?m=h were also searched against the genome (Yoon *et al.*, 2015). The sequence was also subjected to BAGEL 3 (http://bagel.molgenrug.nl/index.php/bagel3) which is a web-based automated bacteriocin mining tool to detect known bacteriocin in the sequence (van Heel *et al.*, 2013).

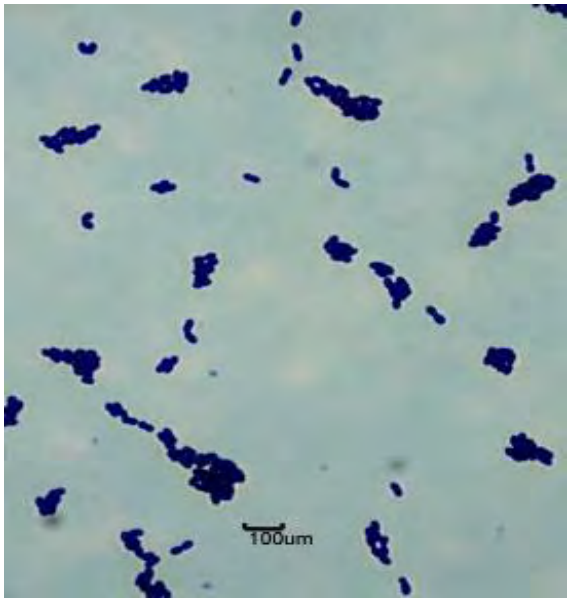
3.25 Application of the bacteriocin and LAB culture in milk

1 % of BacA3 (100 mg/ml) from HPLC, 1 % of BacC1 (100 mg/ml) from HPLC, 1 % *W. comfusa* A3 culture with O.D. of 0.1, 1 % of *E. faecium* C1 culture with O.D. of 0.1, 1 % of nisin (100 mg/ml) (Sigma-Alrich, USA) were added separately into pasteurised milk in triplicates. A blank milk sample was used as control. All treatments and replicates were incubated at 25 °C for 10 days. During the incubation period, 0.1 ml of the milk was plated on plate count agar (PCA) and incubated at 37 °C for 24 hours. The total CFU count of the bacteria in the milk samples was plotted on a daily basis over a period of 10 days.

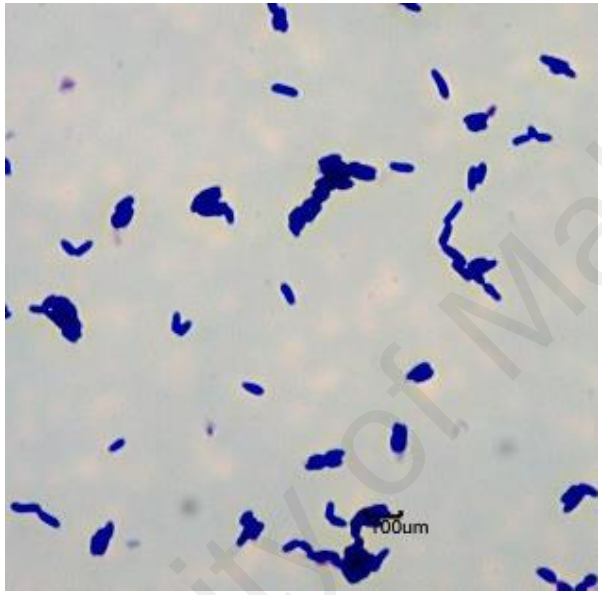
CHAPTER 4: RESULTS

4.1 Identification of lactic acid bacteria

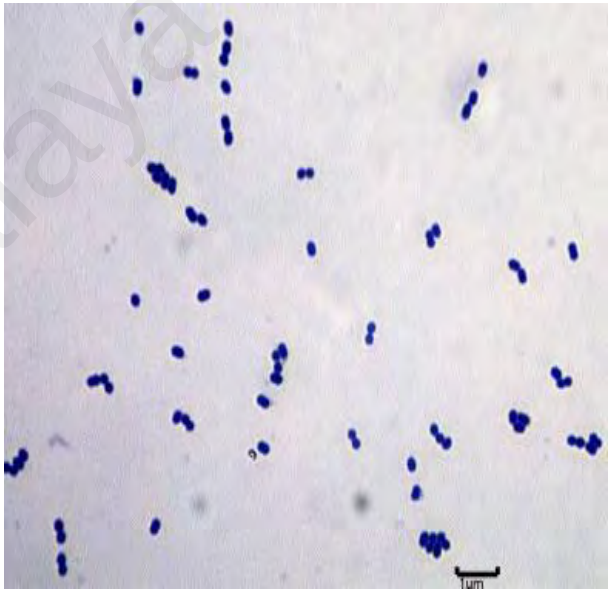
Different colonies were isolated from three different fermented raw milk samples randomly collected from two retail outlets in Petaling Jaya. Initial antimicrobial assay was done to eliminate bacteria which did not produce any antimicrobial metabolites and the bacteria that showed inhibition were chosen for identification. Out of 30 colonies isolated, the fermented supernatant of 3 isolates showed significant antibacterial effect on the target bacteria. They are A1, A3 and C1. The colony morphology on MRS agar plates showed that A1 and C1 were both small in size and A3 relatively bigger in size. All of them are milky-white in colour, circular shaped, convex elevated and with entire margins (Table 4.1). Catalase test showed no bubbles after addition of 3 % hydrogen peroxide to the cultures. Oxidase test showed no colour changes on addition of the oxidase reagent. Therefore all the isolates were catalase and oxidase negative. Spore staining with malachite green showed that they did not produce spore because no green colour spore was found after stained with malachite green. Table 4.2 summarises the results of Gram staining and biochemical tests of the 3 isolates. There are three isolates of which two isolates (A1 and C1) were coccal shaped and one isolate (A3) was short rod-shaped (Figure 4.1). Bile esculin test reviewed that A1 and A3 were able to hydrolyse esculin weakly in the presence of bile because the agar was only partially changed to black colour whereas C1 can hydrolyse esculin better than A1 and A3 as the whole plate turned black colour after incubation. Figure 4.2 shows strong hydrolysis caused by C1.



A1



A3



C1

Figure 4.1: Bacterial shape viewed under light microscope (1000× magnification). A1 and C1 are coccoid-shaped; A3 are short rod-shaped.

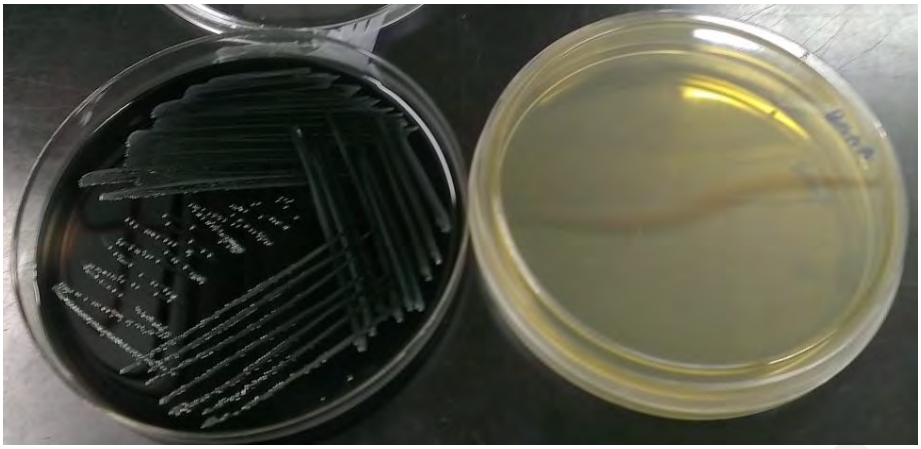


Figure 4.2: Bile esculin test for C1. Plate on the right: before inoculation with test bacteria. Plate on the left: after inoculation with test bacteria wherein medium colour turned black.

Table 4.1: Colony morphology of the isolates cultured on MRS agar

Isolate	Colony morphology				
	Colour	**Size**	**Form**	**Elevation**	**Margin**
A1	Milky-white	Small	Circular	Convex	Entire
A3	Milky-white	Big	Circular	Convex	Entire
C1	Milky-white	Small	Circular	Convex	Entire

Table 4.2: Gram staining and biochemical tests of the 3 isolates

Isolate	Gram stain	Shape under light microscope	Catalase test	Oxidase test	Bile esculin test
A1	Positive	Coccus	Negative	Negative	Weak hydrolysis
A3	Positive	Short rod	Negative	Negative	Weak hydrolysis
C1	Positive	Coccus	Negative	Negative	Strong hydrolysis

4.2 Preliminary antimicrobial assays

The fermented supernatant of the A3 and C1 showed significant antimicrobial activity against *Bacillus cereus* ATCC14579, *Escherichia coli* UT181, *Pseudomonas aeruginosa* PA7, *Staphylococcus aureus* RF122 and *Micrococcus luteus* ATCC10240. On the other hand, A1 was only active against Gram-positive bacteria namely *Bacillus cereus* ATCC14579, *Micrococcus luteus* ATCC10240 and *Staphylococcus aureus* RF122 (Table 4.3).

Table 4.3: Antimicrobial assay of the three isolates against selected test bacteria

Isolate / Test bacteria	*W. confusa* A3	*E. faecium* C1	*L. lactis* A1
***Bacillus cereus* ATCC14579**	+++	+++	+++
***Escherichia coli* UT181**	++	++	-
***Listeria monocytogenes* NCTC10890**	-	-	-
***Pseudomonas aeruginosa* PA7**	+++	+++	-
***Staphylococcus aureus* RF122**	-	-	++
***Micrococcus luteus* ATCC10240**	+++	+++	++

Positive signs (+++) indicate antimicrobial activity with inhibition zone >12mm, (++) indicate <12mm. Negative signs (-) indicate lack of antimicrobial activity.

4.3 Molecular assay to identify bacteria

The purified 16S rRNA gene PCR products showed 1500 bp bands on agarose gel. The sequencing results when compared with the database in National Center for Biotechnology Information (NCBI database) using the Basic Local Alignment Search Tool (BLAST) showed that A1 was 99 % similar to *Lactococcus lactis*, A3 was 99 % similar to *Weissella confusa* and C1 was 99 % similar to *Enterococcus faecium*. The sequences were deposited in NCBI gene bank with accession numbers KJ476186.1 and KL675503.1 for *Weissella confusa* A3 and *Enterococcus faecium* C1 respectively as shown in Appendices A and B. The total DNA bands are shown in Figure 4.3. The PCR products are shown in Figure 4.4 and the PCR products after purification in Figure 4.5.

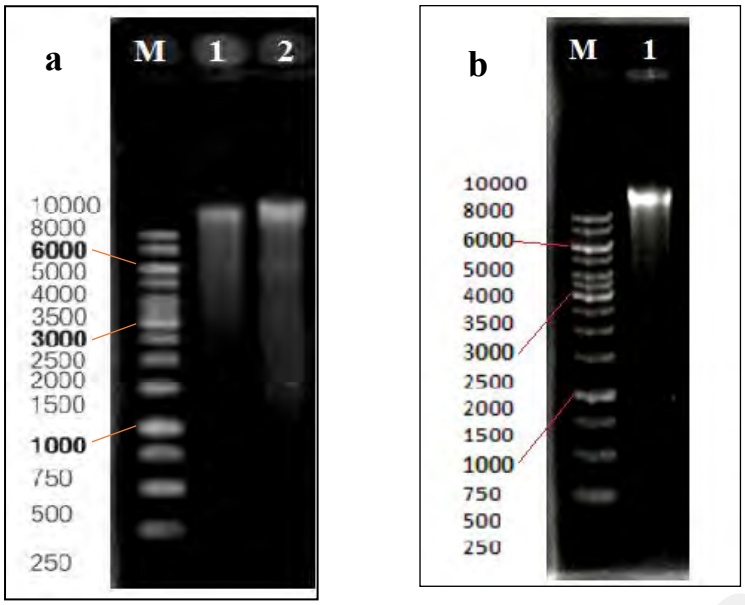


Figure 4.3: Total DNA of A1, A3 (a) and C1 (b). Lane M is DNA ladder; lane 1 is A1, lane 2 is A3 (a); lane 1 is C1 (b).

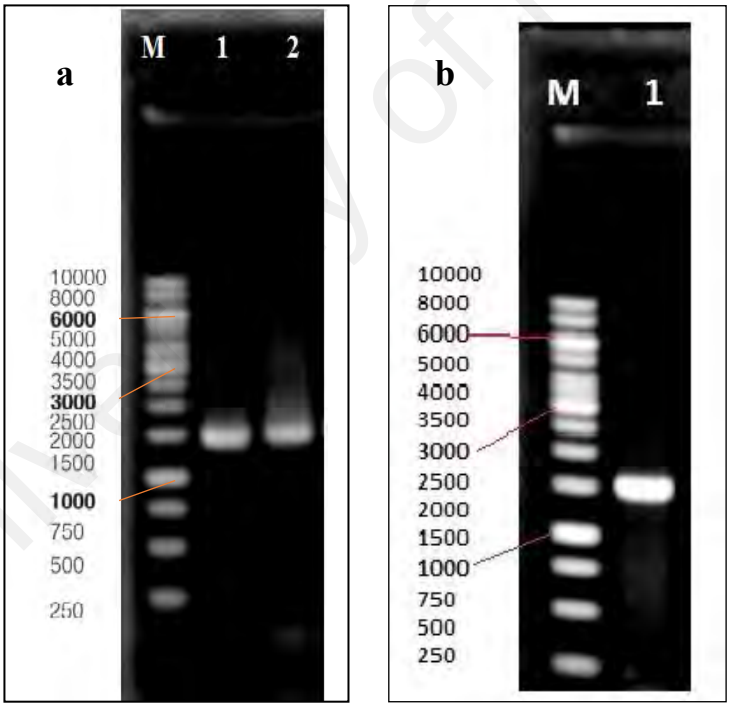


Figure 4.4: PCR products of 16S rRNA genes of A1, A3 (a) and C1 (b). Lane M is DNA ladder; lane 1 is A1, lane 2 is A3 (a); lane 1 is C1 (b).

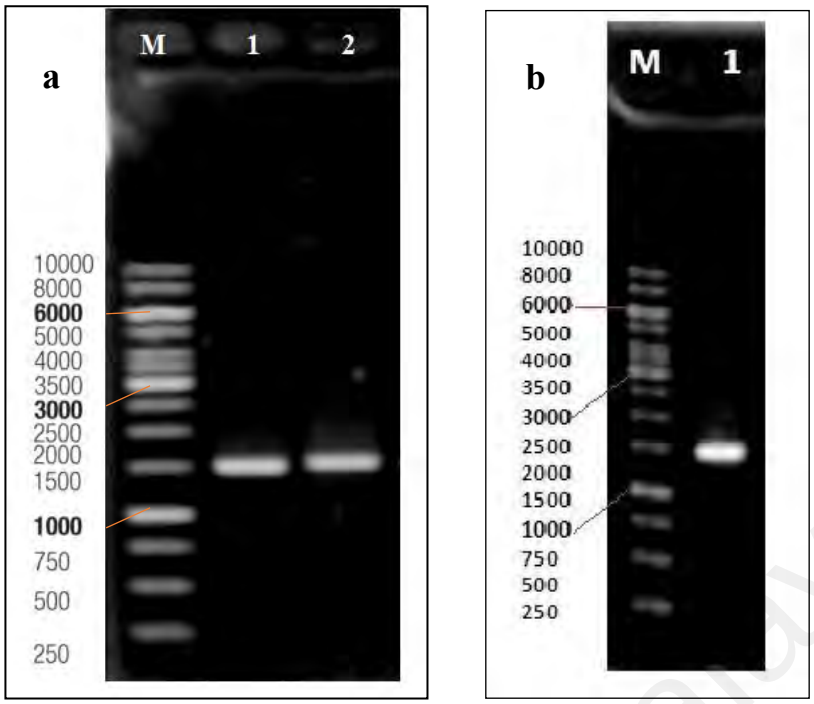


Figure 4.5: PCR products of 16S rRNA genes after purification step. Lane M is DNA ladder; lane 1 is A1, lane 2 is A3 (a); lane 1 is C1 (b).

4.4 API 50 CHL and API 20 strep bacteria identification

Based on interpretation from the API database, the carbohydrate fermentation profile of A3 was shown to be 99.9 % similar to that of *Weissella confusa* and A1 was 84.2% similar to that of *Lactococcus lactis* sub species *lactis 2*. The result of API 20 strep showed that C1 showed 99.4 % similarity to *Enterococcus faecium*. Table 4.4 summarises the API 50 CHL profile of the 3 isolates.

Table 4.4: API 50 CHL carbohydrate fermentation patterns of the bacteria.

Substrate	Isolate			Substrate	Isolate		
	A1	A3	C1		A1	A3	C1
Control	-	-	-	Esculin ferric citrate	+	+	+
Glycerol	-	-	-	Salicin	+	+	+
Erythritol	-	-	-	D-celiobiose	+	+	+
D-arabinose	-	-	-	D-maltose	+	+	+
L-arabinose	-	-	+	D-lactose(bovine origin)	+	-	+
D-ribose	+	-	+	D-melibiose	-	-	+
D-xylose	-	+	-	D-saccharose(sucrose)	+	+	+
L-xylose	-	-	-	D-trehalose	+	-	+
D-adonitol	-	-	-	Inulin	-	-	-
Methyl-βD-xylopyranoside	-	-	-	D-melezitose	-	-	-
D-galactose	+	+	+	D-raffinose	-	-	+
D-glucose	+	+	+	Amidon(starch)	-	-	-
D-fructose	+	+	+	Glycogen	-	-	-
D-mannose	+	+	+	Xylitol	-	-	-
L-sorbose	-	-	-	Gentiobiose	+	+	+
L-rhamnose	-	-	-	D-turanose	-	-	-
Dulcitol	-	-	-	D-lyxose	-	-	-
Inositol	-	-	-	D-tagatose	-	-	+
D-manitol	-	-	+	D-fucose	-	-	-
D-sorbitol	-	-	-	L-fucose	-	-	-
Methyl-αD-mannopyranoside	-	-	+	D-arabitol	-	-	-
Methyl-αD-glucopyranoside	-	-	-	L-arabitol	-	-	-
N-acethylglucosamine	+	+	+	Potassium gluconate	-	+	-
Amygdalin	-	+	+	Potassium 2-ketogluconate	-	-	-
Arbutin	+	-	+	Potassium 5-ketogluconate	-	-	-

The API 50 CHL test only provided the information of the ability of isolate C1 to hydrolyse different carbohydrates but was unable to identify C1 which was shown to be *Enterococcus faecium* from 16S rRNA gene sequencing. Therefore, API 20 strep test was performed to confirm the identity of C1. The results of API 20 strep to identify *Enterococcus* spp. is summarised in Table 4.5. The isolate C1 was positive to 12 tests and negative to 9 tests in API 20 strep.

Table 4.5: API 20 strep of the isolate C1

Test	**Reactions/ Enzymes**	**Result**
VP	Acetoin production (Voges Proskauer)	+
HIP	Hydrolysis (hippuric acid)	+
ESC	B-glucosidase hydrolysis (esculin)	-
PYRA	Pyrrolidonyl arylamidase	+
αGAL	α-galactosidase	-
βGUR	β-glucuronidase	-
βGAL	β-galactosidase	+
PAL	Alkaline phosphate	-
LAP	Leucine amino peptidase	+
ADH	Arginine dihydrolase	+
RIB	Acidification (ribose)	+
ARA	Acidification (arabinose)	+
MAN	Acidification (mannitol)	+
SOR	Acidification (sorbitol)	-
LAC	Acidification (lactose)	+
TRE	Acidification (trehalose)	+
INU	Acidification (inulin)	-
RAF	Acidification (raffinose)	+
AMD	Acidification (amidon)	-
GLYG	Acidification (glycogen)	-
βHEM	β-haemolysis	-

4.5 Growth study

The growth curves of the bacteria showed that both *Lactococcus lactis* A1 and *Weissella confusa* A3 reached stationary phase after 20 hours of incubation. Therefore the bacteriocins should be harvested between 18 to 20 hours during the log phase. On the other hand, C1 reached stationary phase after 18 hours incubation period. Hence the bacteriocins should be harvested between 16 to 18 hours. The growth curves of A1, A3 and C1 are shown in Figures 4.6, 4.7 and 4.8 respectively.

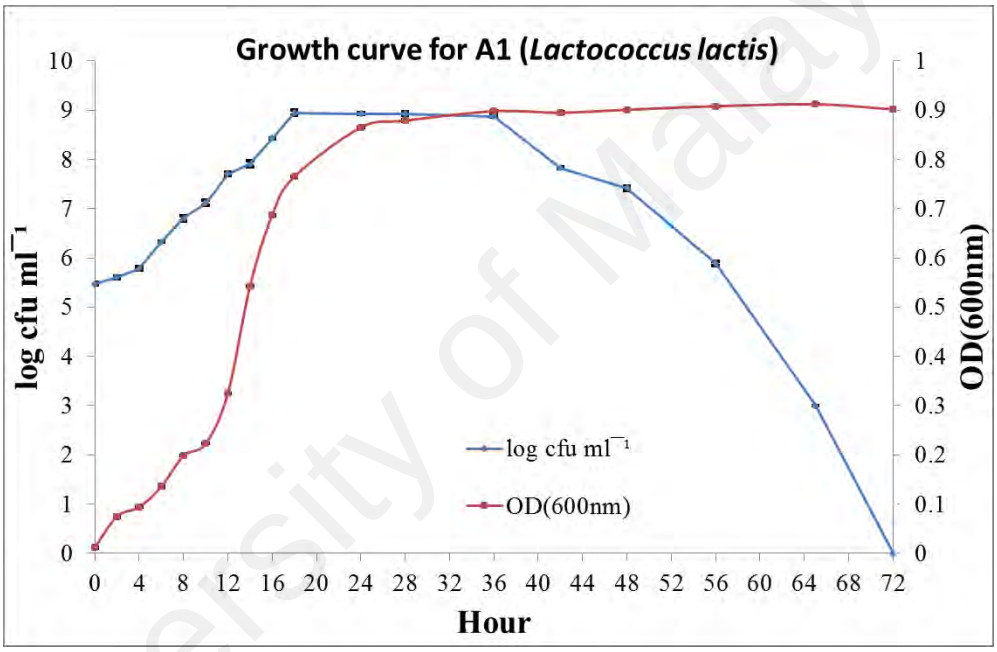


Figure 4.6: Growth profile of A1 (*Lactococcus lactis*). The blue colour line indicates the bacterial concentration measured in log cfu ml^{-1} and the red line indicates the turbidity of the growth measured at wavelength 600nm. N=3 and error bars indicate standard deviation (no error bars if the standard deviations are too small to be plotted).

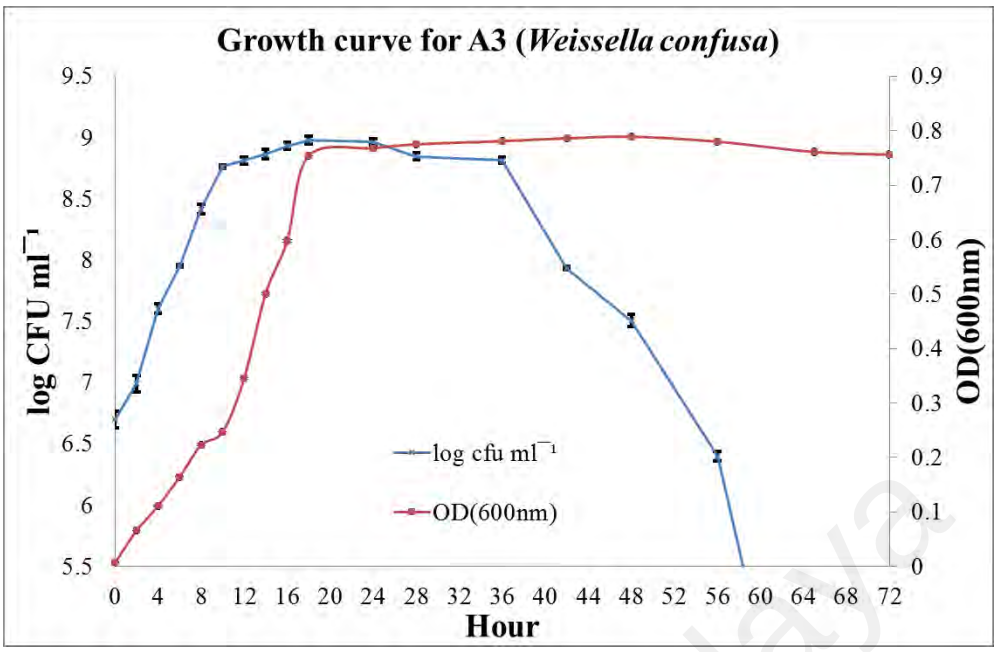


Figure 4.7: Growth profile of A3 (*Weissella confusa*). The blue colour line indicates the bacterial concentration measured in log cfu ml^{-1} and the red line indicates the turbidity of the growth measured at wavelength 600nm. N=3 and error bars indicate standard deviation (no error bars if the standard deviations are too small to be plotted).

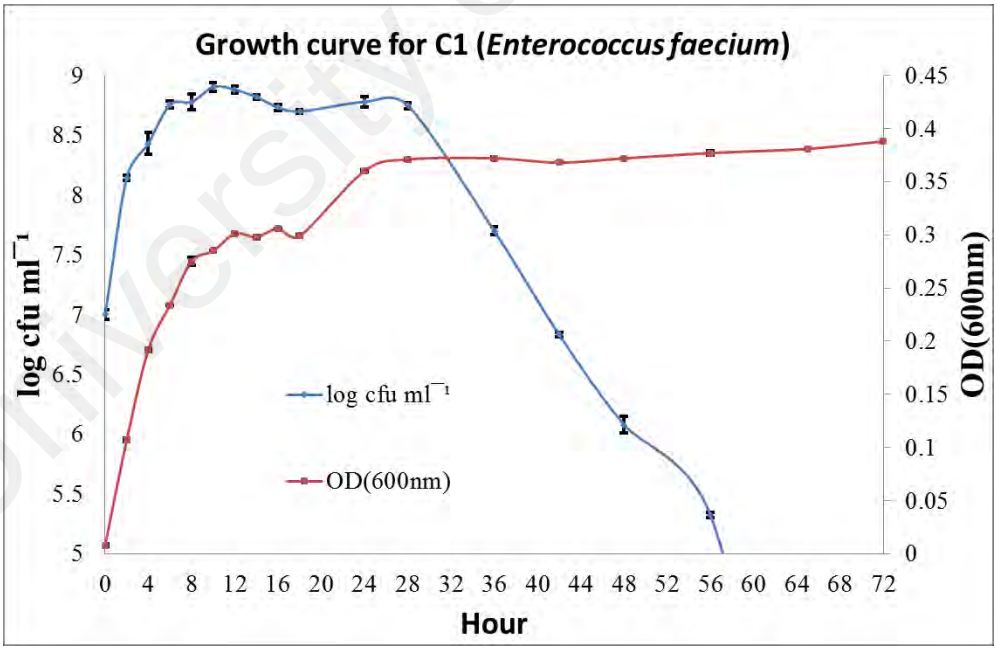
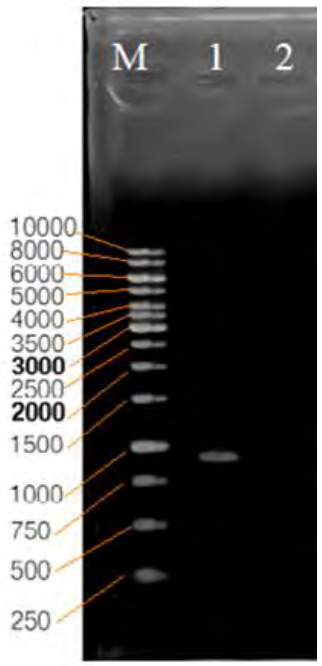


Figure 4.8: Growth profile of C1 (*Enterococcus faecium*). The blue colour line indicates the bacterial concentration measured in log cfu ml^{-1} and the red line indicates the turbidity of the growth measured at wavelength 600nm. N=3 and error bars indicate standard deviation (no error bars if the standard deviations are too small to be plotted).

4.6 PCR amplification to detect genes encoding known bacteriocins

Among the 3 structural genes namely nisin, lactococcin A and lacticin 481, only the gene encoding nisin can be detected from the isolate A1 *Lactococcus lactis*. The amplified gene contained around 900 base pairs as shown in Figure 4.9. There was no DNA band which indicated genes encoding lactococin A and lacticin 481. This suggested that the isolate does not contain genes encoding lactococin A and lacticin 481. After comparing the sequence of the PCR product of the gene encoding nisin with the NCBI database using the Basic Local Alignment Search Tool (BLAST), the gene was shown to be 97 % similar to Nis Z gene with 99 % coverage (Figure 4.10). For *E. faecium* C1, the PCR products of gene encoding entrocin A, B and P showed no band on the agarose gel. None of the primers were able to align and amplify with the DNA template of *E. faecium* C1 in the PCR reaction. This suggested that the *E. faecium* C1 did not harbour gene for enterocin A, B and P production and the antimicrobial activity was from other antimicrobial agent. Since nisin has been well investigated and published in the past, further tests only focused on bacteriocins produced by *W. confusa* A3 and *E. faecium* C1. Figure 4.9 shows the PCR product of the nisin gene and the seqeuce generated from the PCR product.



TTTCTCAGAGTTTGACGCCCAATAACTTAGATTAAA
ATCACCGTCACCTTATTTTTAGGCACGTTCGGCAGT
AACCTTATCAAATGTATCTCAGTCATTGAGTTTCAT
GATAGTATTTACTATTTTGATGGTTTTTGTTATTATCC
AATCGTTAAAATGACAAAAACAAATAGATAAATAG
ATAAATAGATAAATATTTATGGGGAGGACAAGTGAA
CTTATCATGATTAATTGTAAACGATTGAGTTCTCAAT
GTTTCAAATTATGAGGAACAACAGAAGTTGGACTA
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TCTCTTTTACAAAAAATATTTATTTAAGTTATAGTTG
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AGTTCCTGAATAATATAGAGATAGGTTTATTGAGTC
TTAGACATACTTGAATGACCTAGTCTTATAACTATAC
TGACAATAGAAACATTAACAAATCTAAAACAGTCT
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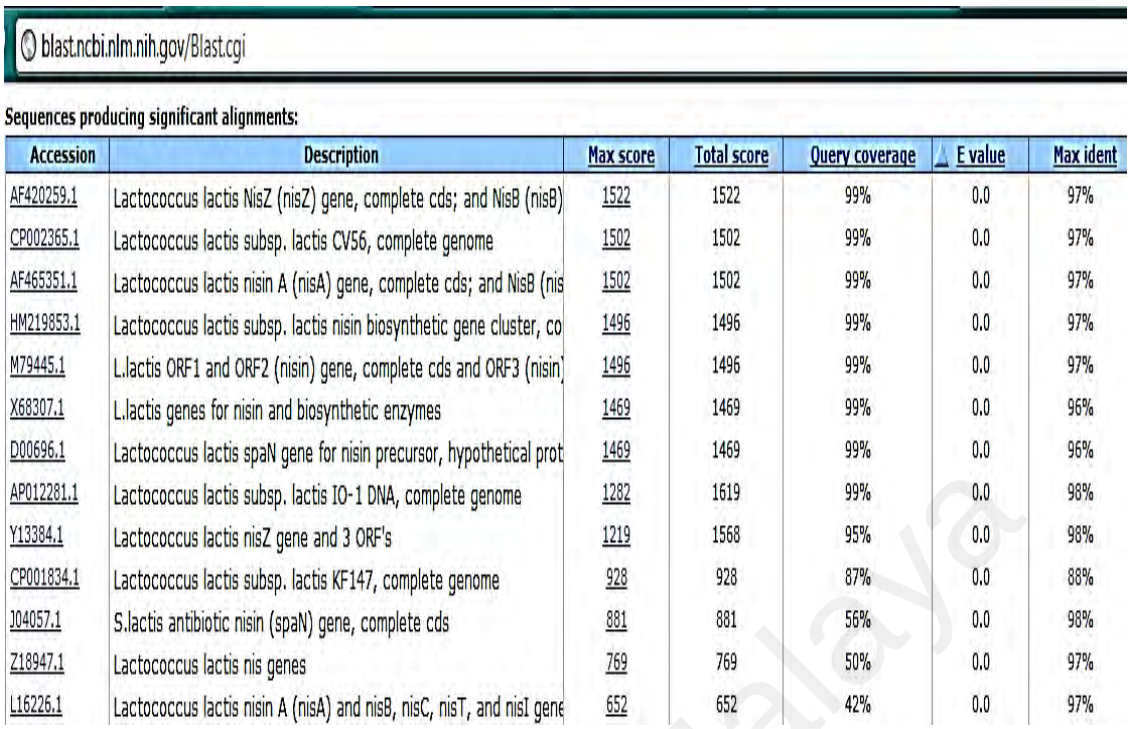
TATCGATAACGCGAGCATAATAAACGGCTCTGATTA
AATTCTGAAGTTTGTTAGATACAAAGATTTCGTTCG
AAGGAACTACAAAATAAATTATAAGGAGGCACTCA
AAATGAGTACAAAAGATTTTAACTTGGATTTGGTAT
CTGTTTCGAAGAAAAATTCAGGTGCATCACCACGC
ATTACAAGTATTTCGCTATGTACACCCGGTTGTAAA
ACAGGAGCTGTGATGGGTTGTAACATGAAAACAG
CAACTTGTAATTGTAGTATTCACGTAAGCAAATAAC
CAAATCAAAGGATAGTTTTTTGTTATTCCAGACCAT
GGAA

Figure 4.9: PCR products of nisin genes after purification step and the sequencing result of the cleaned up PCR product. Lane M is DNA ladder; lane 1 is A1 (*Lactococcus lactis*), lane 2 is negative control.



Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AF420259.1	Lactococcus lactis NisZ (nisZ) gene, complete cds; and NisB (nisB)	1522	1522	99%	0.0	97%
CP002365.1	Lactococcus lactis subsp. lactis CV56, complete genome	1502	1502	99%	0.0	97%
AF465351.1	Lactococcus lactis nisin A (nisA) gene, complete cds; and NisB (nis	1502	1502	99%	0.0	97%
HM219853.1	Lactococcus lactis subsp. lactis nisin biosynthetic gene cluster, co	1496	1496	99%	0.0	97%
M79445.1	L.lactis ORF1 and ORF2 (nisin) gene, complete cds and ORF3 (nisin)	1496	1496	99%	0.0	97%
X68307.1	L.lactis genes for nisin and biosynthetic enzymes	1469	1469	99%	0.0	96%
D00696.1	Lactococcus lactis spaN gene for nisin precursor, hypothetical prot	1469	1469	99%	0.0	96%
AP012281.1	Lactococcus lactis subsp. lactis IO-1 DNA, complete genome	1282	1619	99%	0.0	98%
Y13384.1	Lactococcus lactis nisZ gene and 3 ORF's	1219	1568	95%	0.0	98%
CP001834.1	Lactococcus lactis subsp. lactis KF147, complete genome	928	928	87%	0.0	88%
J04057.1	S.lactis antibiotic nisin (spaN) gene, complete cds	881	881	56%	0.0	98%
Z18947.1	Lactococcus lactis nis genes	769	769	50%	0.0	97%
L16226.1	Lactococcus lactis nisin A (nisA) and nisB, nisC, nisT, and nisI gene	652	652	42%	0.0	97%

Figure 4.10: The PCR sequence of nisin gene detected from *Lactococcus lactis* A1 compared with NCBI database.

4.7 Antibiotic susceptibility and haemolytic tests of the bacteria

4.7.1 Haemolytic test of the bacteria

Both *W. confusa* A3 and *E. faecium* C1 showed γ- haemolysis by which the blood agar remained unchanged. No clear zone surrounding the colony can be seen after 24 hours of incubation. The colonies of *W. confusa* A3 appeared as white colour whereas colonies of *E. faecium* C1 in light brown colour (Figure 4.11).

Figure 4.11: Bacterial colonies grown on blood agar.

4.7.2 Antibiotic susceptibility test

From the results obtained, *E. faecium* C1 was not vancomycin resistant and is sensitive to most of the antibiotics listed on CLSI standard. For *W. confusa* A3, no standard for interpretation is available, but the inhibition zones obtained from amoxycillin, ampicillin, chloramphenicol, clindamycin, ofloxacin and tetracycline were more than 20 mm that indicated it was sensitive to these antibiotics (Table 4.6). *W. confusa* A3 was also shown to be resistant to vancomycin. Therefore no inhibition zone can be observed from the vancomycin disc. The results of antibiotic disc test revealed

that *E. faecium* C1 was not vancomycin and ampicillin resistant and was susceptible to most of the antibiotics listed in the CLSI standard (Table 4.7). The breakpoint of clindamycin and ofloxacin were obtained from *Streptococcus* spp which was the most closely related species with *Enterococcus* spp. According to the breakpoint, the strain is susceptible to ofloxacin but resistant to clindamycin. However, amoxicillin, novobiocin and penicillin G tested in this study was not listed in CSLI standard. But the inhibition zones for amoxicillin and novobiocin showed that the strain was sensitive to both antibiotics. On the other hand, the inhibition zone for penicillin G was much smaller compared to other antibiotics and hence the quantity of penicillin G (1 unit) used was not enough to kill the bacteria.

Table 4.6: Antibiotic susceptibility test of *W. confusa* A3

Antibiotic	Inhibition zone (mm)	Interpretation
Amoxycillin 10 µg	22.26±0.14	N.A.
Ampicillin 10 µg	21.68±1.00	N.A.
Chloramphenicol 30 µg	21.88±0.10	N.A.
Clindamycin 2 µg	21.30±0.46	N.A.
Gentamicin 10 µg	16.3±0.24	N.A.
Novobiocin 5 µg	11.22±0.07	N.A.
Ofloxacin 5 µg	21.28±0.11	N.A.
Penicillin 1 unit	18.54±0.27	N.A.
Penicillin V 10 µg	19.88±0.14	N.A.
Streptomycin 10 µg	13.24±0.36	N.A.
Tetracycline 30 µg	22.72±0.13	N.A.
Vancomycin 30 µg	0	N.A.

The inhibition zone were measured in mm±standard deviation, n= 3 and $P<0.05$
N.A. = no guidelines were available for this antibiotic-bacteria combination
Inhibition zones exceding 20 mm are highlighted.

Table 4.7: Antibiotic susceptibility test of *E. faecium* C1

Antibiotic	Inhibition zone (mm)	CLSI breakpoint[1]			Interpretation
		R	I	S	
Amoxicillin 10 µg	17.46±0.09	-	-	-	N.A.
Ampicillin 10 µg	17.62±0.18	≤16	-	≥17	S
Chloramphenicol 30 µg	18.30±0.21	≤12	13-17	≥18	S
Clindamycin 2 µg	7.30±0.16	≤15[2]	16-18[2]	≥19[2]	R
Gentamicin 10 µg	12.16±0.09	6[3]	7-9[3]	≥10[3]	S
Novobiocin 5 µg	14.20±0.30	-	-	-	N.A.
Ofloxacin 5 µg	16.08±0.02	≤12[2]	13-15[2]	≥16[2]	S
Penicillin G 1 unit	7.06±0.04	-	-	-	N.A.
Penicillin V 10 µg	15.92±0.12	≤14	-	≥15	S
Streptomycin 10 µg	12.22±0.06	6[3]	7-9[3]	≥10[3]	S
Tetracycline 30 µg	22.26±0.14	≤14	15-18	≥19	S
Vancomycin 30 µg	18.34±0.33	≤14	15-16	≥17	S

The inhibition zone were measured in mm±standard deviation, n= 3 and $P<0.05$

N.A.= no guidelines were available for this antibiotic-bacteria combination

[1] S: susceptible, I: intermediate, R: resistant

[2]CLSI breakpoint obtained from *Streptococcus* spp

[3]Breakpoint for High-Level Aminoglycoside Resistance (HLAR)

4.8 Bacteriocin production in different media

Several commercially available media were tested for supporting the growth and production of bacteriocins. For the production of bacteriocin by *W. confusa* A3, among the 10 media used, MRS gave the highest activity followed by LAPTg and M17 supplemented with different carbohydrates. The other 5 media (BHI, TSB with 1 % tween 80, Brucella broth, NB with 1.5 % glucose and Miller's LB broth) can also be used to grow the bacteria but the production of bacteriocin was lower and no bacteriocin could be recovered from the culture grown in Brucella broth. On the other hand, the maximum production of bacteriocin by *E. faecium* C1 was obtained in LAPTg media followed by MRS media. Lower amount of bacteriocin were produced in BHI, TSB with 1 % tween 80, Brucella broth, NB with 1.5 % glucose and Miller's LB broth. No bacteriocin can be produced when Brucella broth was used to grow *E. faecium* C1. The inhibition zone is shown in Table 4.8.

Table 4.8: Antimicrobial activity of bacteriocin recovered from different media

Media	MRS	M17+1.5 % sucrose	M17+1.5 % glucose	M17+1.5 % lactose	BHI	LAPTg	TSB+1% tween 80	NB+1.5% glucose	LB+1.5 % glucose	Brucella broth
***W. confusa* A3**	11.62±0.17	9.68±0.22	9.22±0.13	7.88±0.05	7.02±0.09	9.52±0.37	6.84±0.07	6.18±0.05	7.86±0.31	-
***E. faecium* C1**	11.32±0.12	10.28±0.19	10.46±0.12	9.82±0.13	8.46±0.26	12.68±0.10	8.60±0.13	8.68±0.11	9.08±0.07	-

Inhibition zone measured in millimeters (mm±standard deviation), n= 3 and $P<0.05$

4.9 Time plot of bacteriocin production

For *W. confusa* A3, the antimicrobial assay from the crude bacteriocin purified from the supernatant showed that the bacteriocin production started at 8 hours of incubation and reached an optimum production at 18 hours of incubation. The antimicrobial activity remained almost the same between 18 to 24 hours of growth. However, the activity of bacteriocin started to decrease at 28 hours of growth (Figure 4.12). Therefore the bacteriocin was best harvested between 18 to 24 hours to get optimum production. For *E. faecium* C1, the bacteriocin activity started to be detected at 12 hours of growth. The maximum antimicrobial activity was detected at 24 hours of growth and reduced activity was observed after that (Figure 4.13).

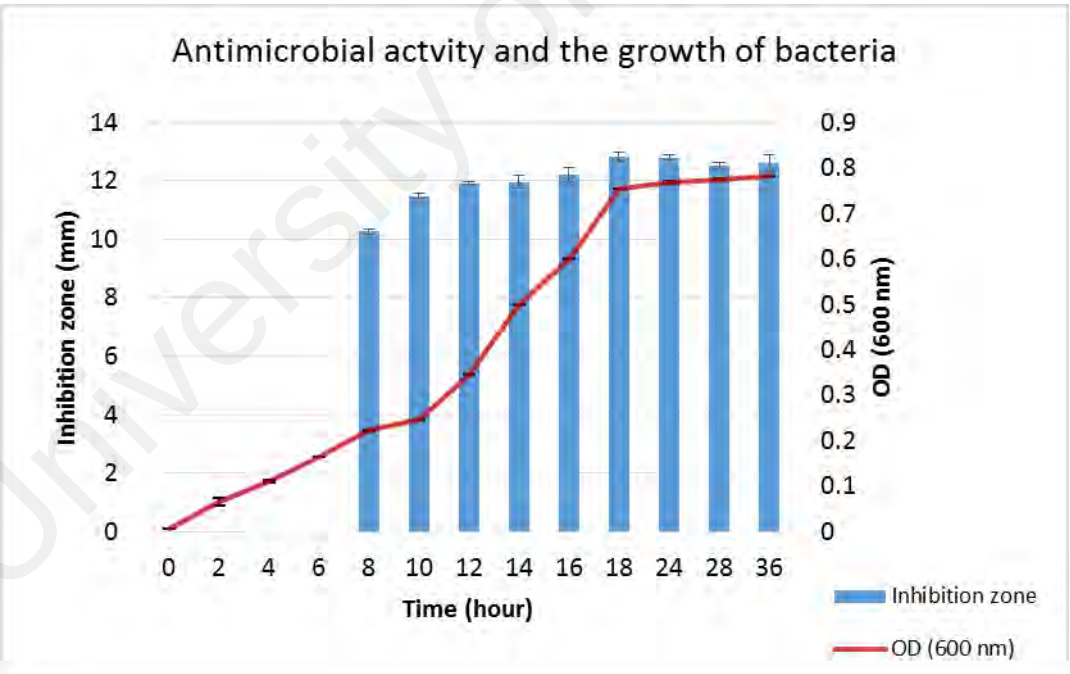


Figure 4.12: Growth curve and bacteriocin biosynthesis of *W. confusa* A3 cultured in MRS broth. The OD_{600} were measured in triplicate and the error bars absent if the standard deviations were too small to be plotted on the graph. The inhibition zones were measured in mm±standard deviation, n= 3 and $P<0.05$.

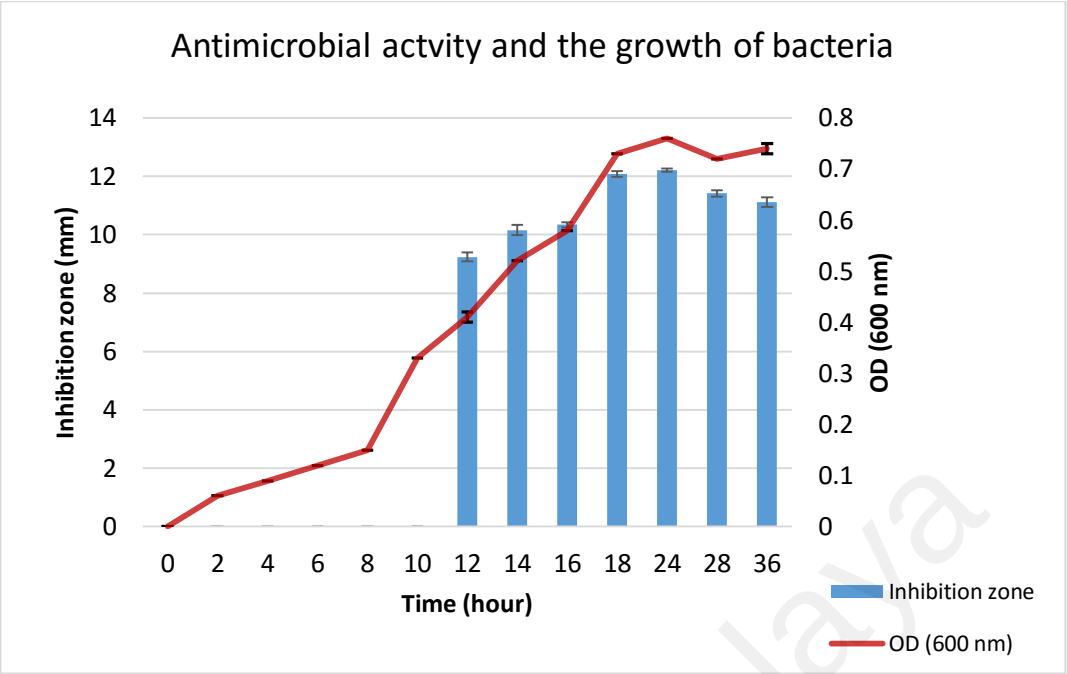


Figure 4.13: Growth curve and bacteriocin biosynthesis of *E. faecium* C1 cultured in LAPTg broth. The OD_{600} were measured in triplicate and the error bars absent if the standard deviations were too small to be plotted on the graph. The inhibition zones were measured in mm±standard deviation, n= 3 and $P<0.05$.

4.10 Effect of carbon sources

Effect of the three different carbohydrates (glucose, lactose and sucrose) supplemented in MRS showed that there was no significant contribution of the different carbohydrates on the inhibition. But 2 % of glucose gave slight better inhibition zones for *W. confusa* A3 ($P=1.19\times10^{-5}$, $P<0.05$). The MRS supplemented with lactose showed the least antimicrobial activity. However, no significant difference ($P=0.11$, $P>0.05$) was observed for antimicrobial activity from *E. faecium* C1 cultured in MRS supplemented with different carbohydrates. Another observation was MRS supplemented with glucose that showed darkest colour after autoclave sterilisation and the crude bacteriocins appeared to be darker compared to MRS supplemented with sucrose and lactose. Figure 4.14 shows the growth of bacteria in MRS supplemented

with different carbohydrates and Figure 4.15 shows the well diffusion assays of the different carbohydrates supplemented in MRS tested against *B. cereus*.

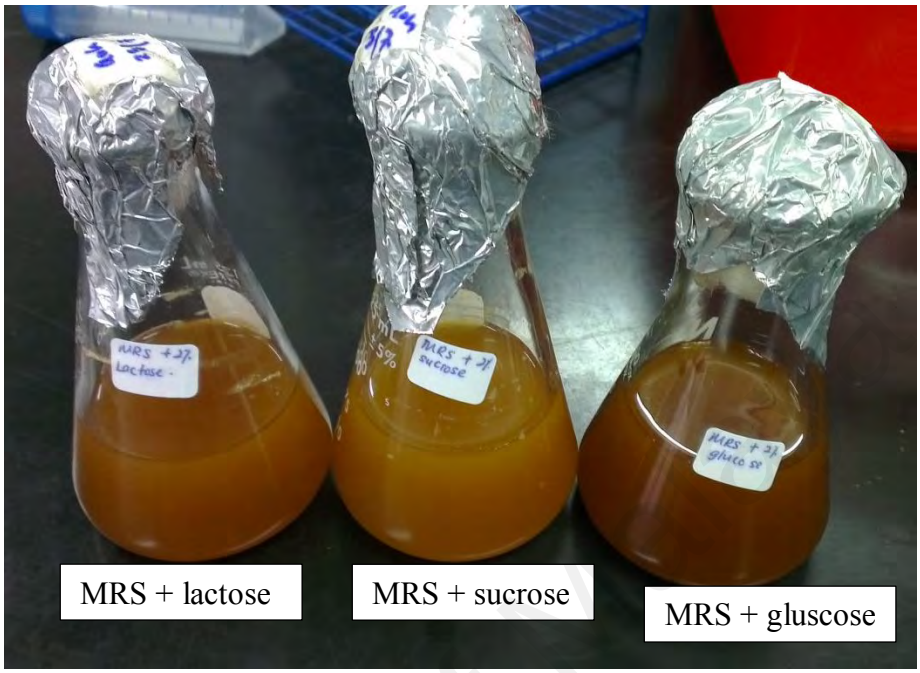


Figure 4.14: The growth of bacteria in MRS supplemented with different carbohydrates.

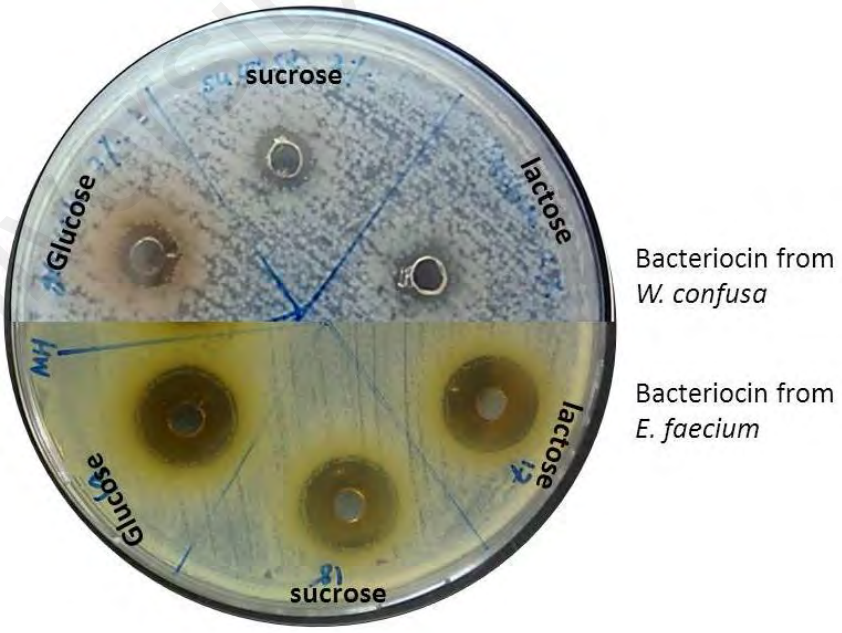


Figure 4.15: Antimicrobial assay of different carbohydrates supplemented in MRS tested against *Bacillus cereus*.

4.11 Amberlite XAD 16 hydrophobic interaction column & Vivaspin centrifugal filter and size separation concentrator

The precipitates from ammonium sulphate from both *W. confusa* A3 and *E. faecium* C1 were found to be active against several test bacteria (*Bacillus cereus* ATCC14579, *Escherichia coli* UT181, *Pseudomonas aeruginosa* PA7 and *Micrococcus luteus* ATCC10240). Three fractions were obtained from the Amberite XAD 16 column. For *W. confusa* A3 and *E. faecium* C1 only fractions eluted out with 50 % and 90 % of acetonitrile gave activity against *B. cereus* (Figure 4.16 and Figure 4.17). The antimicrobial assays of *W. confusa* A3 and *E. faecium* C1 at different purification stages are summarised in Tale 4.9 and 4.10 respectively. For *W. confusa* A3 the 50 % fraction was fractionated with Vivaspin and sample fraction of size 2-5 kDa showed positive result when tested against *B. cereus* (Figure 4.18). On the other hand, the result of Vivaspin showed that the size of bacteriocin from *E. faecium* C1was between 5-50 kDa (Figure 4.19).

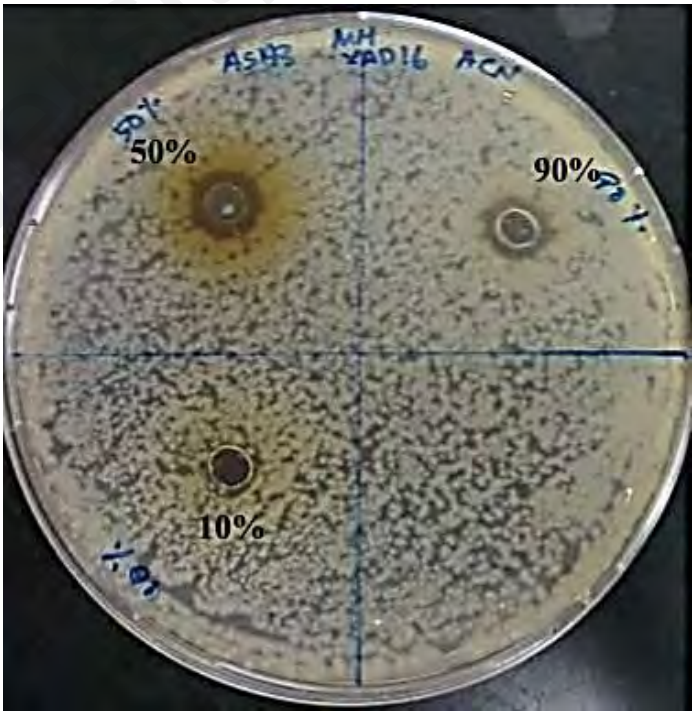


Figure 4.16: Antimicrobial activity of different fractions from Amberlite XAD 16 column. Clockwise from bottom left are samples from *W. confusa* A3 eluted out with 10 %, 50 % and 90 % acetonitrile.

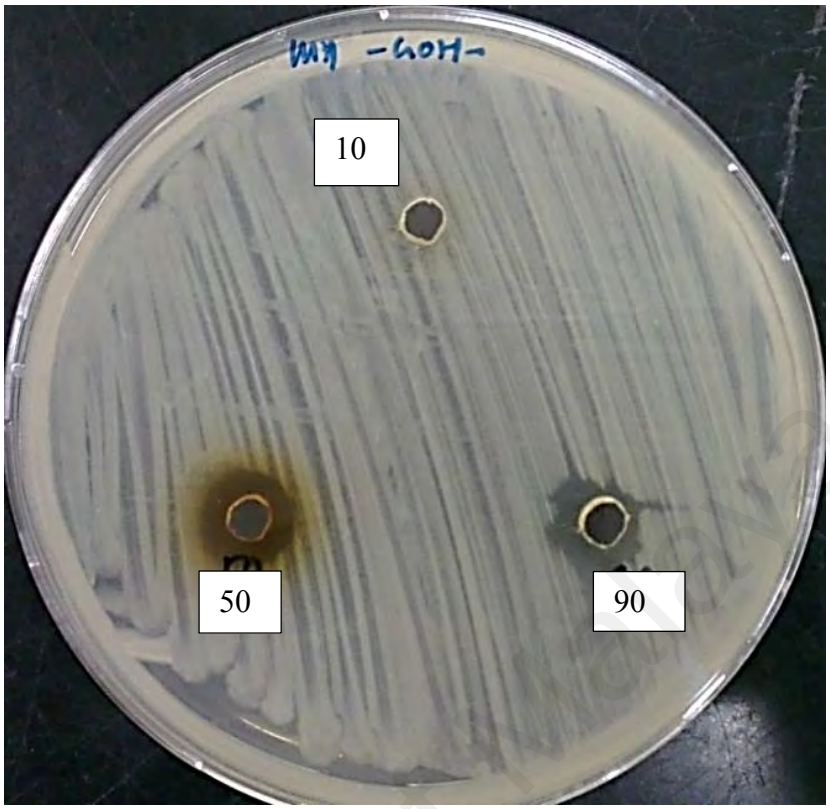


Figure 4.17: Antimicrobial activity of different fractions from Amberlite XAD 16 column. Anti-clockwise from top are samples from *E. faecium* C1 eluted out with 10 %, 50 % and 90 % acetonitrile against *B. cereus*.

Table 4.9: Antimicrobial assay of *W. confusa* A3 at different purification stages

Test bacteria	Ammonium sulphate precipitation*	Amberlite XAD 16 (10 % ACN)	Amberlite XAD 16 (50 % ACN)*	Amberlite XAD 16 (90 % ACN)*
Bacillus cereus ATCC14579	10.67±0.58	0	11.89±0.06	8.69±0.1
Escherichia coli UT181	7.98±0.06	0	8.49±0.25	8.2±0.08
Listeria monocytogenes NCTC10890	0	0	0	0
Pseudomonas aeruginosa PA7	11.83±0.89	0	11.59±0.26	9.03±0.08
Staphylococcus aureus RF122	0	0	0	0
Micrococcus luteus ATCC10240	8.59±0.03	0	11.57±0.10	8.79±0.14

*Inhibition zones measured in ±standard deviation millimeter (mm), n=3 and P<0.05.

Table 4.10: Antimicrobial assay of *E. faecium* C1 at different purification stages

Test bacteria	Ammonium sulphate precipitation*	Amberlite XAD 16 (10 % ACN)	Amberlite XAD 16 (50 % ACN)*	Amberlite XAD 16 (90 % ACN)*
Bacillus cereus ATCC14579	11.18±0.16	0	12.27±0.27	10.39±0.14
Escherichia coli UT181	8.42±0.2	0	8.53±0.28	7.58±0.10
Listeria monocytogenes NCTC10890	0	0	0	0
Pseudomonas aeruginosa PA7	11.31±0.28	0	11.26±0.29	9.10±0.39
Staphylococcus aureus RF122	0	0	0	0
Micrococcus luteus ATCC10240	9.31±0.11	0	12.05±0.50	8.92±0.77

*Inhibition zones measured in ±standard deviation millimeter (mm), n=3 and P<0.05.

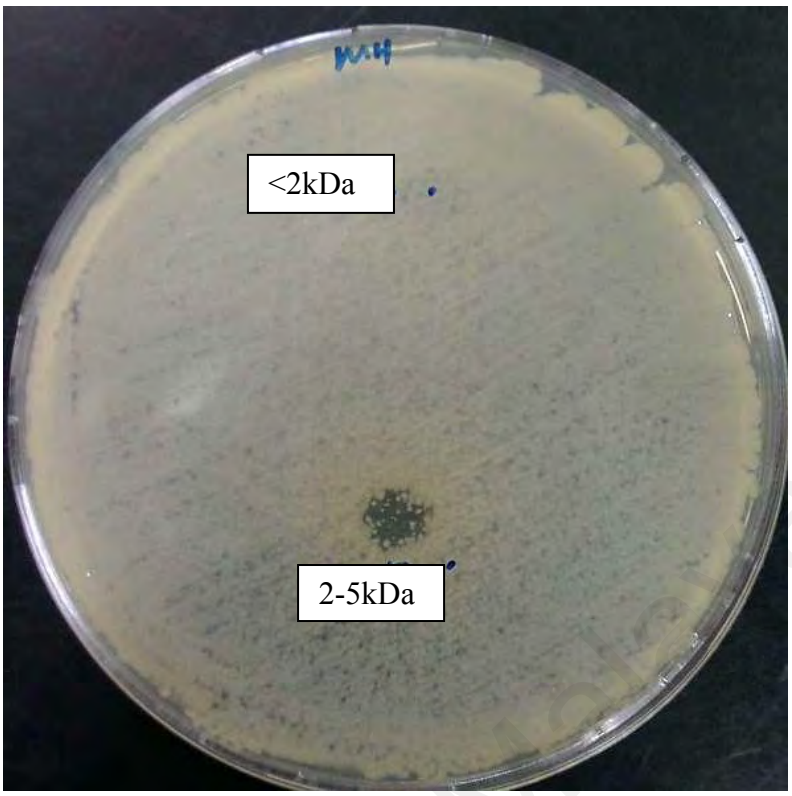


Figure 4.18: Antimicrobial activity of two different fractions of bacteriocins from *W. confusa* A3 obtained from Vivaspin. The top spot is < 2 kDa and the bottom spot is 2-5 kDa.

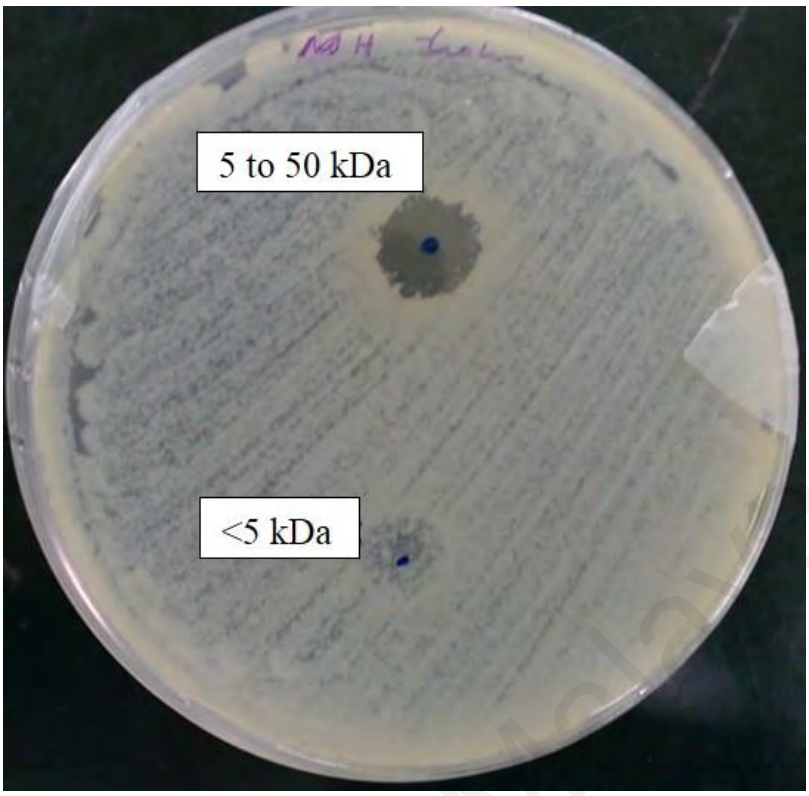


Figure 4.19: Antimicrobial activity of two different fractions of bacteriocin from *E. faecium* C1 obtained from Vivaspin. The top spot is 5-50 kDa and the bottom spot is < 5 kDa.

4.12 Reverse-phase high-performance liquid chromatography (RP-HPLC)

The active fractions of both bacteriocin from *W. confusa* A3 and *E. faecium* C1 obtained from vivaspin column was injected into HPLC system equipped with Chromolith SemiPrep RP-18e column (Merck, Germany). The fractions were collected and tested against *B. cereus*. The HPLC profile for the peptide from *W. confusa* A3 is shown in Figure 4.20. The active fraction was eluted out from 36 to 39 minutes and tested to be active against *B. cereus*.

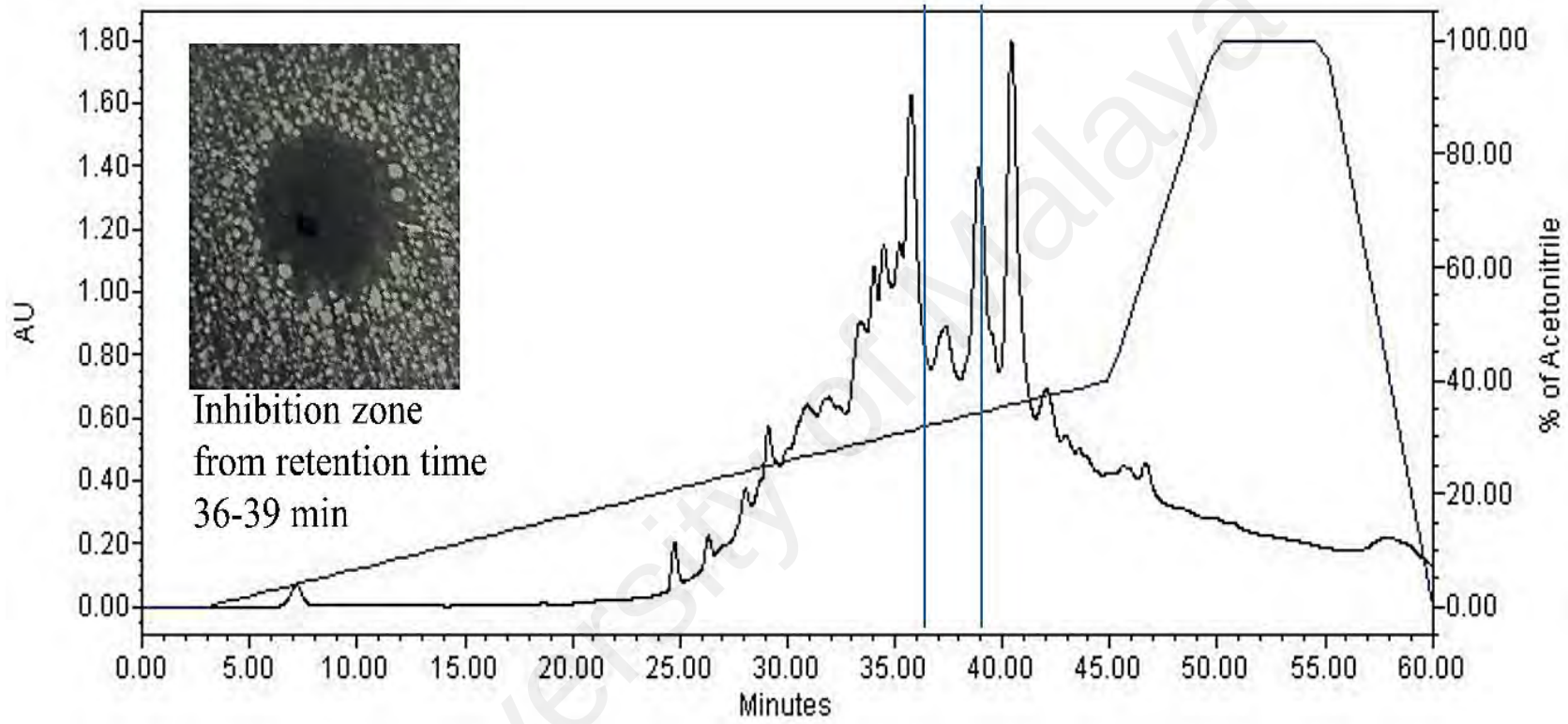


Figure 4.20: RP-HPLC profile of active faction isolated from *Weissella confusa* A3. The antimicrobial activity was found between 36 to 39 minute elution periods. The straight line indicates the percent concentration of acetonitrile.

For *E. faecium* C1, all fractions from the HPLC run were tested and the fraction obtained at retention period between 33-36 minutes was shown to inhibit the growth of *B. cereus*. This fraction consisted of 4 peaks and was eluted out with 30-32 % acetonitrile (Figure 4.21). The antimicrobial assay is also shown in Figure 4.21 also. The runs were repeated to collect replicate samples of the bacteriocin and then lyophilised using the same retention time for subsequent tests. The active fractions were named BacA3 and BacC1 in the subsequence tests.

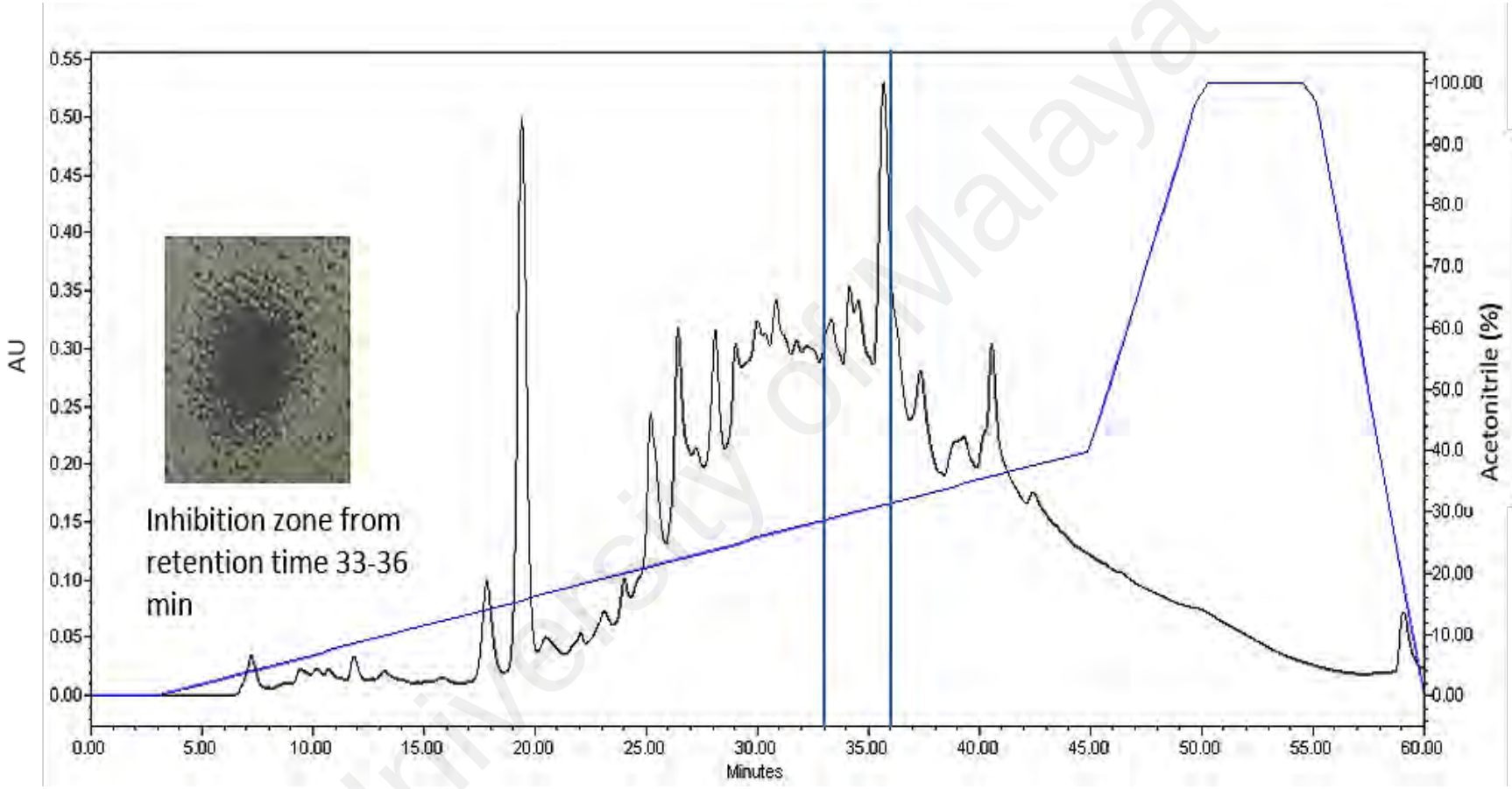


Figure 4.21: RP-HPLC profile of active faction isolated from *Enterococcus faecium* C1. The antimicrobial activity was found between 33 to 36 minute elution periods. The straight line indicates the percent concentration of acetonitrile.

4.13 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC value of HPLC fraction from *W. confusa* A3 worked out to be 9.25 µg/ml against both *B. cereus* and *M. luteus*. However, the MIC value increased to 18.5 µg/ml against *E. coli* and *P. aeruginosa*. After plating 10 µl of the overnight bacteriocin with the bacterial solution from the wells but without any bacterial growth after incubation, the MBC obtained for *B. cereus* and *M. luteus* was 37 µg/ml. The MBC for *E. coli* and *P. aeruginosa* was 74 µg/ml. The MBC of XAD 16 and HPLC fraction were 1.25 mg/ml against all the target bacteria. For *E. faecium* C1, the MIC values for HPLC fraction was 9.81 µg/ml against *B. cereus*, *E. coli* and *M. luteus*. The MIC increased to 19.63 µg/ml against *P. aeruginosa*. On the other hand, the MIC for XAD 16 was 1.25 mg/ml against all test bacteria. Table 4.11 summarises MIC and MBC results obtained from the 2 bacteriocins and Figure 4.22 illustrates the result of MIC obtained by agar dilution method. The MIC for XAD 16 purification was higher because it was measured by the dry weight of the peptides whereas the MIC for HPLC purification was done by using the concentration of peptide from Bradford protein quantification assay.

Table 4.11: MIC and MBC values of bacteriocins produced by *W. confusa* A3 and *E. faecium* C1

	Bacteriocin from *W. confusa* A3			**Bacteriocin from *E. faecium* C1**		
	MIC (HPLC fraction)	**MIC (XAD 16)**	**MBC (HPLC fraction)**	**MIC (HPLC fraction)**	**MIC (XAD 16)**	**MBC (HPLC fraction)**
B. cereus	9.25	625	37	9.81	1250	39.25
E. coli.	18.5	1250	74	9.81	1250	39.25
M. luteus	9.25	625	37	9.81	1250	39.25
P. aeruginosa	18.5	625	74	19.63	1250	78.5

All concentrations in µg/ml, n= 3, standard deviation =0

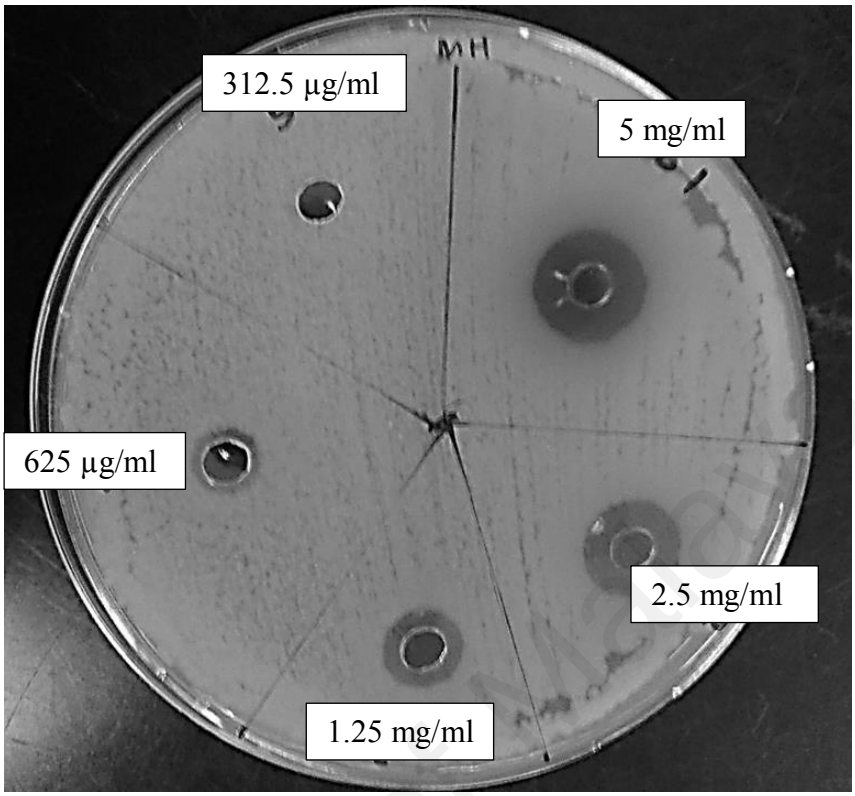


Figure 4.22: Antimicrobial assay of different concentrations of bacteriocin of *W. confusa* A3 purified from Amberlite XAD 16 tested against *B. cereus*. Clockwise from right are crude bacteriocin with concentration 5 mg/ml, 2.5 mg/ml, 1.25 mg/ml, 625 µg/ml and 312.5 µg/ml.

4.14 Effect of temperature, pH and enzyme on activity of bacteriocin

4.14.1 Heat stability test

The heat stability test showed that the bacteriocin from *W. confusa* A3 (BacA3) was heat stable and retained its inhibitory activity after heating at 100 °C for 20 minutes. The bacteriocin from *E. faecium* C1 (BacC1) was also heat stable and remained active against the test bacteria after heating to 40 °C and 60 °C for 20 min. However, the activity of the bacteriocin reduced after heating to 80 °C and 100 °C. Table 4.12 summarises the inhibition zones produced on *B. cereus* lawn and the residue activity after each treatment.

4.14.2 pH stability test

Both bacteriocins BacA3 and BacC1 only function at a low pH range of 2 to 6 and no inhibition activity can be observed at bacteriocins treated with pH higher than 6. The inhibition zones are shown in Table 4.12.

Table 4.12: Heat stability test of bacteriocin produced by *W. confusa* A3 and *E. faecium* C1 against *B. cereus*

	Heat treatment	**Inhibition zone (mm) ***	**Residue activity (%)**	**pH stability**	**Inhibition zone (mm) ***
BacA3	Untreated	11.64±0.23	100	2	11.56±0.56
	40 °C	11.42±0.07	96.69	3	11.30±0.08
	60 °C	11.14±0.06	92.47	4	11.25±0.14
	80°C	11.12±0.09	92.17	5	11.25±0.05
	100 °C	11.00±0.14	90.36	6	11.27±0.09
				7-10	0
BacC1	Untreated	12.36±0.05	100	2	12.50±0.11
	40 °C	12.04±0.10	95.65	3	12.26±0.12
	60 °C	11.94±0.09	94.28	4	12.24±0.22
	80°C	9.12±0.07	55.98	5	12.61±0.11
	100 °C	8.16±0.11	42.93	6	12.31±0.29
				7-10	0

*Inhibition zones measured in mm±standard deviation, n=3 and $P<0.05$.

4.14.3 Effect of enzymes

For enzyme stability test, the bacteriocins BacA3 and BacC1 were stable after treatment with lysozyme, lipase, catalase and lyticase but showed reduction of activity after treatment with proteinase K and peptidase. The reduction of activity by proteolytic enzymes confirmed that the antimicrobial substance was not of lipid nature but was proteinaceous in nature. The result of the enzyme treatments and their residue activity is tabulated in Table 4.13.

Table 4.13: Effect of enzymes on the bacteriocin activity against *B. cereus*

	Treatment	**Inhibition zone (mm)***	**Residue activity (%)**
BacA3	Untreated control	11.50±0.13	100
	Proteinase K	8.78±0.17	58.12
	Lysozyme	11.48±0.36	99.69
	Lipase	11.44±0.13	99.08
	Catalase	11.50±0.40	100
	Lyticase	11.44±0.18	99.69
	Peptidase	8.86±0.28	59.38
BacC1	Untreated control	12.2±0.16	100
	Proteinase K	9.54±0.11	49.17
	Lysozyme	12.02±0.11	97.5
	Lipase	12.24±0.07	100.56
	Catalase	12.08±0.06	98.33
	Lyticase	12.12±0.02	99.34
	Peptidase	9.68±0.06	55.28

*Inhibition zones measured in mm ±standard deviation, n=3 and $P<0.05$.

4.15 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

When the BacA3 was subjected to SDS-PAGE, only a single band was detected confirming its high purity. The purified bacteriocin showed molecular weight approximately 2.5 kDa compared to the marker used (Figure 4.23). The SDS-PAGE gel stained with SimplyBlue showed a clear band with molecular mass at around 10 kDa for BacC1 (Figure 4.24).

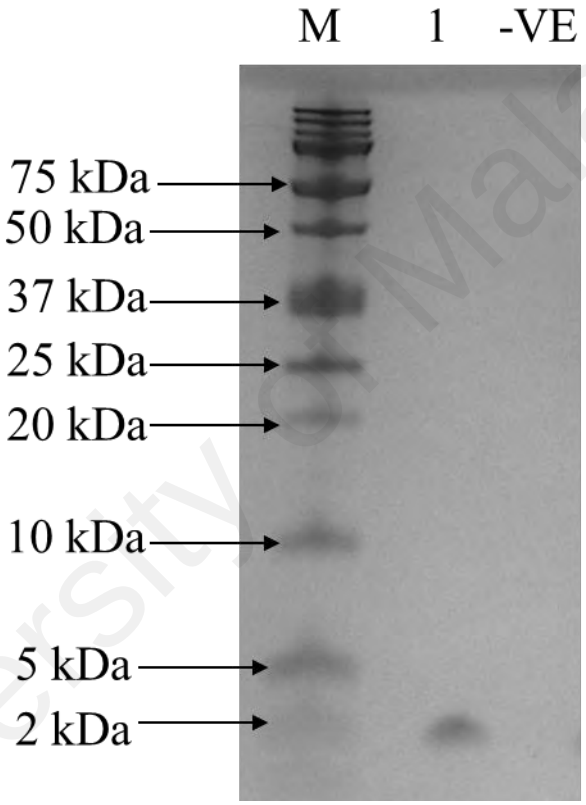


Figure 4.23: SDS-PAGE gel picture. Lane M is Precision Plus Protein™ Dual Xtra Standards (Bio-Rad, USA), lane 1 is HPLC fraction BacA3 purified from *W. confusa* A3 and –VE is negative control with only sample buffer.

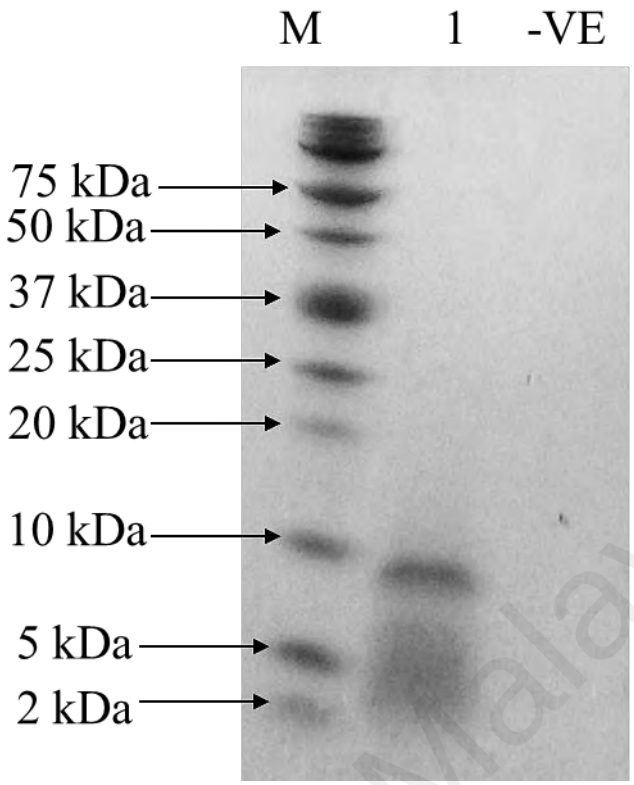


Figure 4.24: SDS-PAGE gel picture. Lane M is Precision Plus Protein™ Dual Xtra Standards (Bio-Rad, USA), lane 1 is HPLC fraction of BacC1 purified from *E. faecium* C1 and –VE is negative control with only sample buffer.

4.16 MALDI-TOF analysis

4.16.1 Sample in liquid form, without digestion

The MALDI-TOF chromatogram of BacA3 isolated from *Weissella confusa* A3 showed that the sample had a high density peak at 2706.6855 Da and no other peaks were detected subsequently (Figure 4.25). Therefore the possible molecular weight of the sample was approximately 2.7 kDa.

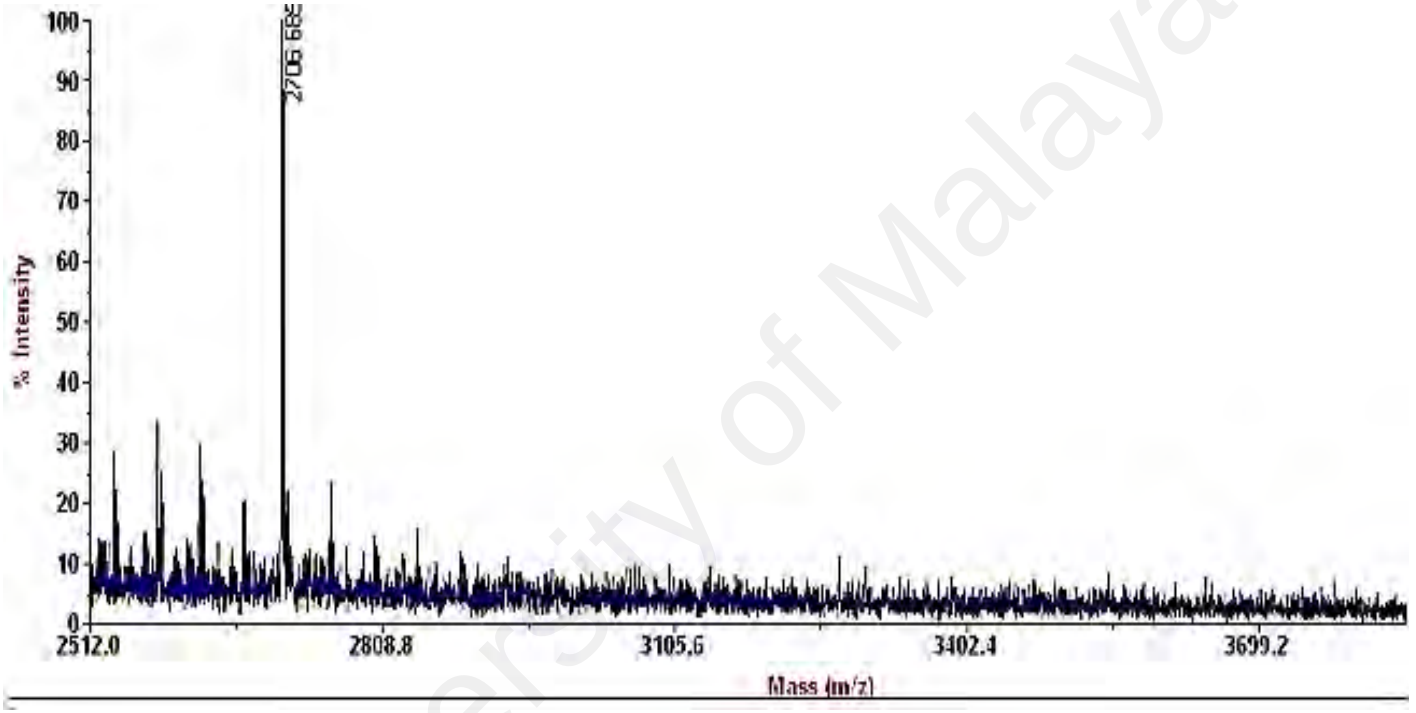


Figure 4.25: MALDI-TOF MS analysis of BacA3. The possible molecular weight was 2706.6855 Da.

4.16.2 Sample in gel form after digestion

Gel digestion was performed for BacC1 because the molecular weight is larger and cannot be analysed in solution form. The SDS-PAGE gel stained with SimplyBlue showed a clear band with molecular mass at around 10 kDa (Figure 4.26). The band was cut and digested with trypsin. The bacteriocin masses were sent to SWISS-PROT using the MASCOT (Matrix Science) Peptide Mass Finger printing program. The mono-isotopic masses and significance threshold of $p<0.05$ were used. No significant matching to the protein database was available that indicated it may be an unidentified novel protein. The following peak list in the m/z ratio was available: 813.7020, 820.5125, 838.5165, 842.4891, 860.5031, 876.4777, 892.4455, 931.5768, 931.5768, 971.5665, 987.5413, 1003.5074, 1042.6436, 1082.6335, 1098.6091, 1114.5751, 1153.7107, 1193.6996, 1209.6763, 1225.6375, 1264.7808, 1304.7681, 1320.7424, 1333.7998, 1336.7086, 1375.8496, 1415.8337, 1431.8047, 1444.8575, 1447.7788, 1486.9182, 1512.6608, 1526.9003, 1542.8745, 1558.8341, 1597.9824, 1637.9673, 1653.9443, 1666.9948, 1669.9137, 1709.0535, 1749.0332, 1765.0128, 1778.0536, 1780.9816, 1820.1227, 1860.1025, 1876.0800, 1892.0282, 1931.1957, 1971.1656, 1987.1464, 2042.2665, 2082.2375, 2098.2070, 2153.3352, 2193.3013, 2264.4048, 2304.3635, 2375.4788, 2415.4316, 2486.5437, 2526.4990, 2597.6130, 2637.5752, 2708.6965

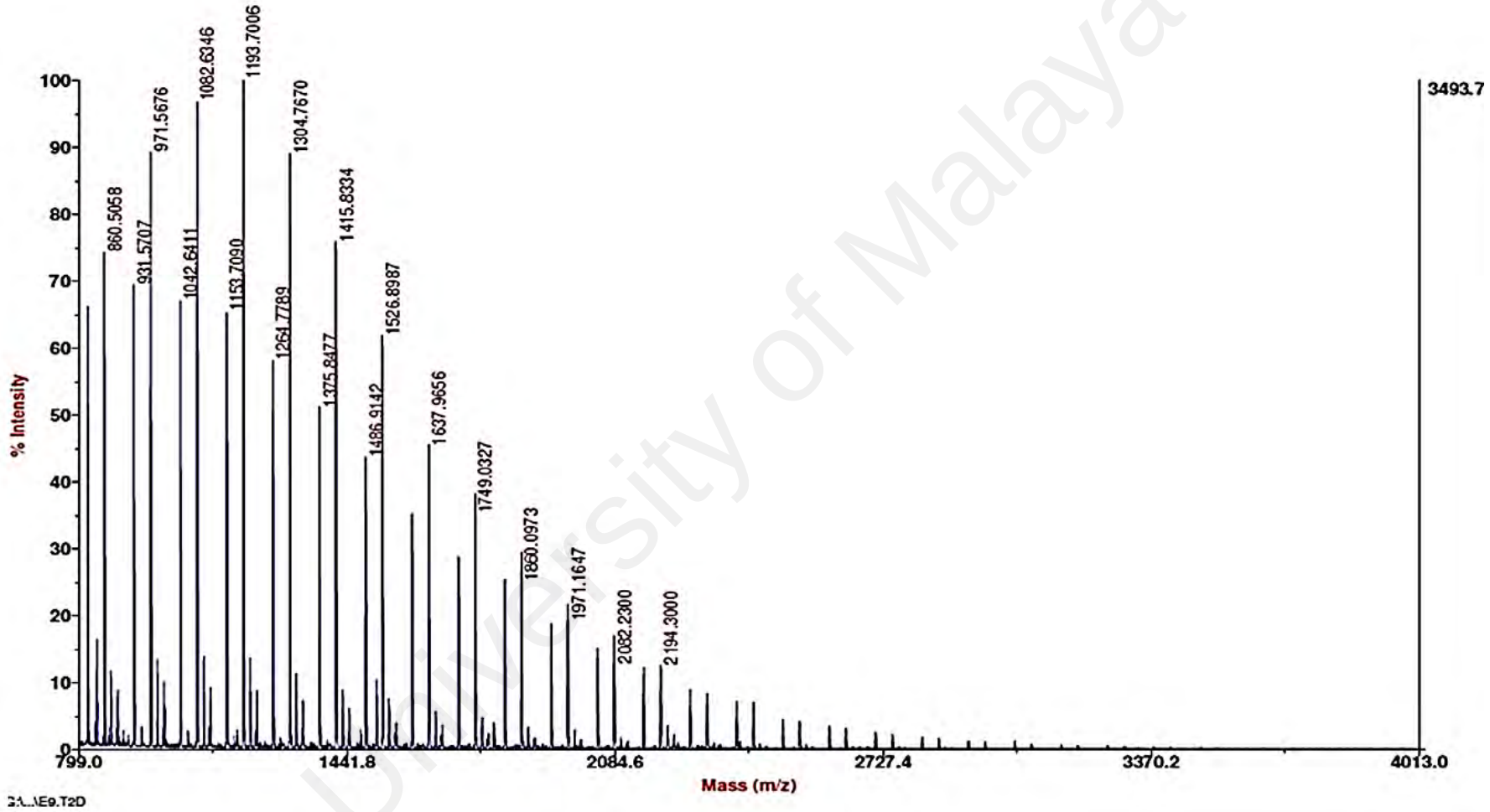


Figure 4.26: MALDI-TOF MS analysis of BacC1 after trypsin digestion.

4.17 Membrane permeabilization test using Real-Time PCR

The real-time PCR fluorescence traces showed that the bacteria treated with BacA3 at dilution 2:1 had the highest fluorescence density after that of the positive control (NaOH, 1M). The fluorescence density decreased when the dilution factor increased. Negative control without adding any bacteriocin and bacteria with tetracycline added showed the lowest fluorescence density (Figure 4.27). This was because tetracycline acted by inhibiting protein synthesis in bacteria and did not break the bacterial membrane. Hence, no penetration of the SYTOX® green nucleic acid stain into the bacterial cell and the same for negative control whereby no substance was present to disrupt the bacterial membrane and allow the fluorescence dye to bind with the bacterial DNA. The fluorescence dye only bound to the inner DNA of the bacteria and cannot penetrate the cell membrane. So once the bacteriocins permeabilized the cell membrane, the SYTOX® green nucleic acid stain will bind to the inner DNA and the fluorescence can be detected by real-time PCR. This was the evidence that the bacteriocins permeabilized the cell membrane.

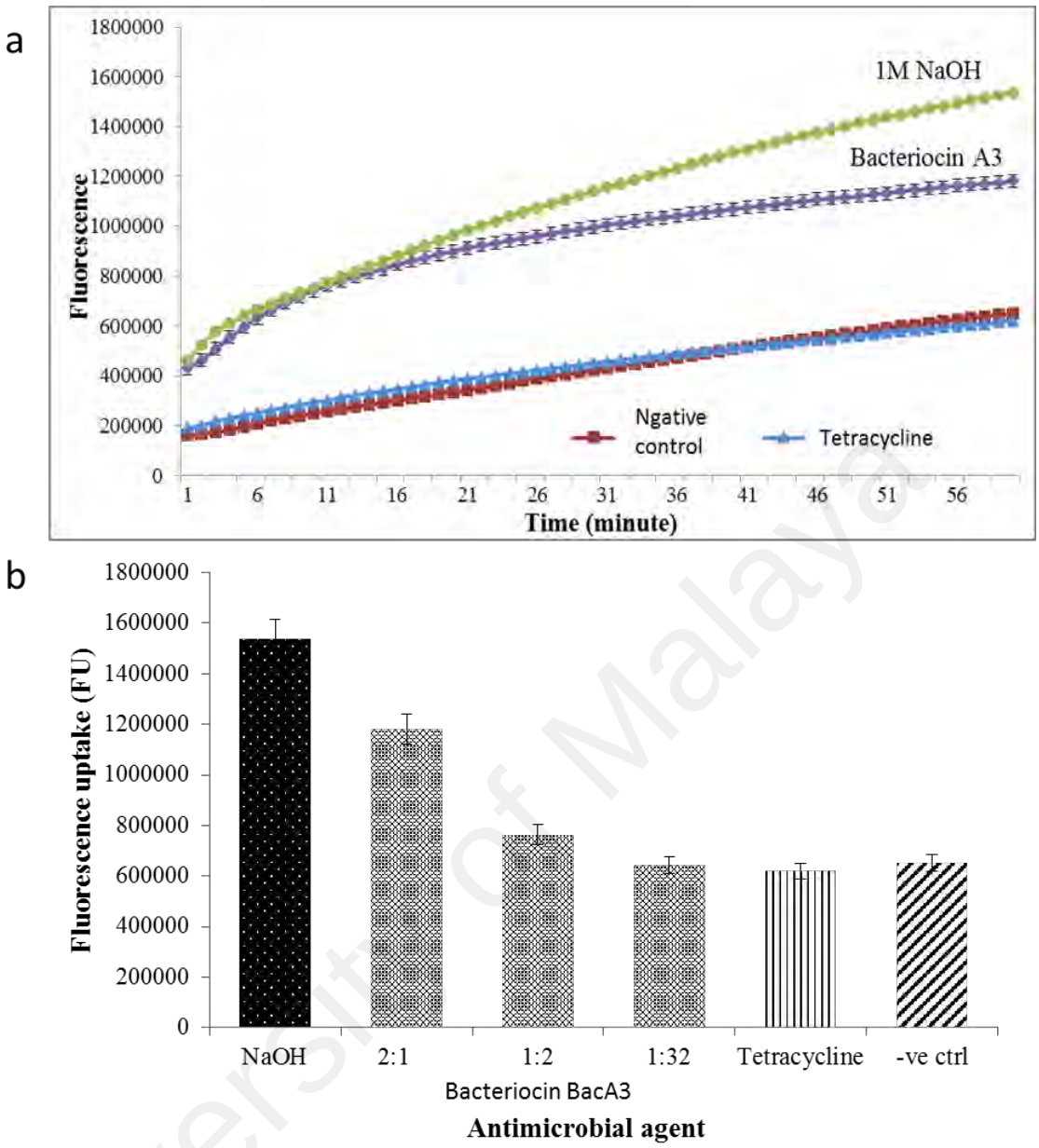


Figure 4.27: Real-time PCR fluorescence traces for BacA3, negative control, positive control (NaOH, 1M) and tetracycline (a). Fluorescence uptake after one hour treatment with bacteriocin from *W. confusa* A3 at dilutions 2:1, 1:1, 1:32; negative control, positive control (NaOH, 1M) and tetracycline (b). N=4, the error bars indicate standard deviation and $P<0.05$.

In Figure 4.28, the membrane permeabilization test of BacC1 with SYTOX® green nucleic acid dye showed significant difference between positive (700 KFU) and negative controls (280 KFU) in permeabilizing bacterial cell membranes. Sodium hydroxide used as the positive control showed high fluorescence unit. The two negative controls used in this study namely tetracycline and blank (without adding anything) showed low fluorescence. The florescence unit for BacC1 from *E. faecium* C1 also indicated membrane disruption on *B. cereus*. The fluorescence uptake decreased when the dilution of the bacteriocin increased. The disruption of the bacterial membrane allowed the SYTOX® green dye to pass through the membrane and incorporated with the bacterial DNA with detection of fluorescence signal.

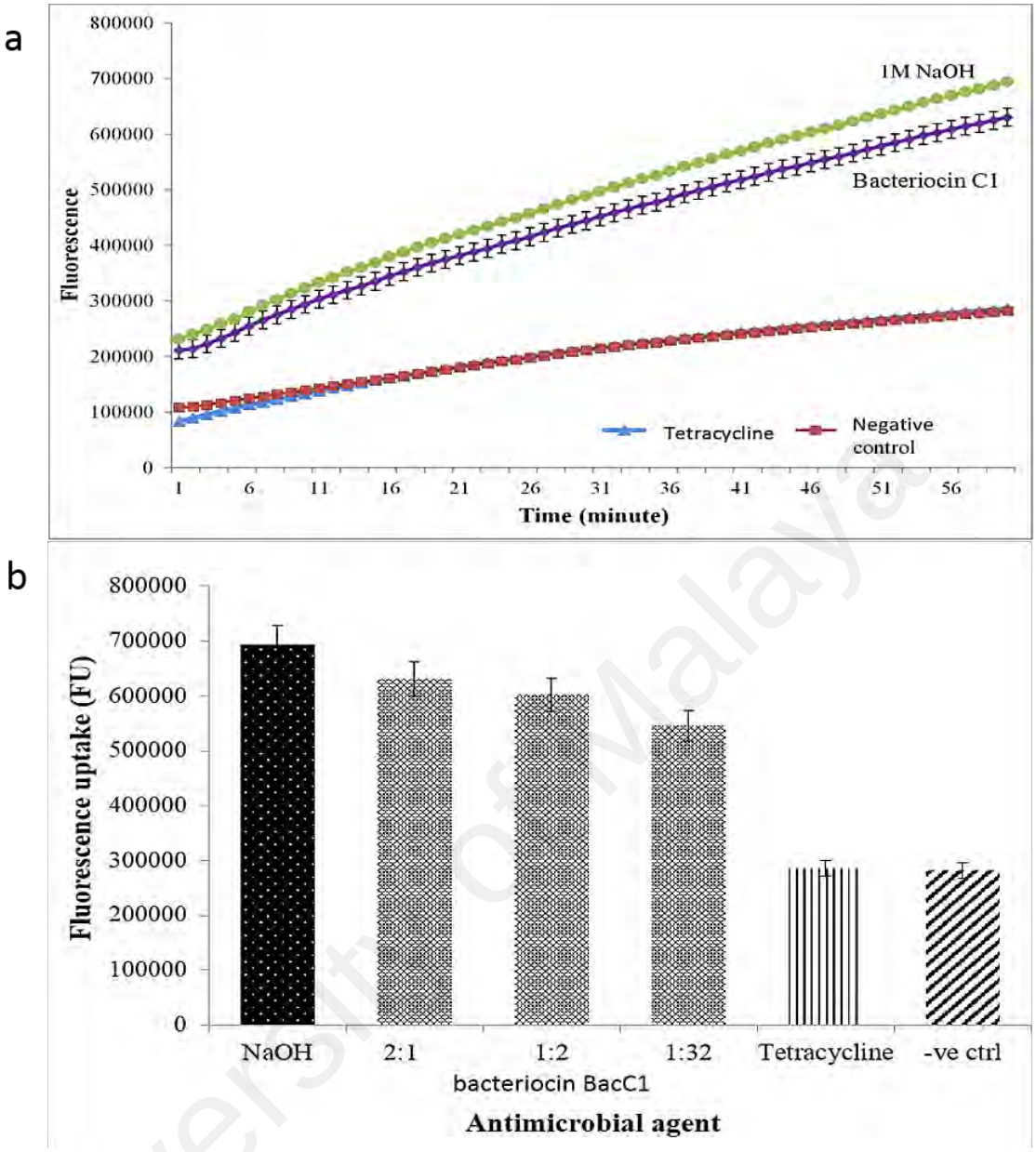


Figure 4.28: Real-time PCR fluorescence traces for BacC1, negative control, positive control (NaOH, 1M) and tetracycline (a). Fluorescence uptake after one hour treatment with bacteriocin from *E. faecium* C1 at dilutions 2:1, 1:1, 1:32; negative control, positive control (NaOH, 1M) and tetracycline (b). N=4, the error bars indicate standard deviation and $P<0.05$.

4.18 Effect of bacteriocin on bacteria examined by electron microscopy methods

4.18.1 Scanning electron microscope (SEM)

Bacillus cereus cells treated with bacteriocin and untreated control were observed under the scanning electron microscope. The untreated cells under SEM showed smooth surfaces without any crack lines (Figures 4.29 (a) & 4.30 (a), same control was used). On the other hand, the bacteria cell treated with bacteriocins BacA3 and BacC1 (Figures 4.29 (b) & 4.30 (b)) showed roughening and cracking of the bacterial surfaces. The treated bacteria also became shrunken after the treatment. This confirmed that the both the bacteriocins were effective in disrupting the outer membrane of the test bacteria *B. cereus.*

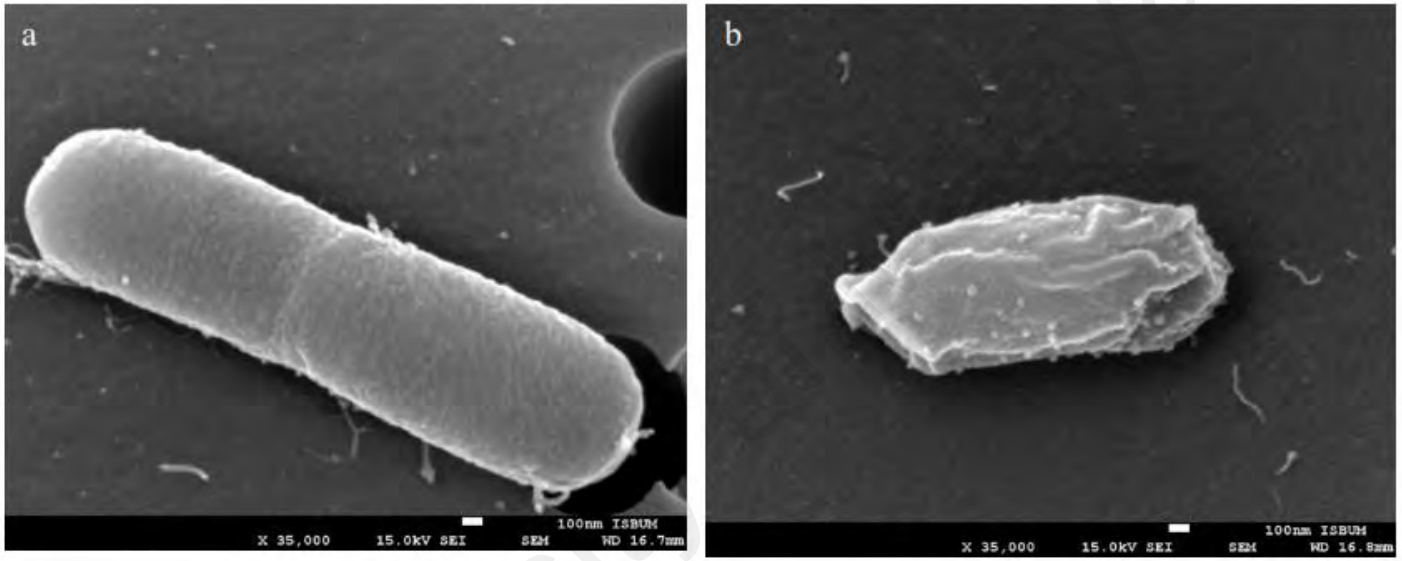


Figure 4.29: Scanning electron microscopic images of *B. cereus* cells before (a) and after (b) treatment with BacA3 from *W. confusa* A3, 35,000 X magnification. Treated bacterial cell shows shrinking effect.

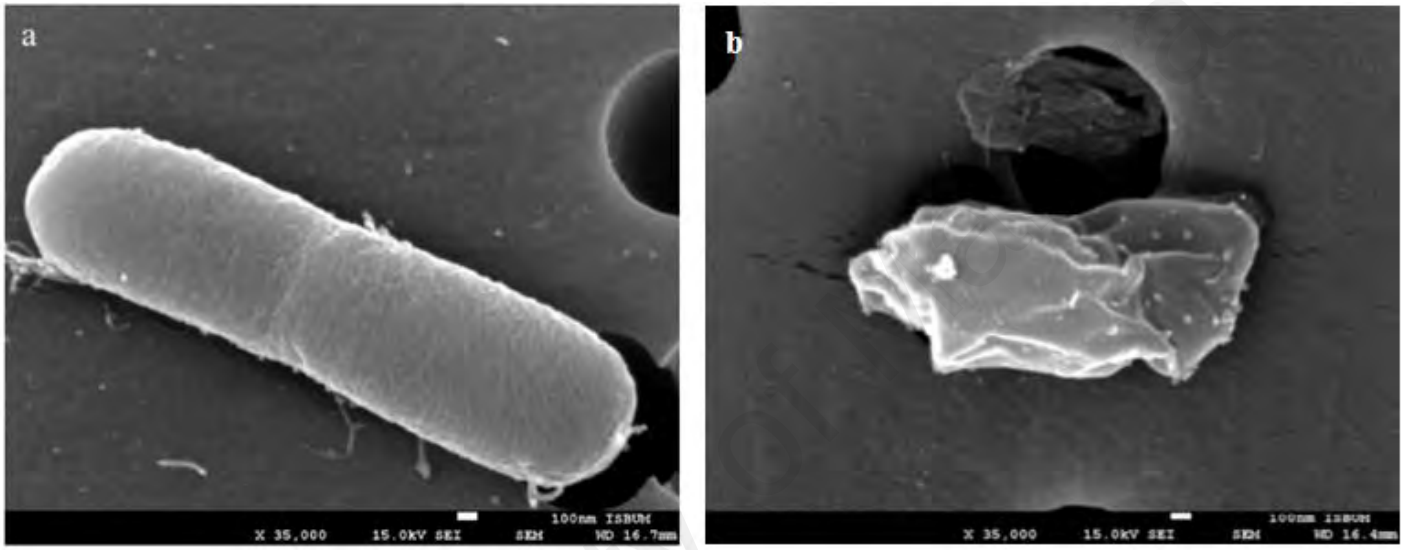


Figure 4.30: Scanning electron microscopic images of *B. cereus* cells before (a) and after (b) treatment with BacC1 from *E. faecium* C1, 35,000 X magnification. Treated bacterial cell shows shrinking effect.

4.18.2 Transmission electron microscope (TEM)

Under the transmission electron microscope, the untreated control cells showed completely intact membrane without any disruption (Figures 4.31 (a) & 4.32 (a), same control was used) whereas the bacterial cells after treatment with bacteriocin showed membrane disruption. In Figure 4.31 (b), the bacterial cell membrane treated with BacA3 was sloughed off and Figure 4.31 (c) shows a pore formation on the cell membrane. After treatment with bacteriocin BacC1, the cell wall of some of the test bacteria was broken (Figure 4.32 (b)) while others appeared as dense cytoplasm without any cell wall (Figure 4.32 (c)).

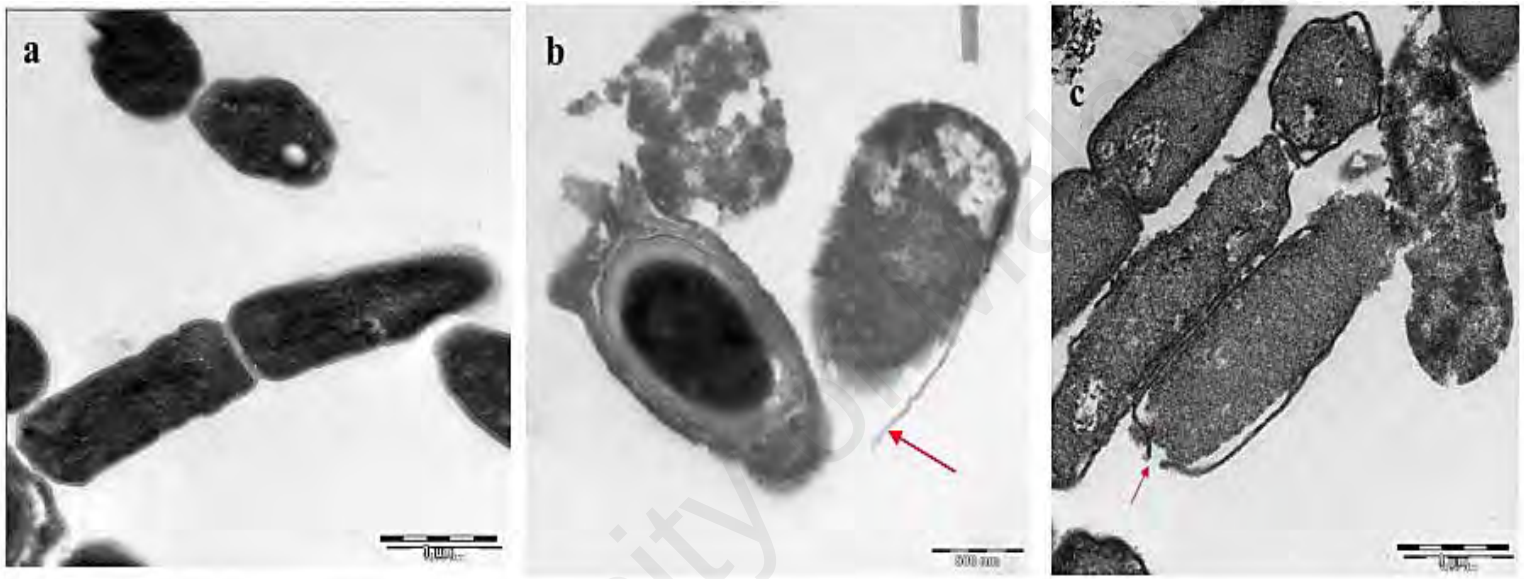


Figure 4.31: Transmission electron microscopic images of *B. cereus* cells before (a) and after (b & c) treatment with BacA3 from *W. confusa* A3. Bar indicates 1 µm for (a & c) and 500 nm for (b). Arrows indicate membrane disruption.

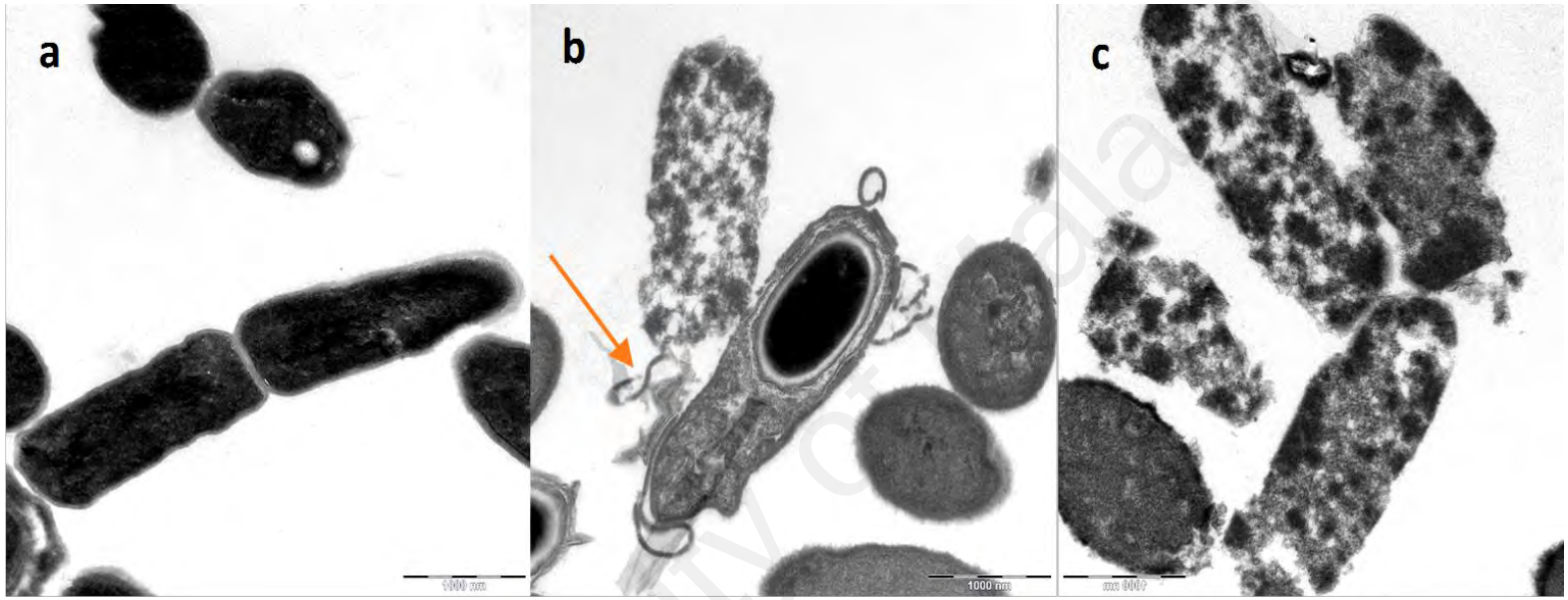


Figure 4.32: Transmission electron microscopic images of *B. cereus* cells before (a) and after (b & c) treatment with BacC1 from *E. faecium* C1. Bar indicates 1 µm. Arrows indicate membrane disruption.

4.19 Amino-terminal sequence analysis

The N-terminal sequence obtained by Edman degradation of BacA3 was VAPGEIVESL (V = valine, A = alanine, P = proline, G = glycine, E = glutamate, I = isoleucine, S = serine, L = leucine). The N-terminal sequencing chromatograph was embedded in Appendix C. The sequence showed E-value of 9.7, score of 65 and 90 % identity with an uncharacterised protein (*Bionectria ochroleuca*) in UniProt database. The length of the original protein sequence was 354 amino acids, and the N-terminal sequence of BacA3 matched with 10 amino acids in the middle of the sequence (Appendix E).

N-terminal analysis by Edman degradation of the bacteriocin BacC1 determined the sequence of the first eight amino acids as follows: GPXGPXGP (G = glycine, P = proline, X = hydroxyproline). Hydroxyproline is a structurally modified amino acid derived from proline (Refer to Appendix D for the N-terminal sequencing chromatograph). The sequence obtained did not match with any known bacteriocins in the database. When searched against the genome of *E. faecium* C1, no significant similarity was detected.

4.20 Genome sequencing and analysis

The DNA libraries prepared showed a size range of about 370 to 680 base pairs in the Bioanalyzer profile (Appendix F). The effective insert sizes of the libraries is in the range of ~250 to 560 bp and the inserts were flanked by adaptors whose combined size is ~120 bp. Based on these population as seen on the profile and the yield by qubit nucleic acid concentration, the library prepared was suitable for sequencing on Illumina platform. Table 4.14 summarises the nucleic acid concentration from Qubit.

Table 4.14: Qubit readings of the prepared library

No	Sample ID	Qubit nucleic acid Concentration (ng/µl)	Volume (µl)	Yield (ng)
1	*W. confus*a A3	55.2	15	828.0
2	*E. faecium* C1	80.8	15	1212.0

4.20.1 De-novo whole genome sequencing for *W. confusa* A3

The raw data QC showed that *W. confusa* A3 produced 773040 (0.7 million pairs) of raw reads from 2 times paired end reads with a total of 232685040 bases (232.6 Mb) being sequenced (Table 4.15). The summary report of *W. confusa* A3 genome analysis, genome sequence data QC statistics, read length distribution and assembly QC statistics are recorded in Appendices G, H, I and J.

Table 4.15: Summary table depicting raw reads vs processed reads statistics

Sample Name	**Platform**	**Type of reads**	**Total number of raw reads**	**Total number of processed reads**
Weisella confusa A3	Illumina MiSeq	2 x Paired end (301bp max)	773040 (0.77 million pairs)	767913 (0.76 million pairs)

Table 4.16 summarises the number of contigs generated and the number of contigs according to their lengths. The number of contigs generated was 63 with average lengths between 507 and 237103 bp.

Table 4.16: De-novo assembly QC statistics at scaffolding step

Sample	***Weisella confusa* A3**
Contigs Generated	63
Maximum Contig Length	237103
Minimum Contig Length	507
Average Contig Length	35,986.7 ± 51,038.4
Median Contig Length	1531
Total Contigs Length	2267162
Total Number of Non-ATGC Characters	5
Percentage of Non-ATGC Characters	0
Contigs ≥100 bp	63
Contigs ≥200 bp	63
Contigs ≥ 500 bp	63
Contigs ≥ 1 Kbp	54
Contigs ≥10 Kbp	35
Contigs ≥1 Mbp	0
N50 value	79432

Out of 63 scaffolds, 2149 genes were predicted and 2098 were annotated. The genome contained 96 unannotated genes (Table 4.17). Microsatellite search of simple sequence repeat (SSR) is shown in Tables 4.18 and 4.19.

Table 4.17: Gene annotation summary of *W. confusa* A3

Gene Annotation Summary	**Genes**
Total Scaffolds	63
Total Predicted genes	2149
Total Annotated genes	2098
Total Unannotated genes	96

Table 4.18: Statistics of SSR identified for *W. confusa* A3

Total number of scaffolds examined	63
Total size of examined sequences (Mb)	2267162 bases (2.2 Mb)
Total number of identified SSRs	5
Number of SSR containing scaffolds	5
Number of SSRs with 1 unit	0
Number of SSRs with 2 units	1
Number of SSRs with 3 units	3
Number of SSRs with 4 units	1
Number of SSRs with 5 units	0
Number of SSRs with 6 units	0
Number of complex SSRs	0

Table 4.19: *W. confusa* A3 SSR identification

Scaffold ID	Type of SSR	Pattern of SSR	Size (bp)	SSR start position	SSR end position
scaffold29|size23800	Tetra-nucleotide repeats	(GTTG) 24	96	2825	2920
scaffold33|size14264	Tri-nucleotide repeats	(TTC)5	15	8841	8855
scaffold8|size79432	Di-nucleotide repeats	(GA)6	12	69899	69910
scaffold13|size57209	Tri-nucleotide repeats	(ATA)5	15	15686	15700
scaffold3|size134030	Tri-nucleotide repeats	(AAT)5	15	38416	38430

4.20.2 Re-sequencing for *E. faecium* C1

The sequence alignment of *E. faecium* C1 was done by using Bowtie 2-2.0.5 and a total of 17040 variants were generated using Samtools – 0.1.18 & SnpEFF 3.4 (Table 4.20). *Enterococcus faecium* CRL1879 was used as the reference strain and 78.67 % of reads were mapped as shown in Table 4.21.

Table 4.20: Sequence data information for *E. faecium* C1

Analysis Steps	
Alignment	Bowtie 2-2.0.5
Variant Detection	Samtools – 0.1.18 & SnpEFF 3.4
Variant filter cutoff	
Mapping Quality	≥30
Read Depth	≥20
Variant Summary	
Total number of variants	17040
Variant Annotation Summary	
Total number of SNPs	160446
Total number of Indels	6019

Table 4.21: Coverage Analysis of resequencing of *E. faecium* C1

Reference	*Enterococcus faecium* CRL1879
Total number of Reads	2681988
Percentage of Reads Mapped	78.67380466
1X Coverage	78.9390965
10X Coverage	78.11124425
20X Coverage	77.54406604

4.20.3 Analysis of the genome data

4.20.3.1 *Weissella confusa* A3

The draft genome includes 2,289,873 bp with 120 contigs and 274 subsystems in RAST. The number of coding sequences (CDS) was 2104 and number of RNAs was 113. The analysis with RAST server showed that 43 % of the genome was covered in the subsystems of RAST (Figure 4.33). Among the subsystem coverage, no gene can be detected from the category of bacteriocins that are ribosomally synthesised antibacterial peptides. For virulence factor determination, no gene can be seen in the category of toxins and superantigens and virulence, disease and defense. The gene hsp 33, chaperonin heat shock protein was detected in the adhesion system. The same gene was found in *Streptococcus pyogenes* recombinatorial zone. Although hsp 33 is present in the genome, the other genes such as Spy0135, SOF, RofA, Nra and PrtF2 involved in the cluster of adhesion were absent that might cause the gene to dysfunction. The same gene was also found in other species of LAB in RAST database as shown in Figure 4.34. In the annotated data, 11 genes involved in probiotic function were detected (Table 4.22). Four genes were detected for the production of Sortase A, LPXTG

specific, 3 genes for predicted cell-wall-anchored protein SasA (LPXTG motif), one gene each for putative peptidoglycan bound protein (LPXTG motif) Lmo1799 homolog, ABC-type antimicrobial peptide transport system, permease component, possible colicin V production protein and cell wall surface anchor family protein.

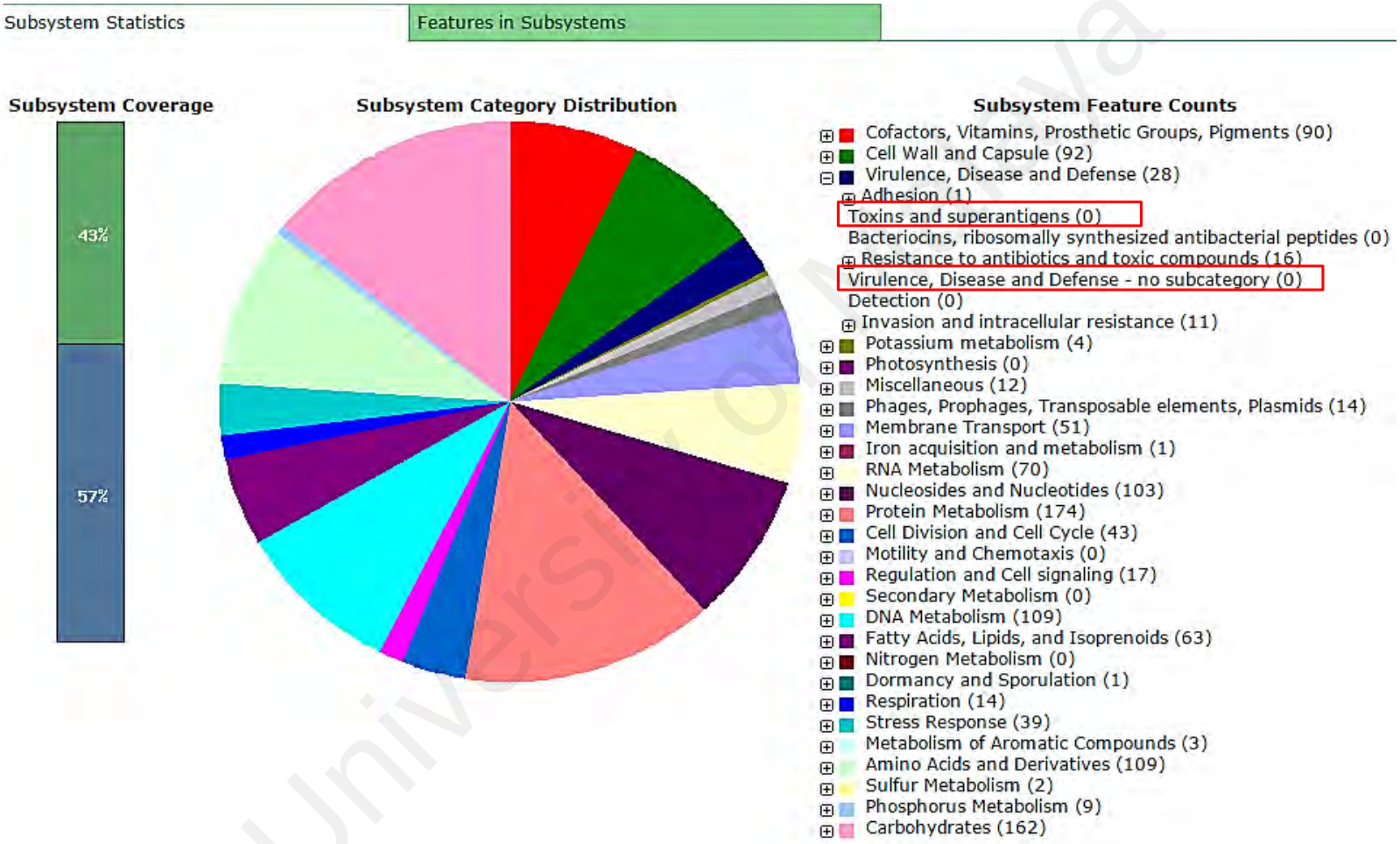


Figure 4.33: Subsystem information of *W. confusa* A3 using SEED viewer in RAST server. The red boxes showed no gene detected in the category of toxins and superantigens and virulence, disease and defense.

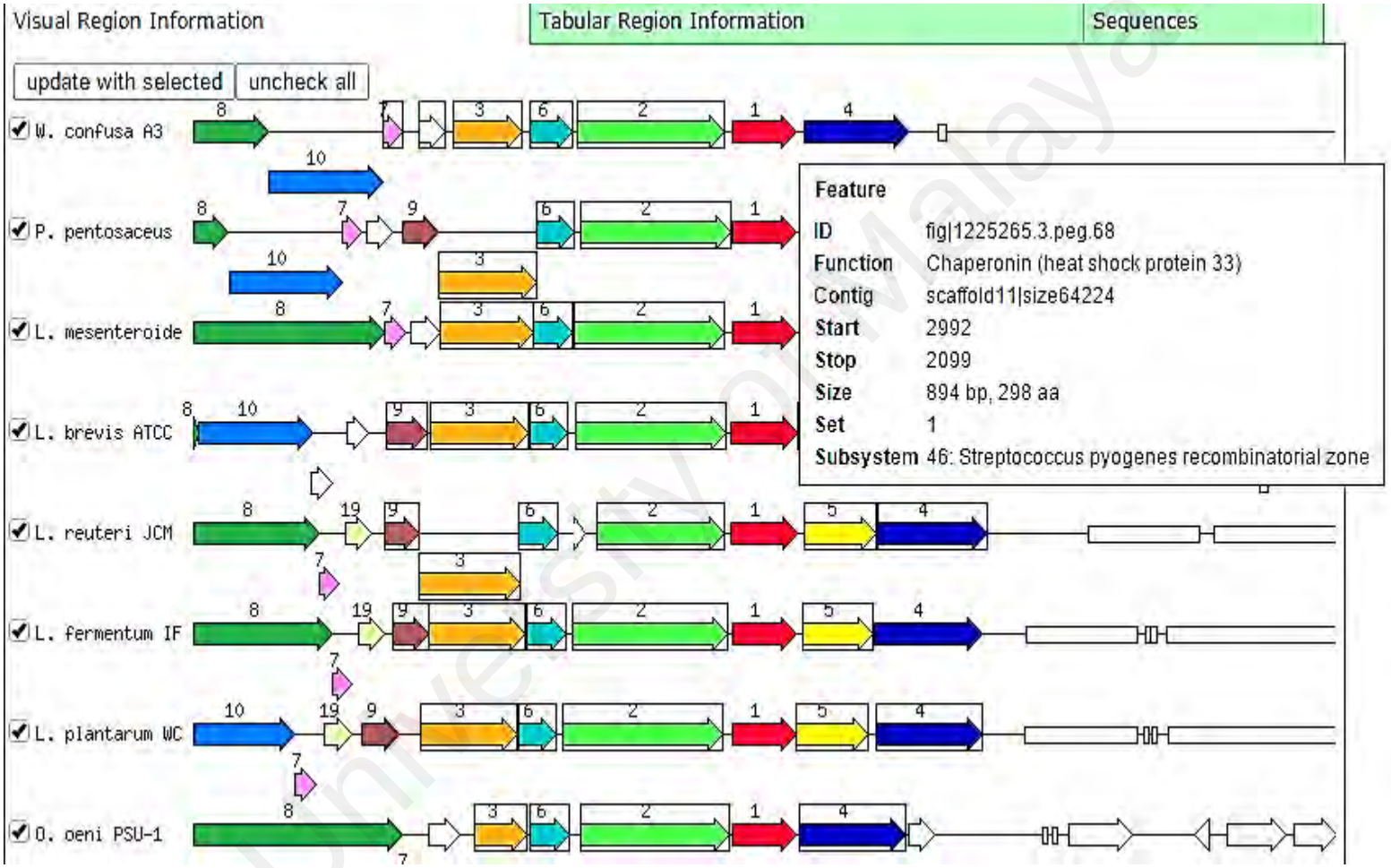


Figure 4.34: Heat shock protein 33 which is red and numbered 1 found in other LAB.

Table 4.22: List of genes with probiotic functions in *W. confusa* A3

No	Location	Gene Size (bp)	Annotation
fig|1225265.3.peg.677	scaffold21|size34135	780	Sortase A, LPXTG specific
fig|1225265.3.peg.995	scaffold2|size207902	897	Sortase A, LPXTG specific
fig|1225265.3.peg.1288	scaffold3|size134030	726	Sortase A, LPXTG specific
fig|1225265.3.peg.2103	scaffold9|size73525	804	Sortase A, LPXTG specific
fig|1225265.3.peg.193	scaffold13|size57209	2442	Predicted cell-wall-anchored protein SasA (LPXTG motif)
fig|1225265.3.peg.276	scaffold14|size56814	1332	Predicted cell-wall-anchored protein SasA (LPXTG motif)
fig|1225265.3.peg.1378	scaffold42|size4760	1017	Predicted cell-wall-anchored protein SasA (LPXTG motif)
fig|1225265.3.peg.583	scaffold1|size189855	12234	Putative peptidoglycan bound protein (LPXTG motif) Lmo1799 homolog
fig|1225265.3.peg.924	scaffold29|size23800	1788	ABC-type antimicrobial peptide transport system, permease component
fig|1225265.3.peg.942	scaffold2|size207902	543	Possible colicin V production protein
fig|1225265.3.peg.42	scaffold10|size65582	681	cell wall surface anchor family protein

4.20.3.2 *Enterococcus faecium* C1

The draft genome includes 2,283,839 bp with 56 contigs and 325 subsystems in RAST. The number of coding sequences (CDS) was 2420 and number of RNAs was 66. The analysis with RAST server showed that 53 % of the genome was covered in the subsystems of RAST (Figure 4.35). In the analysis of genes encoding bacteriocin production with RAST server, one gene cluster with colicin production was detected from *E. faecium* C1. A bacteriocin of more than 5 kDa that was a phage lysin was also detected. After checking with Bactibase database (http://bactibase.pfba-lab-tun.org/blast), the sequence showed 14 % similarity to enterolysin A. Under virulence, disease and defence category, no toxin and superantigen genes were detected. The heat shock protein 33 under the category of adhesion was also detected by RAST.

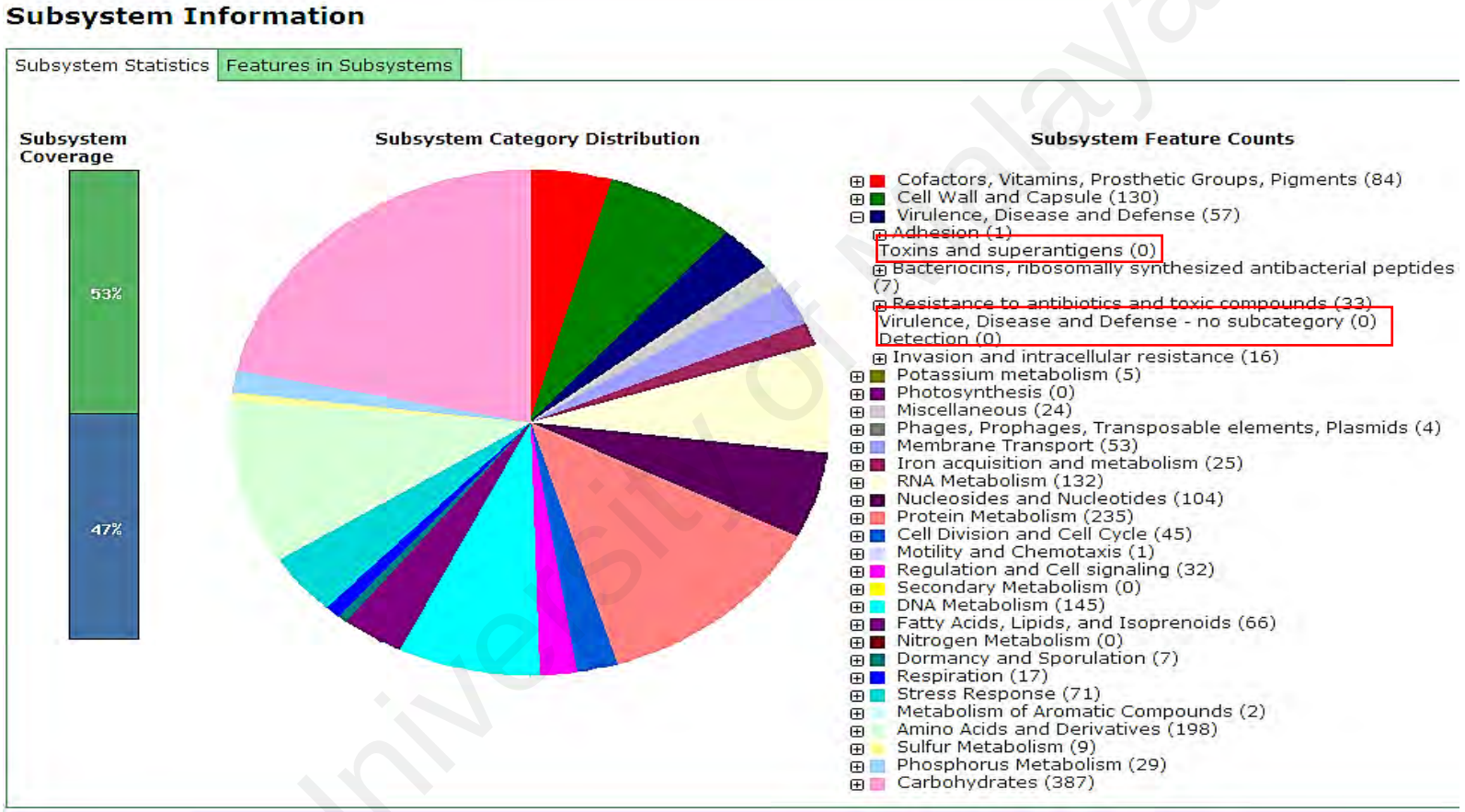


Figure 4.35: Subsystem information of *E. faecium* C1 using SEED viewer in RAST server. The red boxes showed no gene detected in the category of toxins and superantigens and virulence, disease and defense.

Furthermore, 16 genes involved in probiotic functions were detected in the genome of *E. faecium* C1. Seven genes were detected for sortase A, LPXTG specific, 4 genes for ABC-type antimicrobial peptide transport system, permease component, 2 genes for cell wall surface anchor family protein, LPXTG motif, one gene each for phage lysin and colicin V production protein. Table 4.23 listed the probiotic genes with its location and gene size on *E. faecium* C1.

Table 4.23: List of genes with probiotic functions in *E. faecium* C1

No	Location	Size (bp)	Annotation
fig|333849.24.peg.110	Contig_141_16802_16026	777	Sortase A, LPXTG specific
fig|333849.24.peg.127	Contig_141_33240_32677	564	Sortase A, LPXTG specific
fig|333849.24.peg.1370	Contig_288_6552_7223	672	Sortase A, LPXTG specific
fig|333849.24.peg.1372	Contig_288_9267_10019	753	Sortase A, LPXTG specific
fig|333849.24.peg.1851	Contig_382_89436_89891	456	Sortase A, LPXTG specific
fig|333849.24.peg.2188	Contig_73_94496_95656	1161	Sortase A, LPXTG specific
fig|333849.24.peg.2288	Contig_86_51029_50136	894	Sortase A, LPXTG specific
fig|333849.24.peg.1843	Contig_382_79966_80673	708	ABC-type antimicrobial peptide transport system, permease component
fig|333849.24.peg.1844	Contig_382_80685_81038	354	ABC-type antimicrobial peptide transport system, permease component
fig|333849.24.peg.2095	Contig_71_64732_64601	132	ABC-type antimicrobial peptide transport system, permease component
fig|333849.24.peg.2096	Contig_71_64868_64725	144	ABC-type antimicrobial peptide transport system, permease component
fig|333849.24.peg.2212	Contig_73_115518_117320	1803	ABC-type antimicrobial peptide transport system, permease component
fig|333849.24.peg.1387	Contig_288_23850_25055	1206	Phage lysin (EC:3.2.1.17)
fig|333849.24.peg.897	Contig_231_97574_97035	540	Colicin V production protein
fig|333849.24.peg.2289	Contig_86_51575_51300	276	Cell wall surface anchor family protein, LPXTG motif
fig|333849.24.peg.2290	Contig_86_52612_51575	1038	Cell wall surface anchor family protein, LPXTG motif

The genome of the bacteria was compared with complete genome of the probiotic strain *E. faecium* T110 with accession number CP006031 in NCBI (Natarajan & Parani, 2015). The comparisons between both strains are listed in Table 4.24. A total of 43 virulence genes related to *Enterococcu*s species was examined and probiotic strain *E. faecium* T110 contained 8 virulence genes out of the 43 genes. On the other hand, the strain in this study harboured 6 virulence genes.

Table 4.24: List of virulence genes between *E. faecium* C1 and *E. faecium* T110

Category	**Gene**	**Function**	***E. faecium* C1**	***E. faecium* T-110**
Adherence	*Acm*	Adhesion of collagen from *E. faecium*	+	+
	Scm	Second collagen adhesion of *E. faecium*	-	+
	EcbA	*E. faecium* collagen binding protein	-	-
	SagA	Aggregation substance	+	+
	efaA	endocarditis specific antigen	-	-
	*EfaAfm**	Cell wall adhensins	+	+
	Esp	Surface protein precursor	-	-
	Esp	surface protein	-	-
	Ace	Adhesion of collagen from *E. faecalis*	-	-
	Asa1	Aggregation substance	-	-
	SalA	Secreted lipase	-	-
	SalB	Secreted antigen	-	-
	prgB/asc10	Aggregation substance	-	-
	EF0149	Aggregation substance	-	-
	EF0485	Aggregation substance	-	-
Antiphagocytosis	*CdsA**	Host immune evasion	+	+
	*UppS**	Host immune evasion	+	+
	cpsK	Host immune evasion	-	-

Table 4.24 continued

	cpsJ	Host immune evasion	-	-
	cpsI	Host immune evasion	-	-
	cpsH	Host immune evasion	-	-
	cpsG	Host immune evasion	-	-
	cpsF	Host immune evasion	-	-
	cpsE	Host immune evasion	-	-
	cpsD	Host immune evasion	-	-
	cpsc	Host immune evasion	-	-
	glf	Host immune evasion	-	-
Biofilm formation	*BopD*	Biofilm formation	+	+
	fsrA	Biofilm formation	-	-
	fsrB	Biofilm formation	-	-
	fsrC	Biofilm formation	-	-
Exoenzyme	**Gelatinase**			
	gelE	Exoenzyme, Biofilm formation	-	-
	Hyaluronidase			
	EF3023	Exoenzyme, Biofilm formation	-	-
	EF0818	Exoenzyme, Biofilm formation	-	+
	SprE	Exoenzyme, Serine protease	-	-
Toxin	**Cytolysin**			
	cylA	Toxin	-	-
	cylB	Toxin	-	-
	cylI	Toxin	-	-
	cylLl	Toxin	-	-
	cylLs	Toxin	-	-
	cylM	Toxin	-	-
	cylR1	Toxin	-	-
	cylR2	Toxin	-	-

List of virulence factors were taken from the genomes of *Enterococcus* species at virulence factor database (http://www.mgc.ac.cn/VFs/).
*amino acid sequence from UniProt (http://www.uniprot.org/)
Differences between the two strains are highlighted.

After comparing 4 genes related to pathogenicity islands and 8 genes related to resistance islands of *E. faecium* listed in pathogenicity island database (PAI DB), no gene can be detected from strain C1.

Table 4.25: Genes involved in pathogenicity islands (PAIs) and antimicrobial resistance islands (REIs)

Name	**GenBank Accn**	**Function**	**Host strain**	***E. faecium* C1**
virE	YP_006376742.1	virulence-associated E family protein	*E. faecium* DO	-
esp	AAL40269.1	Virulence-associated surface protein (*esp*), *ara*C, *Uve*2, muramidase	*E. faecium* E300	-
esp	AAQ89938.1	Virulence-associated surface protein (*esp*), *ara*C, *Uve*2, muramidase	*E. faecium* E470	-
esp	AAQ89936.1	Virulence-associated surface protein (*esp*), *ara*C, *Uve*2, muramidase	*E. faecium* E734	-
traA	YP_008394233.1	conjugal transfer relaxase *Tra*A	*E. faecium* AUS0085	-
vanRB	YP_008394254.1	DNA-binding response regulator VanRB		-
vanSB	YP_008394255.1	DNA-binding response regulator VanRB		-
vanYB	YP_008394256.1	VanY protein, D-alanyl-D-alanine carboxypeptidase		-
vanWB	YP_008394257.1	vancomycin B-type resistance protein VanW		-
vanHB	YP_008394258.1	VanH protein, D-specific alpha-keto acid dehydrogenase		-
vanB	YP_008394259.1	Vancomycin B-type resistance protein vanB, D-alanine-D-lactate ligase		-
vanXB	YP_008394260.1	D-alanyl-D-alanine dipeptidase		-

The list of genes was taken from pathogenicity island database (PAI DB) http://www.paidb.re.kr/about_paidb.php?m=h (Yoon *et al*., 2015)

The search for bacteriocin gene with BAGEL 3 showed that *E. faecium* C1 harboured genes related to bacteriocin /modified peptide at contig 288 (Figure 4.36). Two bacteriocins were found from the genome.

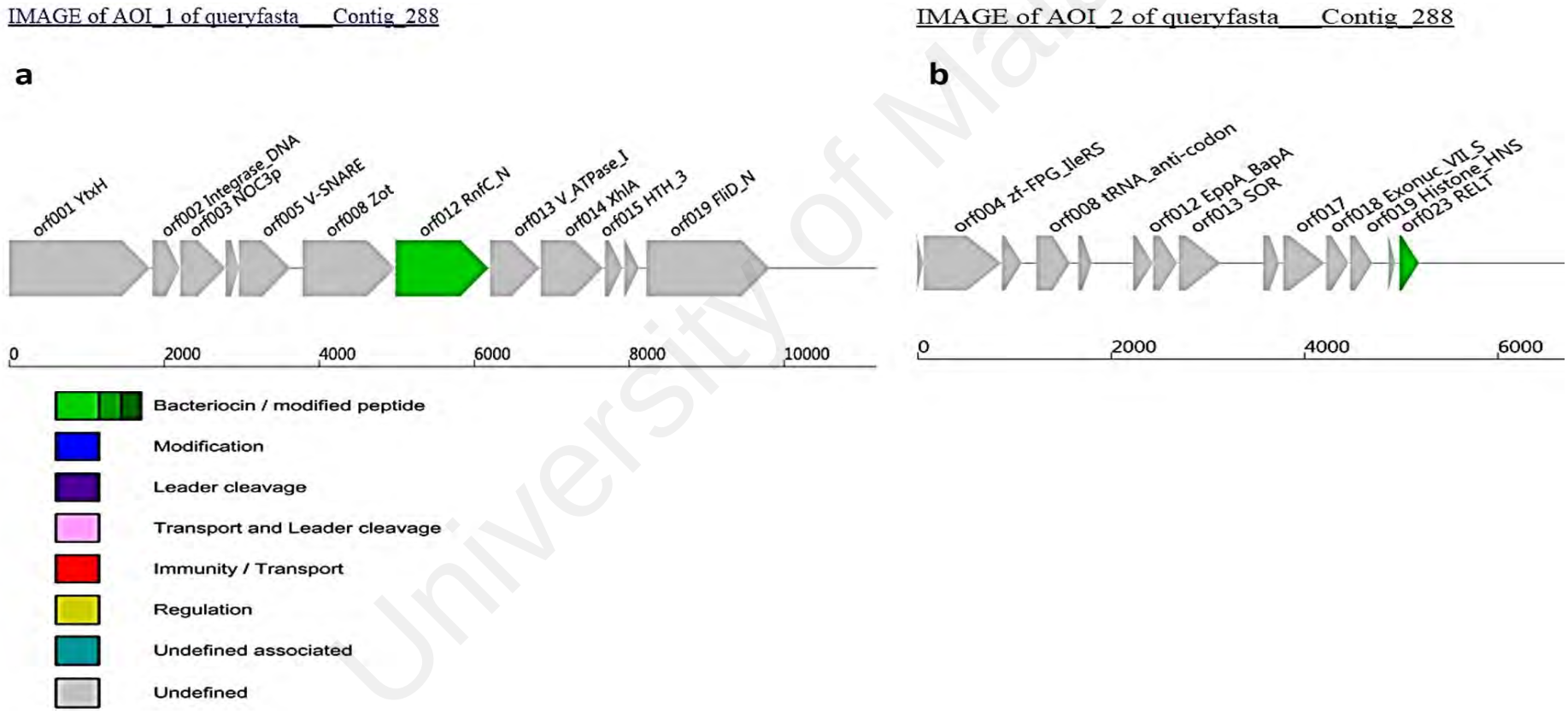


Figure 4.36: *E. faecium* C1 possible bacteriocin gene cluster detected by BAGEL 3. The green colour box indicate bacteriocin gene (BAGEL 3: http://bagel.molgenrug.nl/index.php/bagel3, van Heel *et al*., 2013).

The protein sequence of the two bacteriocin genes present on the genome of *E. faecium* C1 were tabulated in Table 4.26. The sequences were then compared with UniProt database and the class III bacteriocin found on BAGEL 3 showed 99.3 % similarity to peptidase M23B from *E. faecium* and the class II bacteriocin was similar by 100 % to enterocin TW 21 and other enterocin (uncharacterised protein, bacteriocin hiracin-JM79 and enterocin SE-K4) from *E. faecium* (Figure 4.38). The sequences obtained was also compared with the annotated date in RAST and the class III bacteriocin showed 100 % similarity to the sequence of phage lysin in fig|1305849.12.peg.1387. The alignments of the bacteriocin are shown in Figures 4.37 and 4.38.

Table 4.26: Protein sequence of the identified bacteriocin using BAGEL 3

	Type	**Protein ID**	**Protein sequence**	**Blast hit in bacteriocin II database**
a	ClassIII	AOI_1;orf012	MENQNESLIKQYVKRRAKRRLFLWLFGTSAGLITNLITVFVTLFLILAAGSIDNSDSDSSSGGEDFTGEYSEGLPIYKEIKGRGPFSDEIAQYAVGAAVKYKLLPSVILSQYGYESAFGTSASARNDLNYFGITWFDGCLFPKGTARGIGGIEGGWYMKFPNSKAAFSYYGFMVATQSNFNACVGNKSPGASLLILGRGGYAAAGITEDSPYYTGCMSIITSNKLTEYDEFAIKHWGEGNNNNGTITGEWTNPFPGSSLDKSSFSGGQLFGTNPGGEFRPNGFHDGLDFGSVDHPGSEIHAVHGGKVVYVGNPGISGLGACVIVINYDGLNMVYQEFANSTGNSRVKVGDQVKVGQVIGIRDTAHLHLGFTRMDWRQAQGHAFTDDGTWIDPLPFLNSSKK	Enterolysin_A[9e-29]
b	Class II	AOI_2;orf023	MKKKFVSIFMILGIVLLSVSTLGITVDAATYYGNGVYCNTQKCWVDWSRARSEIVDRGVKAYVRMY	Enterocin_SE-K4[1e-30]

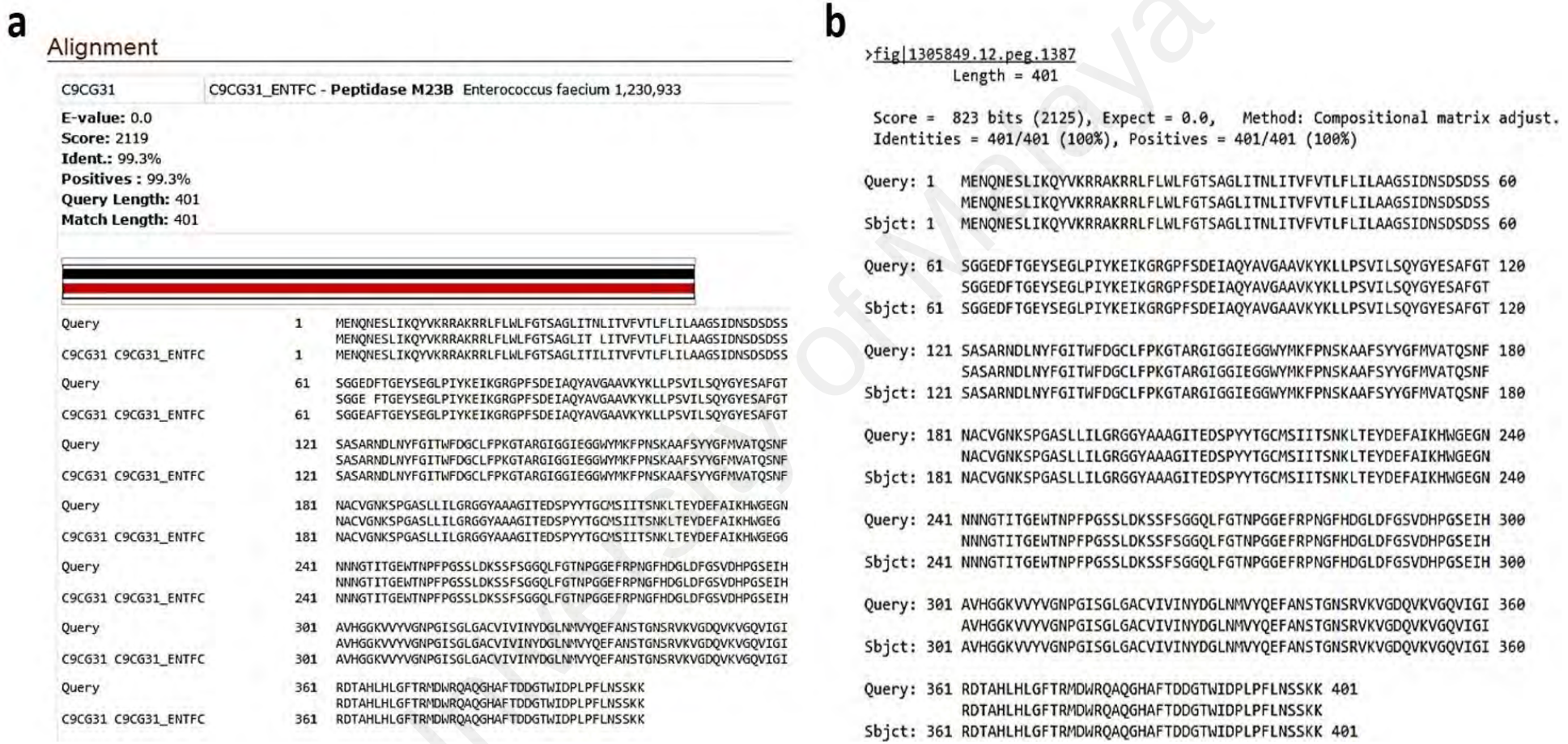


Figure 4.37: The alignment of class III bacteriocin gene detected by BAGEL 3 with UniProt database (http://www.uniprot.org/blast/) (a) and RAST (http://rast.nmpdr.org/) (b).

Alignment

How to print an alignment in color

```
b                       1    MKKKFVSIFMILGIVLLSVSTLGITVDAATYYGNGVYCNTQKCWVDWSRARSEIVDRGVK   60
R9R6P4      R9R6P4_ENTFC    1    MKKKFVSIFMILGIVLLSVSTLGITVDAATYYGNGVYCNTQKCWVDWSRARSEIVDRGVK   60
R2LYU1      R2LYU1_ENTFC    1    MKKKFVSIFMILGIVLLSVSTLGITVDAATYYGNGVYCNTQKCWVDWSRARSEIVDRGVK   60
C2H7J5      C2H7J5_ENTFC    1    ---------MILGIVLLSVSTLGITVDAATYYGNGVYCNTQKCWVDWSRARSEIVDRGVK   51
A0A099R0E5  A0A099R0E5_ENTFC 1   ---------MILGIVLLSVSTLGITVDAATYYGNGVYCNTQKCWVDWSRARSEIVDRGVK   51
C9AKK3      C9AKK3_ENTFC    1    ---------MILGIVLLSVSTLGITVDAATYYGNGVYCNTQKCWVDWSRARSEIVDRGVK   51
                                             **************************************************

b                       61   AYVRMY----------                                                  66
R9R6P4      R9R6P4_ENTFC    61   AYVNGFTKVLGGVGGR                                              76
R2LYU1      R2LYU1_ENTFC    61   AYVNGFTKVLGGVGGR                                              76
C2H7J5      C2H7J5_ENTFC    52   AYVNGFTKVLGGVGGR                                              67
A0A099R0E5  A0A099R0E5_ENTFC 52  AYVNGFTKVLGGVGGR                                              67
C9AKK3      C9AKK3_ENTFC    52   AYVNGFTKVLGGVGGR                                              67
                                 ***. :
```

	Entry	Entry name	Protein names	Organism	Gene name
☐	R9R6P4	R9R6P4_ENTFC	**Enterocin TW21**	Enterococcus faecium (Streptococcus faecium)	
☐	R2LYU1	R2LYU1_ENTFC	**Uncharacterized protein**	Enterococcus faecium EnGen0263	UA3_02563
☐	C2H7J5	C2H7J5_ENTFC	**Bacteriocin hiracin-JM79**	Enterococcus faecium TX1330	**hirJM**, HMPREF0352_0177
☐	A0A099R0E5	A0A099R0E5_ENTFC	**Enterocin**	Enterococcus faecium (Streptococcus faecium)	LK25_14190
☐	C9AKK3	C9AKK3_ENTFC	**Enterocin SE-K4**	Enterococcus faecium Com12	EFVG_02512

Figure 4.38: The alignment of class II bacteriocin gene detected by BAGEL 3 with UniProt database (http://www.uniprot.org/blast/).

4.21 Application of the bacteriocin and LAB culture in milk

The blank control milk sample showed the highest number of bacterial count compared to milk added with BacA3, BacC1 and nisin. The bacteriocin BacA3 and BacC1 showed better inhibitory activity than nisin. In the presence of live *W. confusa* A3 and *E. faecium* C1, the number of bacterial count on day 1 was lower by 5 log CFU/ml compare to control. After 3 days, no bacteria can grow in the milk samples added with either *W. confusa* A3 or *E. faecium* C1. The milk samples added with nisin, BacA3 or BacC1 showed no viable count after 4 days of incubation. On the other hand, bacteria continued to grow until day 10 in the blank milk sample. The result of each treatment was plotted and illustrated in Figure 4.39.

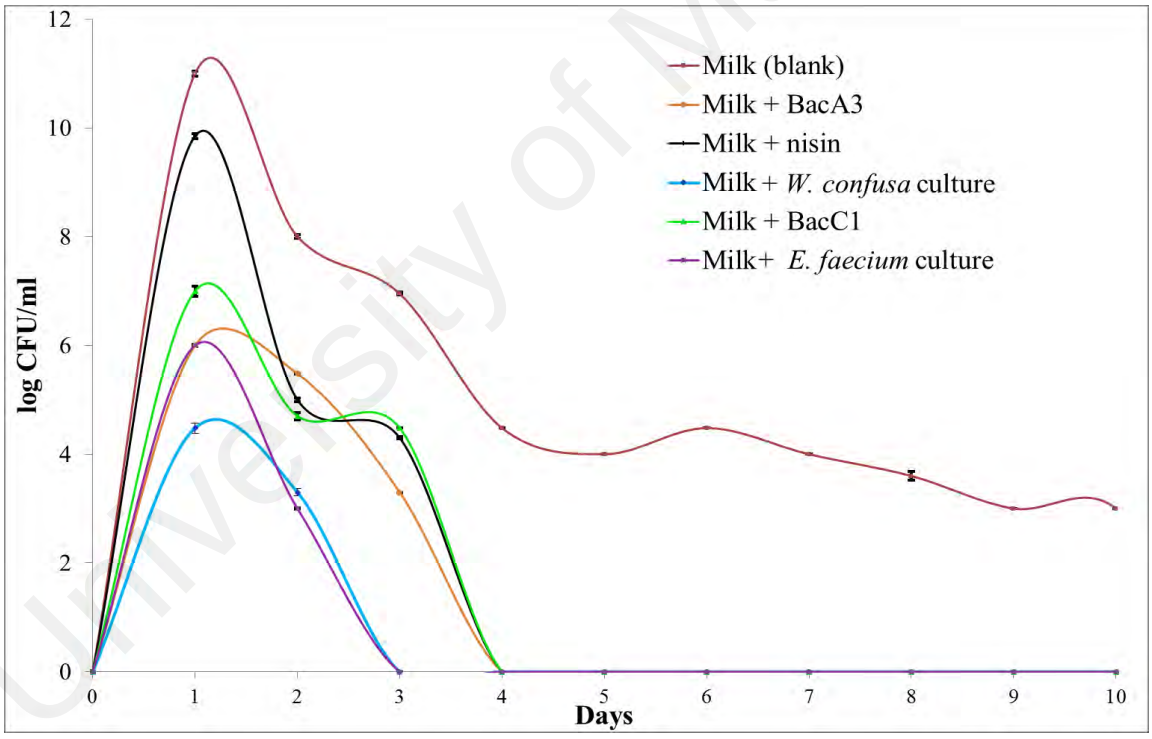


Figure 4.39: The number of bacterial count in the milk with different treatments over number of days, n=3, error bars indicate $\log_{10}$ standard deviations and $P<0.05$ between each treatment.

CHAPTER 5: DISCUSSION

5.1 Isolation, identification and characteristics of LAB

Bacteria are ubiquitous but their modes of propagation may not be visible to the naked eye. It is apparent that a nutrition enriched environment can support the growth of many microorganisms such as lactic acid bacteria because their metabolic pathways and ability to tolerate an acidic condition is conducive for their propagation. Antimicrobial peptides produced by bacteria, termed bacteriocins, play an important role in their survival and domination within a microbial ecosystem. The general consensus is that the bacteriocins or bacteriocin-producers best suited in controlling a problematic spoilage or pathogenic microorganism will often be one that is found in the same environmental niche. This is based on the expectation that bacteriocins provide an advantage in competition for scarce resources in a specific environment (O'Connor *et al*., 2015). Therefore in this study, the LAB isolated from fermented milk which contained both LAB and other spoilage bacteria. Hence, the chance of getting LAB that can produce bacteriocins to inhibit the growth of spoilage bacteria is higher. Before fermentation of the milk, it was pasteurised to kill pathogenic and other harmful bacteria while leaving mainly the good bacteria and heat resistant endospores intact which can withstand high temperature (Holsinger *et al*., 1997). These good bacteria comprise mostly of the LAB which are essential for human life and form a barricade against disease-causing bacteria (Sharma *et al*., 2014). During fermentation process, the bacteria utilise the nutrients in the milk for further propagation. In this study, most of the bacteria isolated from the fermented milk formed colonies that were in milky white colour with circular shape which matched with the characteristic of lactic acid bacteria.

MRS agar was used to isolate the lactic acid bacteria because it was designed to support the growth of LAB which produce lactic acid. MRS medium contain sodium

acetate and ammonium citrate to inhibit Gram-negative bacteria, fungi and moulds. With the addition of both of these inhibiting agents, the growth of LAB in MRS is improved. Three LAB strains with antimicrobial property were successfully isolated from the fermented milk. These were identified by 16S rRNA gene sequencing and either API 50 CHL or 20 Strep as *L. lactis*, *W. confusa* and *E. faecium* with a similarity of 84.2 %, 99.9 % and 99.4 % respectively from API database and 99 % similarity for 16S rRNA gene sequence using the NCBI BLAST database. All the 3 isolates showed LAB characteristics whereby they did not produce catalase and oxidase enzymes. Besides, they also showed negative result for spore staining test with malachite green. *E. faecium* C1 from the *Enterococci* group gave the darkest colour change in bile esculin test compared to the other 2 isolates (Figure 4.2). This proved that *E. faecium* C1 was able to grow in the presence of bile salts and hydrolyse the glycoside esculin in the medium to glucose and esculetin which then reacted with ferric citrate to form a dark brown or black diffusible phenolic iron complex. This test is commonly used to presumptively identify Group D *Streptococci* but also showed positive reaction for *L. lactis* A1 and *W. confusa* A3 in this study.

The API 50 CHL carbohydrate fermentation in Table 4.4 shows that the 3 isolates can utilize D-galatose, D-glucose, D-fructose, D-mannose, N-acethylglucosamine, esculin ferric citrate, salicin, D-celiobiose, D-maltose, sucrose and gentiobiose. When compared with the other 2 isolates, *L. lactis* A1 was unable to ferment amygdalin and *W. confusa* A3 cannot ferment D-ribose, arbutin, D-lactose and D-trehalose but was able to utilize D-xylose and potassium gluconate. On the other hand, *E. faecium* C1 showed positive for L-ribose, D-manitol, methyl-αD-mannopyranoside, D-melibiose, D-raffinose and D-tagatose where *L. lactis* A1 and *W. confusa* A3 were unable to utilize.

The growth kinetics of the bacteria showed that both *L. lactis* A1 and *W. confusa* A3 reached stationary phase after 20 hours of incubation (Figures 4.6 and 4.7). The growth of *E. faecium* C1 was faster and attained stationary phase at 18 hours of incubation (Figure 4.8). Past research showed that most of the bacteriocins were produced during the exponential phase and decreased after the stationary phase. The decrease in bacteriocin production at the stationary growth phase was mainly due to the conditions of the growth media. Nutrient depletion, deferent effect of low pH on bacteriocin production and/or protein degradation seemed more responsible for this phenomenon (Lee & Paik, 2001; Taheri *et al*., 2012; Zhang *et al*., 2013).

Antibiotic susceptibility and haemolysis tests were done to ensure that the producers are safe to be used as potential antimicrobials candidates in food industry. Both results showed that the LAB isolated were not resistant to most of the antibiotics tested and they did not lyse the blood agar (Figure 4.11). Although there is no available guideline to measure antibiotic resistance for *Weissella* genus, the inhibition zone listed in Table 4.6 shows that strain A3 was sensitive to amoxycillin 10 µg, ampicillin 10 µg, chloramphenicol 30 µg, clindamycin 2 µg, ofloxacin 5 µg and tetracycline 30 µg with inhibition zones between 21 to 22 mm. The inhibition zones for gentamicin 10 µg, novobiocin 5 µg, penicillin 1 unit, penicillin V 10 µg and streptomycin 10 µg were between 11 to 20 mm. *Weissella confusa* A3 was resistant to vancomycin because according to literature *Weissella* genera are intrinsically resistant to vancomycin (Björkroth & Holzapfel, 2006). The results showed that the *E. faecium* C1 was non-haemolytic bacteria and sensitive to ampicillin, chloramphenicol, penicillin V, tetracycline and vancomycin based on CLSI standards (Table 4.7). Although no interpretation is available for amoxicillin, but according to CLSI, *Enterococcus*, which is susceptible to penicillin, is predictably susceptible to ampicillin and amoxicillin. CLSI also states that clindamycin may appear active *in vitro* but is not effective

clinically and should not be reported as susceptible. Therefore resistance to clindamycin is not an issue in this strain. However, the strain was resistant to one unit of penicillin G (1 unit is equivalent to 0.6 μg) and this is most probably due to the lower concentration of penicillin G used compared to the higher sensitivity of 10 μg of penicillin V used. Many *Enterococci* aquire various pathogenic traits such as haemolytic activity in clinical environment and have been reported to be involved in nosocomial infection. Besides, the resistance of this genus to vancomycin is also of particular concern since vancomycin is often the last resort for treatment of enterococcal infection. Due to the opportunistic nature of the *Enterococci* spp, it is crucial for the industry to select only non-virulent and non-antibiotic resistant strains of *Enterococci* to be incorporated directly into food products and for use in the food industry (Franz *et al*., 2003; Kayser 2003).

5.2 Bacteriocin purification and characterisation

L. lactis A1 isolated from this study was not further investigated after the detection of Nis Z gene in this strain. This is because nisin is already well studied in past research and well published (Cheigh *et al*., 2004; Mitra *et al*., 2007; Park *et al*., 2003). *E. faecium* C1 isolated in this study did not produce the common enterocin A, B and P and was therefore chosen for further investigation. Research on species like *W. confusa* is very limited and no genes encoding bacteriocin production has been done from this strain. Therefore it was worthwhile to investigate the characteristics of the antimicrobial peptides and study its mode of action on target microorganisms.

Before the purification step, the bacteriocin production in different media was checked in order to choose the best media for optimum yield of bacteriocin production. The bacteriocin production is strongly dependent on the nutrient sources on the growth media and higher levels of bacteriocin production are often obtained at conditions lower

than required for optimal growth (Todorov & Dicks, 2004; Todorov *et al*., 2000). The highest yield of bacteriocin production by *W. confusa* A3 was recorded in MRS broth with inhibition zone of 11.62 mm and low bacteriocin activity was recorded when the strain was grown in BHI broth, TSB broth with 1 % tween 80, Brucella broth, NB with 1.5 % glucose and Miller's LB broth (inhibition zone reduce to 7 mm or less). It was notable that no bacteriocin activity can be detected when both strain A3 and C1 were grown in Brucella broth. This phenomenon may be due to low concentration of carbohydrate which is only 0.1 % of dextrose present in the medium.

In the case of *E. faecium* C1, the highest activity was seen in LAPTg media with inhibition zone of 12.68 mm followed by MRS media, 11.32 mm and lower in other media (Table 4.8). The inhibition zones when M17 supplemented with different sugars were around 9.82 to 10.46 mm. The simultaneous presence of a carbon source, a nitrogen source and salts play significant role in the production of bacteriocin. Therefore, in the subsequent purification steps, MRS was used to cultivate *W. confusa* A3 and LAPTg was used for culturing *E. faecium* C1. Different carbohydrates added to the media also interfered with the bacteriocin production. For example, the effect was shown when MRS was added with lactose, the antimicrobial activity decreased (Figure 4.15). It might be because *W. confusa* A3 was unable to ferment lactose as was recorded in the ealier API 50 CHL test (Table 4.4).

In order to harvest the bacteriocin at the right time and to get the highest production, the bacteriocin activity was monitored during the growth of the producer. It was shown that both strains A3 and C1 started to produce bacteriocin during exponential growth phase (Figures 4.12 and 4.13). For *W. confusa* A3 and *E. faecium* C1, the maximum bacteriocin production was detected between late exponential to early stationary phase. *W. confusa* A3 started to produce bacteriocin as early as 8 hours of incubation period while *E. faecium* C1 only produced at 12 hours of incubation time.

After stationary phase, both strains showed decease in antimicrobial activity. Therefore the accurate time of incubation for both bacteria to obtain the optimum amount of bacteriocin was between 18 to 24 hours. Similar trends of bacteriocin production was also reported in previous studies for nisin and other bacteriocins (de Arauz *et al*., 2009; Zamfir *et al*., 2000). The peptides were harvested during the optimum time range to prevent deactivation of the peptides by protease produced by the isolates itself.

The peptides or bacteriocins produced by other genera of LAB such as *Lactobacillus*, *Enterococcus*, *Leuconostoc*, *Streptococcus* and *Carnobacterium* were reported in previous studies. Nevertheless, there was insufficient studies on bacteriocins from *Weissella* sp especially *W. confusa* (Srionnual *et al*., 2007). The bacteriocins isolated from *W. confusa* was found to belong to class II bacteriocins from past research because the antibacterial activity was stable after exposure to low pH, pepsin, proteinase or heat (Nam *et al*., 2002). In this current study the antibacterial activity of the bacteriocin also showed similar characteristics. Therefore, BacA3 in this study was proposed to be class II bacteriocin.

The bacteriocins extracted from the bacteria in the current study showed good inhibitory activity against *B. cereus*, *E. coli*, *P. aeruginosa* and *M. luteus*. This indicates that the bacteriocin produced by *W. confusa* A3 and *E. faecium* C1 has a broad spectrum of antimicrobial activity inhibiting both Gram-positive and Gram-negative bacteria. Most of the Gram-positive bacteria are susceptible to Nisin or class I bacteriocin but have little or no effect on Gram-negative bacteria. This makes it applicable for use in the food industry as an additive to improve shelf life of many marketable food products. BacA3 was heat stable, functions well at pH range of 2 to 6 and sensitive to proteinase and peptidase. When compared to BacA3, BacC1 showed reduction in activity after heating to 80 and 100 °C (Table 4.12). It also functions at low pH and is sensitive to proteinase and peptidase (Table 4.13). It is notable that large bacteriocins from class III

is sensitive to high temperature. LAB can produce a number of substances which have antimicrobial activities. These include lactic acid, ethanol, hydrogen peroxide, aldehydes and bacteriocins. The most important property of bacteriocins is their proteinaceous nature which distinguishes them from other antimicrobial substances. The reduction of activity by proteolytic enzymes as shown in Table 4.13 confirms that the antimicrobial substance was not of lipid nature but was proteinaceous in nature. Furthermore, the antimicrobial activity was retained in the presence of catalase enzyme eliminating the possibility of antimicrobial effects due to the presence of hydrogen peroxide. Similar results also have been reported by various researchers in the enzymatic test of bacteriocin (Todorov & Dicks, 2006; Belgacem *et al*., 2008).

Since bacteriocins are proteinaceous in nature, they are very difficult to be purified from the complex media used. Furthermore, the bacteriocins are produced in very minute quantities in the broth thereby making it difficult to concentrate and purify the bacteriocin. Therefore, the first step of purification of bacteriocin usually involves concentrating the bacteriocin to reduce the working volume and then followed by a series of purification steps such as ion exchange, gel filtration or PR-HPLC. However, there is loss of bacteriocin activity at each step of the purification and sometimes the entire activity may be lost. Hence, although many strains are reported to produce antimicrobial activities but a few have been purified to homogeneity and characterised (Prada *et al*., 2007).

In this study, the method used to extract the bacteriocin first involved extracting the total protein from the bacteria broth culture by ammonium sulphate precipitation. Ammonium sulphate precipitation was found to be the ideal method to precipitate and concentrate the bacteriocin based on past research (Ghanbari *et al*., 2013; Rajaram *et al*., 2010; Salaiva *et al*., 2014). Then, the ammonium sulphate residues was cleaned up using Amberlite XAD 16 column and at the same time fractionated the hydrophobic

protein into 3 groups through elution by 10 % acetonitrile (weak hydrophobic), 50 % acetonitrile (hydrophobic) and 90 % acetonitrile (strong hydrophobic). Then the active fractions were further purified by size exclusion principle using Vivaspin centrifugal tubes. Vivaspin results in the current study also suggested that the bacteriocin was of small size ranging from 2 to 5 kDa for BacA3 and 5 to 50 kDa for BacC1. The SDS-PAGE also showed that BacA3 was approximately 2.5 kDa (Figure 4.23) and BacC1 around 10 kDa compared to the marker used (Figure 4.24). Furthermore MALDI-TOF result confirmed that the size of the BacA3 was approximately 2.7 kDa (Figure 4.25). The size was different from weissellin-A which was a 4450 Da class IIa bacteriocin purified from *Weissella paramesenteroides* DX (Papagianni & Papamichael, 2011) and weissellicin 110 which was 3,487.8 Da purified from *Weissella cibaria* 110 (Srionnual *et al*., 2007). Bacteriocin with similar molecular weights reported from past studies included pediocin A from *Pediococcus acidilactici* (Bhunia *et al*., 1988) and bacteriocin ST33LD from *Leuconostoc mesenteroides* subsp. *mesenteroides* (Todorov & Dicks, 2005). The N- terminal sequence of BacA3 was VAPGEIVESL. The BLAST search of the amino acid sequence with UniProt (http://www.uniprot.org/blast/) showed that it matched with an uncharacterised protein from *Bionectria ochroleuca* (protein entry: A0A0B7JVE4). The BLAST search was shown in Appendix E. The sequence length of the uncharacterised protein was 354 amino acids, and the N-terminal sequence of BacA3 matched with only 10 amino acids in the middle of the sequence with one amino acid mismatched. The glutamic acid (E) of BacA3 is mismatched with aspartic acid (D) from the protein from *Bionectria ochroleuca*. *Bionectria ochroleuca* is an endophytic fungus which produced secondary metabolites with a broad spectrum of antibacterial, antifungal and anti-dermatophytic activities (Ebrahim *et al*., 2012; Samaga *et al*., 2014).

The *E. faecium* C1 was isolated from milk fermented in the laboratory. *Enterococci* can be found naturally in the gastrointestinal tract and are also widely used

as starter cultures in many kinds of fermented food products. The antimicrobial activity of *E. faecium* was caused by antibacterial peptides called bacteriocins and has been widely investigated and discussed in the past (Kang & Lee, 2005). The bacteriocin produced by the strain in this study was different from the common enterocin reported. The common bacteriocin produced by *E. faecium* are enterocin A with 47 amino acid residues and molecular weight of 4.829 kDa (Aymerich *et al*., 1996), enterocin B with 53 amino acid residues and 5.465 kDa (Casaus *et al*., 1997) and enterocin P with 44 amino acid residues with theoretical molecular weight of 4.493 kDa (Cintas *et al*., 1997). The peptide extracted in this study is larger compared to the known enterocin but smaller than enterolysin which was 34.501 kDa and with 316 amino acids residues (Nilsen *et al*., 2003). The BacC1 was proposed as a class III bacteriocin which is a large and heat-labile peptide.

The same motif discovered in this study (GPXGPX) was reported in a previous study of bacteriocin produced by *E. faecium* FL31 but located in the middle of the sequence (Chakchouk-Mtibaa *et al*., 2014). But in the current study this novel motif was uniquely located at the beginning of the N-terminal of the bacteriocin sequence. Hydroxyproline with a hydroxyl (OH) group attached to the gamma C atom of proline is produced by hydroxylation. Bacteriocin BacFL31 produced by *E. faecium* FL31 is the first bacteriocin reported to contain hydroxyproline residues. Interestingly, these are novel and unique characteristics of bacteriocins produced by this genus and not frequently reported in any other genera (Chakchouk-Mtibaa *et al*., 2014). Hydroxyproline is a major component of the protein collagen and also found in collagen-like proteins in animals and in many glycoproteins of plants. Hydroxyproline plays an important role in maintaining collagen triple-helix structure and stability (Jenkins & Raines, 2002). This modified amino acid is also present in peptide antibiotics such as in β-ring of actinomycins produced by *Streptomyces* sp. and

microbisporicin produced by *Microbispora* sp. (Bitzer *et al*., 2009; Castiglione *et al*., 2008; Foulston & Bibb, 2010). The operon of the bacteriocin was not detectable in the bacterial genome as the operon encoding the production and immunity is harboured within the plasmid or the gaps of the genome which were not completely sequenced. The possibility of the operon being located within the plasmid is very high because many bacteriocins purified from *Enterococci* are frequently associated with plasmids as reviewed by Franz *et al*. (2007).

5.3 Effect of bacteriocins on target bacteria

The study of the mode of action of the bacteriocins was carried out with real-time PCR to detect the fluorescence emitted after different treatments. In this study, SYTOX® green fluorescence dye was used to assess the depolarization of the cytoplasmic membrane of *B. cereus*. In the treatment with tetracycline, low fluorescence density was detected whereby the bacteriocin BacA3 and BacC1 gave high fluorescence density (Figures 4.27 and 4.28). This was attributed to the action of tetracycline by inhibiting protein synthesis in bacteria without breaking the bacterial membrane. Hence, no DNA can be released from the cell. Same result was obtained for the negative control in which no substance was present to destroy the membrane and thereby allowed the detection of bacterial DNA. The mode of action shown by using real-time PCR and SYTOX® green dye binding to nucleic acid of affected target bacteria showed that the cell membrane was disrupted by the action of the isolated bacteriocin. SYTOX® Green dye has high binding affinity toward nucleic acid and easily penetrates those cells with compromised membranes but does not cross the intact membranes of live cells (Lebaron *et al.*, 1998; Roth *et al*., 1997). Instead of using a flow cytometer to quantify the fluorescence emission, real-time PCR was used. So once the bacteriocin permeabilized the cell membrane, the SYTOX® Green dye bound to the inner DNA and the fluorescence can be detected by real-time PCR. This was evidence that the bacteriocin

permeabilized the cells. SYTOX® Green dye appeared to be a reliable molecular probe to detect membrane disruption of targeted sensitive bacterial strains treated with bacteriocins. A similar method was also used in the past to detect membrane disruption of salivaricin 9 purified from *Streptococcus salivarius* (Barbour *et al*., 2013). Figure 4.27 (b) shows that the fluorescence uptake of the target bacteria in the negative control and tetracycline treatment was only about 600 kFU while the fluorescence uptake increased to between 1118 and 640 kFU when concentration of BacA3 was increased. The same trend also appeared in *B. cereus* treated with BacC1 in which the negative control and tetracycline treatments showed 282 and 286 kFU whereas 545, 606 and 631 kFU were recorded for BacC1 with increasing concentrations (Figure 4.28 (b)).

The electron micrographs of the untreated bacterial cells had smooth membranes and uniform shape (Figures 4.29 (a) and 4.30 (a)) but the two treated bacterial cells in this study became shrunken after treatment indicating water and ion losses from the cells (Figures 4.29 (b) and 4.30 (b)). Such an observation was also described in a past study (Raafat *et al*., 2008). At the same time the shape of the cell also became rough and irregular rod shaped. It may be due to the formation of pores that disrupt the proton motive force and the pH equilibrium causing leakage of ions and hydrolysis of ATP resulting in cell death (Benz *et al*., 1991; Roy *et al*., 2014; Sahl *et al*., 1987). In the transmission electron micrograph, the negative control without adding bacteriocin showed complete layers of the membranes (Figures 4.31 (a) and 4.32 (a)). However, after treatment with bacteriocin, the membrane sloughed off (Figures 4.31 (b) and 4.32 (b)) but retained the cytoplasmic content of the cell. Besides, spore formation could also be seen after the treatment. In Figure 4.31 (c) the inner content of the cell oozed out. *B. cereus* formed spores when the surrounding condition was not favourable as spores allowed them to remain dormant for extended periods. This indicated that the

bacteriocin added stress to the *Bacillus cereus* causing it to form spores for survival under an extreme condition.

Many of the lantibiotics exert their antimicrobial activity through interaction with the peptidoglycan precursor lipid II which, in turn, causes disruption of cell wall biosynthesis and/or pore formation thereby compromising the integrity of the cytoplasmic membrane of target cells (Pag & Sahl, 2002). The most well-known and extensively studied lantibiotic Nisin is capable of both mechanisms (Wiedemann *et al*., 2001). The unmodified class II bacteriocins are more diverse and generally function through membrane permeabilization of the target cells, causing dissipation of the proton motive force (Nissen-Meyer *et al*., 2009). Class III large bacteriocins can function directly on the cell walls of Gram-positive targets and cause death and lysis of the target cells. The TEM electron micrographs showed significant membrane disruption on the targeted cell membranes wherein the bacterial cells were completely ruptured after treatments with either BacA3 or BacC1 causing cell death of the target bacteria.

5.4 Genome sequencing of the LAB

The genome sequencing of *W. confusa* A3 generated a total number of 63 scaffolds and 2149 predicted genes. 2048 out of the 2149 are annotated genes, leaving 96 unannotated genes. The annotation with RAST server showed that the genome of *W. confusa* A3 contained 2,289,873 bp with 120 contigs and 274 subsystems in RAST which represent only 43 % of the sequences assigned. Most of the annotated genes are in the category of protein and carbohydrate metabolism. 174 out of a total of 1210 annotated genes are related to protein metabolism while 162 genes for carbohydrate metabolism. The third abundant set of genes encode for amino acids, their derivatives and the genes involved in DNA metabolism (Figure 4.33). For the resequencing of *E. faecium* C1 genome, the annotation with RAST server showed that the size of the

genome was 2,283,839 bp with 56 contigs and covered 325 subsystems which represent 53 % of the sequences assigned. More than 20 % (387 out of 1862) of the genes fall into the category of carbohydrate metabolism followed by 235 genes for protein metabolism and 198 genes for amino acids and their derivatives (Figure 4.35).

Surface proteins play an important role in mediating interactions with the surrounding environment. The sortase enzyme is responsible for covalently coupling a subset of sortase-dependent proteins (SDPs) to the cell wall of Gram-positive organisms through recognition of a conserved C-terminal LPXTG motif. Sequencing of the LAB isolates has allowed for the identification of the gene encoding sortase production. Studies suggest that LPXTG-anchored motifs may play a role in mucus-binding in the gastrointestinal tract (GIT) of the host (Roos & Jonsson, 2002). The genome of *W. confusa* A3 showed 8 genes related to sortase (LPXTG motif) and for *E. faecium* C1, 9 genes were detected from the genome. The use of the LPXTG motif has been investigated for *in vitro* vaccine delivery using food grade probiotic *Lactobacilli* as the presentation vector for the antigen. LAB and sortase-mediated cell wall anchoring have been explored in the display of potential vaccine antigens targeted on the gastrointestinal tract (Call & Klaenhammer, 2013; Vizoso Pinto *et al*., 2009). Although the genes encoding antimicrobial peptide transport system, permease component and possible colicin V production protein were detected in the genome of *W. confusa* A3 from RAST, the other bacteriocin genes were not detected in the current version of RAST. For *E. faecium* C1, 4 genes for ABC-type antimicrobial peptide transport system, permease component, phage lysin and colicin V production protein can be detected by RAST. The search of bacteriocin gene with BAGEL 3 also showed that *E. faecium* C1 harboured 2 genes related to bacteriocin /modified peptide at contig 288 which was listed as phage lysin by RAST (Table 4.23) and another unmodified class II bacteriocin with YGNGV amino acid sequence. Although this class II bacteriocin gene

is present in the genome, it might not be expressed during the fermentation process in LAPTg broth used. Therefore, the 66 amino acid long class II bacteriocin could not be purified from *E. faecium C*1.

In addition, given the importance of the genus *Enterococci* as a potential nosocomial pathogen, a search for virulence factors associated with invasiveness and disease severity was performed. A total of 43 virulence genes listed in virulence factor database and related to *Enterococcu*s species was examined. Out of the 43 virulence genes, 37 of these genes were found absent (Table 4.24). The probiotic strain *E. faecium* T110 contained 8 virulence genes out of the 43 genes. On the other hand, *E. faecium* C1 in this study harboured 6 virulence genes. Among the virulence genes examined, the bacteria showed to contain 3 out of 15 genes related to adherence. When compared with the probiotic strain *E. faecium* T-110, *E. faecium* C1 in this study does not produce *Scm* which is the second collagen adhesion gene. It also showed absence of the gene encoding the production of hyaluronidase in *E. faecium* C1. In the antiphagocytosis category, 13 genes were compared and only 2 genes were present in the bacteria but these 2 genes (*CdsA* and *UppS*) were reported to be safe because they are found to be essential proteins in other bacterial systems (Thurlow *et al*., 2009). In the category for biofilm production, although the *BopD* gene was present, it may not be expressed due to the absence of the *fsrABC* operon (Bourgogne *et al*., 2006; Natarajan & Parani, 2015). The genomic analysis with pathogenicity island database (PAI DB) also showed the absence of genes related to pathogenicity islands and vancomycin resistance in the genome of *E. faecium* C1 (Table 4.25).

5.5 Application of bacteriocin or the producer cultures

The bacteria when added to the milk showed significant reduction of the naturally occurring microorganisms in terms of total CFU count. Therefore, the

bacterial culture can be directly added to the milk in order to reduce or inhibit total microbial count and at the same time enhance the flavour and the taste of the milk. Another possible application of this strain is in cheese making (Gardiner *et al*., 1999; Giraffa, 2003). The bacteriocin produced by the strain can also be used as a commercial additive to extend the shelf-life of food products.

The application of bacteriocins either as additive or *in situ* production by bacteriocin producer is a promising area. When live cultures which produce bacteriocins are used in the food, the bacteriocin producers are either substituted for all or part of the starter or are subsequently acted as a protective culture to improve the safety of the food product (Leroy *et al*., 2003; O'Sullivan *et al*., 2002). Studies also showed that bacteriocins produced *in situ* by its producer or added exogenously were highly effective in inhibiting the growth of other bacteria especially when combined with moderate heat treatment in dairy products (Muñoz *et al*., 2007). In *ex situ* production of bacteriocin, the producer strains are grown in large scale and then the bacteriocins are recovered and added to food in partially purified or purified concentrates which would require approval as an additive. However, low concentration of bacteriocin is usually recovered leading to increase of processing cost.

Although bacteriocin production by inoculation with the producer strain is more cost effective, the production is influenced by many factors which include physicochemical factors and food composition. With optimum conditions and environment, the bacteriocin can be produced ideally in the food (Galvez *et al*., 2007) . The application of the *W. confusa* A3 and *E. faecium* C1 cultures or their bacteriocins in milk gave a positive impact on the milk (Figure 4.39). The milk sample was shown to give low bacteria count on PCA which usually does not support the growth of lactic acid bacteria but provide sufficient nutrient for the enumeration of bacteria in water, wastewater, food and dairy products. The decrease in number of bacteria in milk sample

added with *W. confusa* A3 and *E. faecium* C1 culture or their bacteriocins proved that their presence can inhibit the growth of other bacteria which can cause milk spoilage. The total bacteria count in the blank milk sample dramatically increased on the first day of incubation and then followed by continuous decrease during the incubation period. The reduction in bacteria count may be due to either nutrient depletion in the sample or growth of lactic acid bacteria (which did not appear on PCA) with antagonism effect against the bacteria. The strain also proved to be non-virulent and does not produce toxins and superantigens by genome analysis.

The selection of potential probiotic strains that can perform effectively in the gastrointestinal tract is a significant challenge. One common feature of probiotics is bile tolerance and both strains especially *E. faecium* C1 is highly tolerant to bile as shown in bile esculin test (Begley *et al*., 2006). Nowadays, the consumers pay a lot of attention to the relation between food and health. Since bacteriocins are considered as natural products, the usage of bacteriocins in food systems will surely gain good acceptance from consumers who demand for more natural and safer food products.

Apart from nisin which is FDA approved and used in the food industry for more than 40 years, the use of other new bacteriocin is also consider as an integral part of hurdle technology. Although they are not added to food in pure bacteriocin form, the bacteriocin producers are being incorporated into food to inhibit food pathogens. For example, *L. curvatus* CRL705 produces two peptides (lac705α and lac705β) bacteriocin and has strong inhibitory activity against *L. monocytogenes*. This bacteriocin producer and *L. monocytogenes* were incorporated into wheat gluten film in pilot scale and the anti-*Listeria* activity was observed over 50 days. The finding confirmed the potential of gluten film added with *L. curvatus* CRL705 was effective in preventing the growth of *L. monocytogenes* and thus improving the food quality and safety (Blanco Massani *et al*., 2014). Interestingly, *Weissella* and some other LAB also produce exopolysaccharides

(dextran) and have been introduced into sourdough baking. Research also showed that dextran improved bread softness, neutralized bran-induced volume loss, extend the shelf-life and enhance the nutrition value of bread without the use of additives (Kajala *et al*., 2015; Katina *et al*., 2009; Wolter *et al*., 2014). Therefore, the *W. confusa* A3 isolated from this study not only has antimicrobial properties but also has high industrial value to be used as a starter culture in baking industry. Due to the tedious procedures to obtained approval to use bacteriocin in food, it is more likely to use the bacteriocin producers as starter culture in fermentation to inhibit undesirable bacteria.

A research carried out in Germany by feeding piglets with probiotic *E. faecium* strain NCIMB 10415 showed significant reduction in diarrhea associated with *E. coli* and increase in piglets' body mass. The finding hypothesised that the probiotic present in the gut may influence bacterial colonisation by inhibiting the pathogen binding to the GI tract. The probiotic strains compete with the pathogens for the binding site on the host cell or they bind with the pathogens and block the adhesive surface of the pathogen and eventually prevent binding of the pathogens to the host cell (Bednorz *et al*., 2013). Previous study of *Enterococi* strains on dairy matrices also supports the use of *Enterococci* as potential probiotics. Four *Enterococci* strains isolated from a traditional Portuguese cheese were added to yoghurt and ingested by four healty adults. The results show that *E. faecium* 32 has the highest ability to transiently colonise the human GI tract with the highest number of viable bacterial count in the faeces. This result indicates that the *E. faecium* 32 not only can survive in the milk matrix used but also growth and proliferate in human GI tract (Primental *et al*., 2012). Therefore, the *E. faecium* C1 isolated in this study has high commercial value to be used as probiotic in animal feed and also in dairy products for human consumption.

CHAPTER 6: CONCLUSION

In this study, three LAB strains with antimicrobial properties were isolated from fermented cow milk samples. After the identification by 16S rRNA gene sequencing and API 50 CHL or 20 Strep, they were named as *Lactococcus lactis* A1, *Weissella confusa* A3 and *Enterococcus faecium* C1. The growth study of the bacteria showed that both *L. lactis* A1 and *W. confusa* A3 reached stationary phase after 20 hours of incubation. The growth of *E. faecium* C1 was faster and reached stationary phase at 18 hours incubation. Initial screening of the antimicrobial properties from the three isolates showed that strain A1 was only active against Gram-positive target bacteria which were *B. cereus*, *S. aureus* and *M. luteus* while A3 and C1 were active against both Gram-positive and negative bacteria used in this study namely *B. cereus, E. coli, P. aeruginosa* and *M. luteus*. In this context, it was noted that nisin, a commonly used bacteriocin was generally inactive against Gram-negative bacteria, imposing a limitation on its usage against food-borne pathogens such as *E. coli*, *Salmonella* spp., *Campylobacter* spp. and *Yersinia* spp. In fact, bacteriocins with activity against Gram-negative bacteria are rarely reported. PCR amplification to detect the structural genes encoding bacteriocin production in this study proved that *L. lactis* A1 harboured Nis Z gene that encoded for the production of nisin but *E. faecium* C1 showed absence of the three genes encoding enterocin A, B and P. In order to study novel bacteriocin production, *W. confusa* A3 and *E. faecium* C1 were selected for further bacteriocin purification and characterisation. The purification steps involved were 80 % ammonium sulphate purification, Amberlite XAD 16 hydrophobic interaction column, Vivaspin centrifugal filter and size separation concentrator and finally RP-HPLC. The purified bacteriocins were named BacA3 and BacC1. The results from SDS-PAGE showed that the molecular weight for BacA3 was around 2.5 kDa and matched with the active fraction from Vivaspin between 2-5 kDa. MALDI-TOF analysis suggested that the

molecular weight of BacA3 might be approximately 2.7 kDa. On the other hand, the molecular weight for BacC1 estimated by SDS-PAGE was around 10 kDa. The trypsin digested bacteriocin showed unique molecular weight of the fractions which did not match with any known protein from UniProt database thereby suggesting that it may be a novel protein. BacA3 exhibited thermo-stability when exposed to a temperature of 100 °C but BacC1 showed reduced activity after heating to 80 and 100 °C. Both BacA3 and BacC1 retained their activity when subjected to pH ranging from 2 to 6. When treated with proteinase and peptidase, both bacteriocins showed significant loss in activity. The reduction of activity by proteolytic enzymes confirmed that the antimicrobial component was not of lipid nature but proteinaceous in nature. The membrane permeabilization test by SYTOX® green nucleic acid stain and electron micrograph showed that both bacteriocins acted on the bacterial membrane by depolarising the membrane and leading to cell death. The basic antibiotic test showed that both strains were sensitive to most of the tested antibiotics. The search for genes encoding virulence, superantigens and diseases by RAST showed that both strains did not harbour such genes. The antibiotic resistance genes from *E. faecium* species as pathogenicity island database was also absent in *E. faecium* C1. The virulence genes as detected from virulence factor database showed that 6 genes (*Asm*, *SagA*, *EfaAfm*, *CdsA*, *UppS*, *BopD*) present in C1 were also present in another probiotic strain T110. Both bacterial strains and their bacteriocins showed significant reduction in total bacterial count in milk samples when added to study inhibitory effect. Besides, most bacteriocin-producing LAB is isolated from natural food sources and ideally suited for food industry applications.

Consumers are seeking to avoid foods that undergo extensive processing or which contain chemical preservative. Therefore, the production of bacteriocins by LAB is not only advantageous to the bacteria themselves but could also be exploited by the

food industry as a tool to control undesirable bacteria in a natural manner which is likely to be more acceptable to consumers. The era of the most successful infection prevention and treatment therapies using current antibiotics is coming to an end. The careless regulation and misuse of antibiotics have led to the evolution of antibiotic resistant microorganisms. Bacteriocins from lactic acid bacteria have been of particular interest for the food industry and have also shown promise in the medical industry. In conclusion, researchers and the industry have to continue the effort to discover novel bacteriocins that can control spoilage and pathogenic bacteria more effectively. These novel bacteriocins isolated from indigenous milk sources have potentials as an antimicrobial in the food industry. Further work is being pursued to facilitate its development as a food preservative or a new line of antibiotics.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

Conference presentations:

- Goh, H. F. & Philip, K. (2011). Isolation and identification of lactic acid bacteria with antimicrobial properties from fermented food products. *International Congress of the Malaysian Society for Microbiology*, *I*, pp. 634-638. Batu Ferringhi, Penang (Poster presentation).
- Goh, H. F. & Philip, K. (2014). Mechanism of binding of antimicrobial peptide on targerted bacterial membranes. RMIT, Melbourne (Oral presentation).

Seminar:

- Venue: Faculty of Science, ISB, Unit of Genetics and Molecular Biology, Seminar Room B6, 3 pm, 9th October 2015 (Friday)

  Title: Mechanism of binding of selected antimicrobial peptide on targeted bacterial membranes.

Publications:

- Goh, H. F. & Philip, K. (2015). Isolation and mode of action of bacteriocin BacC1 produced by nonpathogenic *Enterococcus faecium* C1. *Journal of Dairy Science, 98*(8), 5080-5090.
- Goh, H. F. & Philip, K. (2015). Purification and characterization of bacteriocin produced by *Weissella confusa* A3 of dairy origin. *PloS one*, *10*(10), e0140434.

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Isolation and mode of action of bacteriocin BacC1 produced by nonpathogenic *Enterococcus faecium* C1

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ABSTRACT

Lactic acid bacteria are present in fermented food products and help to improve shelf life and enhance the flavor of the food. They also produce metabolites such as bacteriocins to prevent the growth of undesirable or pathogenic bacteria. In this study, *Enterococcus faecium* C1 isolated from fermented cow milk was able to produce bacteriocin BacC1 and inhibit the growth of selected food-spoilage bacteria. The bacteriocin was purified through 4 steps: ammonium sulfate precipitation, hydrophobic interaction column, a series of centrifugal steps, and finally reversed-phase HPLC. A membrane permeability test using SYTOX green dye (Invitrogen, Grand Island, NY) showed that the bacteriocin caused significant disruptions to the test bacterial membrane, as shown by transmission electron microscopy. The molecular weight of the BacC1 obtained from SDS-PAGE was around 10 kDa, and N-terminal sequencing revealed a partial amino acid sequence of BacC1: GPXGPXGP. The bacterial strain was nonhemolytic and not antibiotic resistant. Therefore, it has high potential for application in the food industry as an antimicrobial agent to extend the shelf life of food products.

Key words: lactic acid bacteria, bacteriocin, fermented milk

INTRODUCTION

Lactic acid bacteria (**LAB**) are generally recognized as safe (GRAS) microorganisms and have been used in food fermentation for a long time (Stiles and Holzapfel, 1997). During fermentation, LAB utilize the sugar in food to produce end products such as lactic acid, hydrogen peroxide, diacetyl, and other organic acids that prevent the growth of spoilage bacteria. The production of bacteriocins by some LAB also contributes to the inhibition of spoilage bacteria, thereby extending the shelf life of the food (Deegan et al., 2006). *Enterococcus* is one of the prominent genera among LAB that can produce diverse antimicrobial peptides within a single strain (Izquierdo et al., 2009).

Enterococci are found in many foods such as dairy products, fermented sausages, and olives (Ben Omar et al., 2004). Enterococci are now being used as probiotics but they are also among the most common nosocomial pathogens associated with many human infections. They are known to cause endocarditis, bacteremia, and urinary tract, central nervous system, intraabdominal, and pelvic infections, and can cause multiple antibiotic resistance. The number of vancomycin-resistant enterococci is increasing. However, enterococci that possess virulence genes are only isolated from patients (Franz et al., 2003; Foulquié Moreno et al., 2006). Most of the pathogenic strains are multi-antibiotic resistant and produce adhesins, invasins, pili, and hemolysin, causing bacteremia, endocarditis, and other infections (Franz et al., 2011; Rehaiem et al., 2014).

The LAB are known to produce bacteriocins, which are antimicrobial peptides that can inhibit the growth of closely related microorganisms and certain food-spoilage microorganisms, including *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus subtilis*, and spores of *Clostridium perfringens* (Klaenhammer, 1993; du Toit et al., 2000). Producer strains of bacteriocins have a specific immunity mechanism to protect them from their own bacteriocins. These bacteriocins can also be produced in situ in the human gut by producer strains to combat intestinal infections (Cotter et al., 2013). Bacteriocins can be generally divided into 3 classes. Class I consists of lantibiotics, which are of low molecular weight and include lanthionine and β-methyllanthionine. Class II comprises heat-stable unmodified bacteriocins further sub-divided into 3 classes: namely, class IIa (*Listeria* active peptides), class IIb (2-peptide bacteriocins), and class IIc (cyclic peptides). Class III comprises high-molecular-weight and heat-labile bacteriocins (Yi et al., 2010).

In this paper, we describe a bacteriocin produced by *Enterococcus faecium* C1 isolated from fermented cow milk. This bacteriocin is a high-molecular-weight peptide with a broad inhibitory spectrum capable of permeabilizing the cell walls of target bacteria. In this study, gram-positive and gram-negative bacteria

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RESEARCH ARTICLE

Purification and Characterization of Bacteriocin Produced by *Weissella confusa* A3 of Dairy Origin

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Competing Interests: The authors have declared that no competing interests exist.

Abstract

A dramatic increase in bacterial resistance towards currently available antibiotics has raised worldwide concerns for public health. Therefore, antimicrobial peptides (AMPs) have emerged as a promisingly new group of therapeutic agents for managing infectious diseases. The present investigation focusses on the isolation and purification of a novel bacteriocin from an indigenous sample of cow milk and it's mode of action. The bacteriocin was isolated from *Weissella confusa* A3 that was isolated from the sample and was shown to have inhibitory activity towards pathogenic bacteria namely *Bacillus cereus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Micrococcus luteus*. The bacteriocin was shown to be heat stable and functioned well at low pH (2 to 6). Reduction of activity was shown after treatment with proteinase K, trypsin and peptidase that confirmed the proteinaceous nature of the compound. MALDI-TOF analysis of the sample gave a mass approximating 2.7 kDa. The membrane of the bacteria was disrupted by the bacteriocin causing SYTOX® green dye to enter the cell and bind to the bacterial DNA giving fluorescence signal. Bacterial cell treated with the bacteriocin also showed significant morphological changes under transmission electron microscope. No virulence and disease related genes can be detected from the genome of the strain.

Introduction

Antimicrobial peptides (AMPs) are ubiquitous and natural antibiotics generated by a diverse range of microorganisms, plants, insect and mammalian cells. Recent attention has been drawn to AMPs as new antimicrobials to combat harmful microbes especially those resistant to conventional antibiotics. In the search for new antimicrobial agents, these may be used as templates for the design of novel drugs [1,2]. AMPs are divided into different groups based on their variable structural characteristics [3]. Those AMPs produced by bacteria to kill or inhibit other bacteria are known as bacteriocins [4,5].

In an effort to establish a new antimicrobial agent, attention was focused on lactic acid bacteria (LAB) in this study. This was chosen as some bacteriocins from LAB are well studied in

APPENDIX

Appendix A

***Weissella confusa* A3 isolated in this study deposited in NCBI gene bank**

Weissella confusa strain A3 16S ribosomal RNA gene, partial sequence

GenBank: KJ476186.1

FASTA Graphics

Go to:

```
LOCUS       KJ476186               1447 bp    DNA     linear   BCT 05-MAY-2014
DEFINITION  Weissella confusa strain A3 16S ribosomal RNA gene, partial
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ACCESSION   KJ476186
VERSION     KJ476186.1  GI:625293650
KEYWORDS    .
SOURCE      Weissella confusa
  ORGANISM  Weissella confusa
            Bacteria; Firmicutes; Bacilli; Lactobacillales; Leuconostocaceae;
            Weissella.
REFERENCE   1  (bases 1 to 1447)
  AUTHORS   Koshy,P. and Goh,H.F.
  TITLE     Direct Submission
  JOURNAL   Submitted (20-FEB-2014) Division of Microbiology, Institute of
            Biological Sciences, Faculty of Science, University of Malaya,
            Pantai Valley, Kuala Lumpur, Federal Territory 50603, Malaysia
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                     /country="Malaysia"
                     /collected_by="Goh Hweh Fen"
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                     /product="16S ribosomal RNA"

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      121 ataacatttg gaaacagatg ctaataccgt ataacaatga caaccgcatg gttgttattt
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      241 taatggctta ccaaggcgat gatgcatagc cgagttgaga gactgatcgg ccacaatggg
      301 actgagacac ggcccatact cctacgggag gcagcagtag ggaatcttcc acaatgggcg
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      541 ttattgggcg taaagcgagc gcagacggtt atttaagtct gaagtgaaag ccctcagctc
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     1321 tcgctagtaa tcgcggatca gcacgccgcg gtgaatacgt tcccgggtct tgtacacacc
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     1441 gccgtct
```

Appendix B

***Enterococcus faecium* C1 isolated in this study deposited in NCBI gene bank**

Enterococcus faecium strain C1 16S ribosomal RNA gene, partial sequence

GenBank: KJ675503.1

FASTA Graphics

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LOCUS       KJ675503               1424 bp    DNA     linear   BCT 28-JUN-2014
DEFINITION  Enterococcus faecium strain C1 16S ribosomal RNA gene, partial
            sequence.
ACCESSION   KJ675503
VERSION     KJ675503.1  GI:658159245
KEYWORDS    .
SOURCE      Enterococcus faecium
  ORGANISM  Enterococcus faecium
            Bacteria; Firmicutes; Bacilli; Lactobacillales; Enterococcaceae;
            Enterococcus.
REFERENCE   1  (bases 1 to 1424)
  AUTHORS   Hweh Fen,G. and Philip,K.
  TITLE     Direct Submission
  JOURNAL   Submitted (08-APR-2014) Division of Microbiology, Institute of
            Biological Sciences, Faculty of Science, University of Malaya,
            Pantai Valley, Kuala Lumpur, Federal Territory 50603, Malaysia
COMMENT     ##Assembly-Data-START##
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            Sequencing Technology :: 16S rDNA
            ##Assembly-Data-END##
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      481 cagccgcggt aatacgtagg tggcaagcgt tgtccggatt tattgggcgt aaagcgagcg
      541 caggcggttt cttaagtctg atgtgaaagc ccccggctca accggggagg gtcattggaa
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      661 agatatatgg aggaacacca gtggcgaagg cggctctctg gtctgtaact gacgctgagg
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      781 agtgctaagt gttggagggt ttccgccctt cagtgctgca gctaacgcat taagcactcc
      841 gcctggggag tacgaccgca aggttgaaac tcaaaggaat tgacgggggc ccgcacaagc
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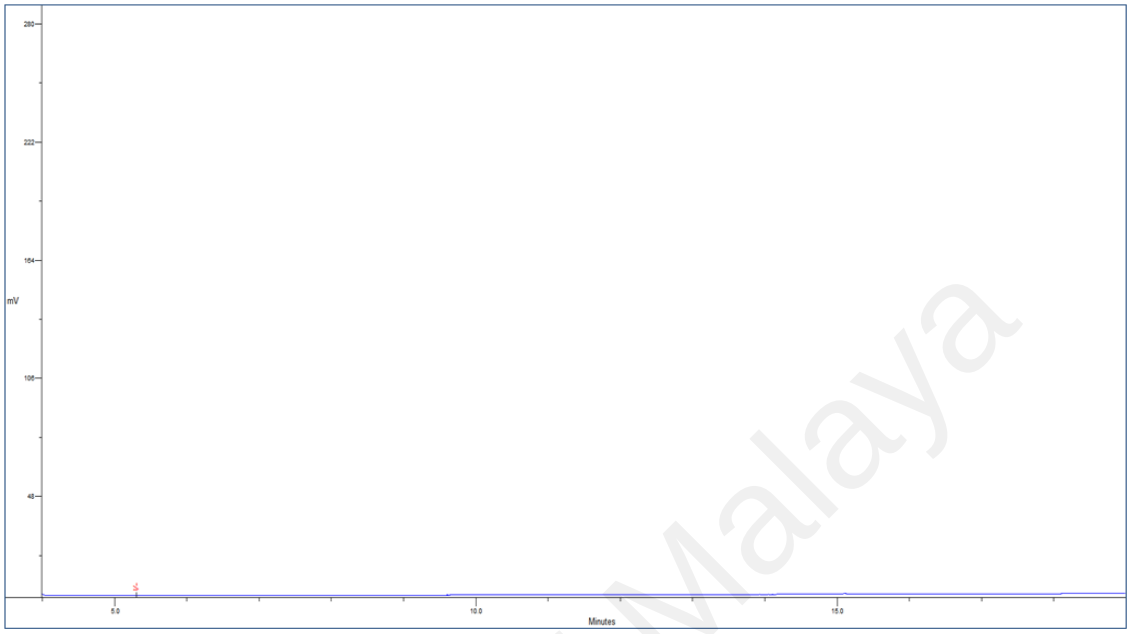
Appendix C

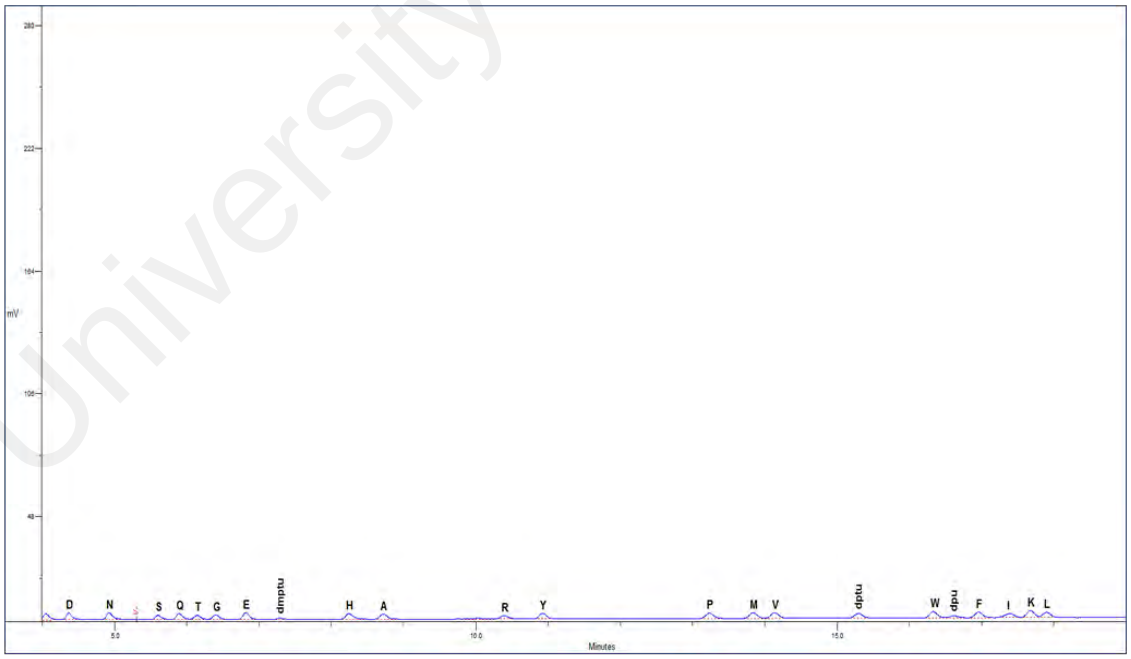
N-terminal sequencing chromatograph of BacA3

1. Blank (-2.0~290mV)



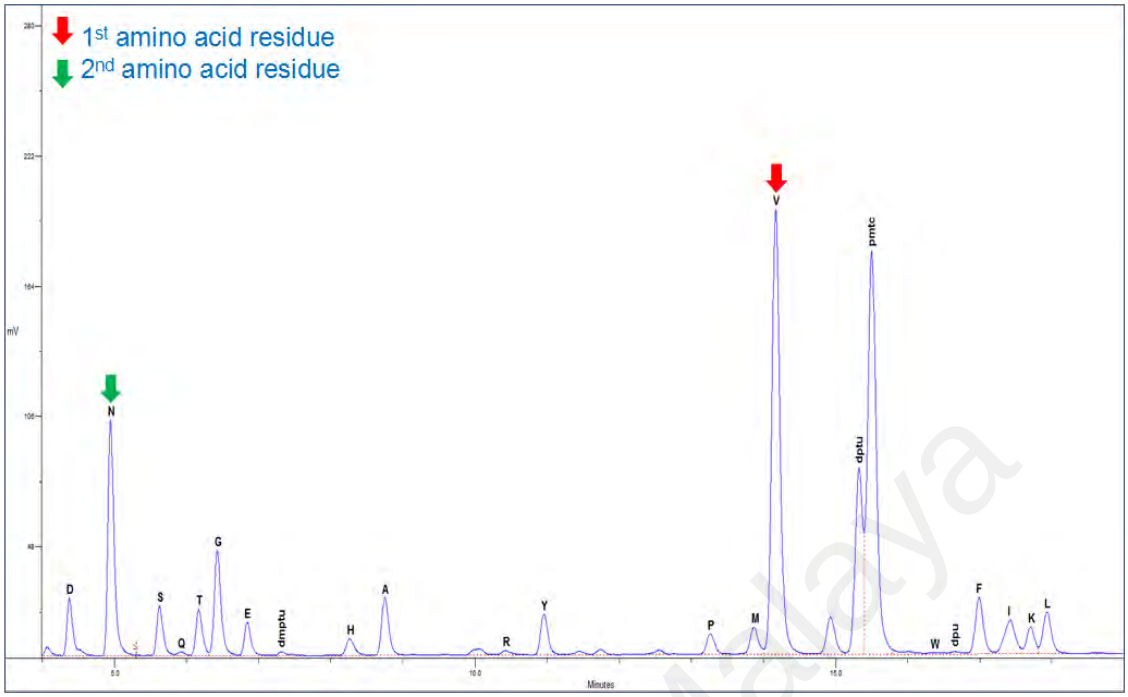
To check column cleaning condition

2. PTH-standard :

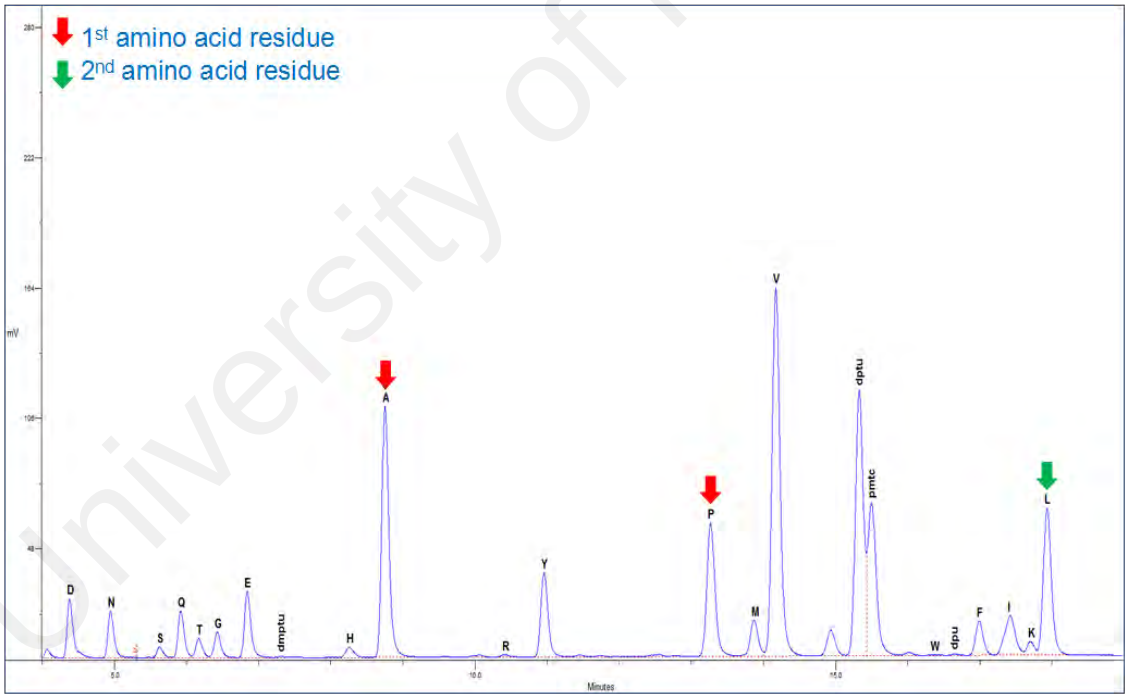


To calculate for retention times about 19 amino acids on PTH column, by loading the standard amino acid mixture.

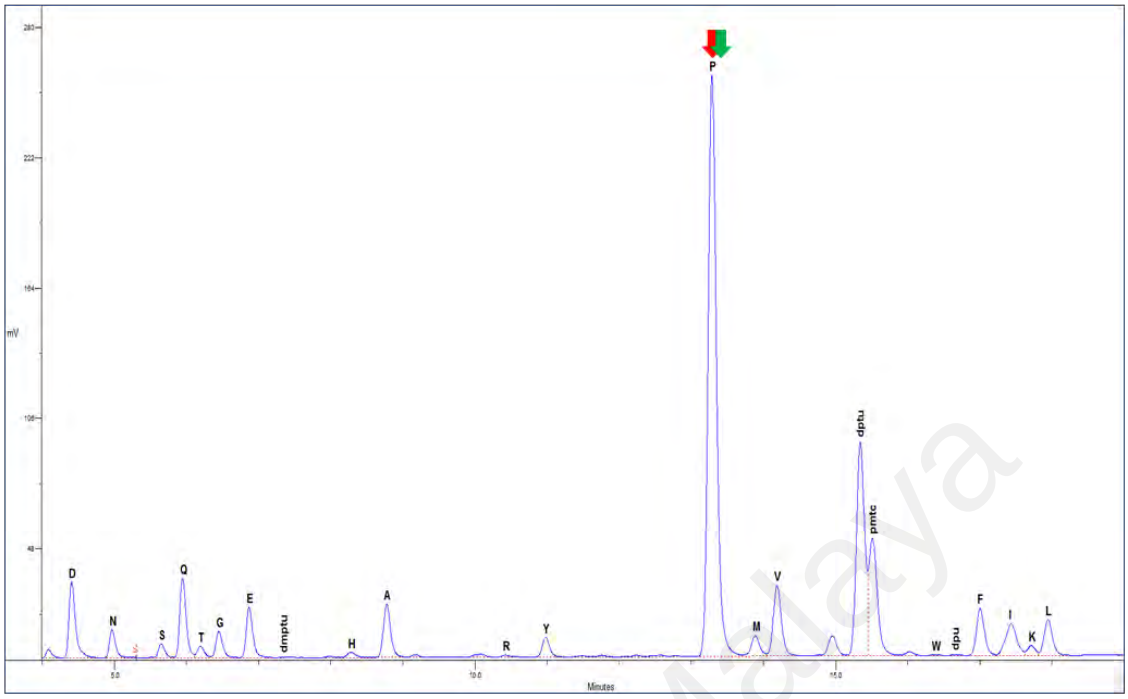
3. 1st residue chromatogram



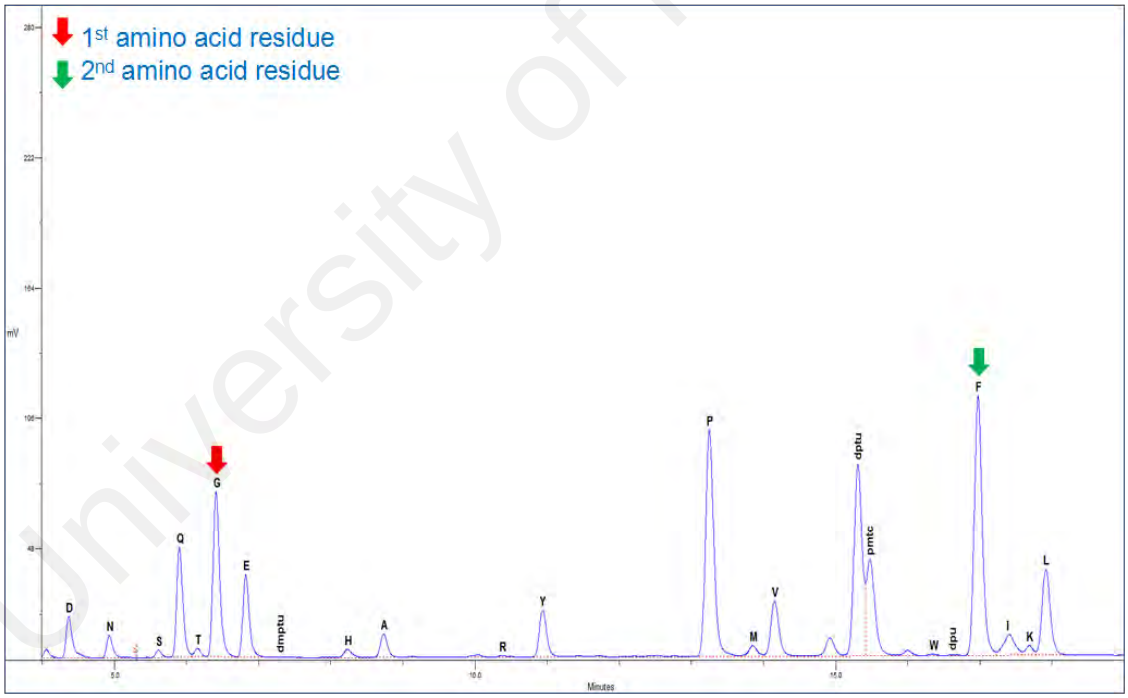
4. 2nd residue chromatogram



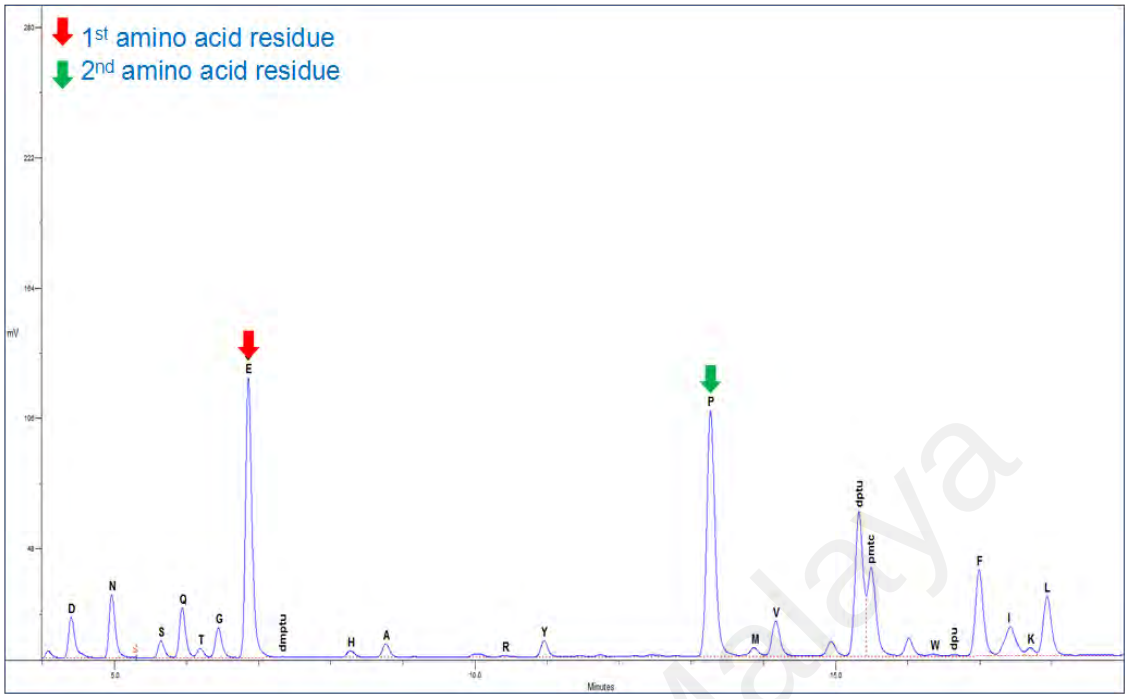
5. 3rd residue chromatogram



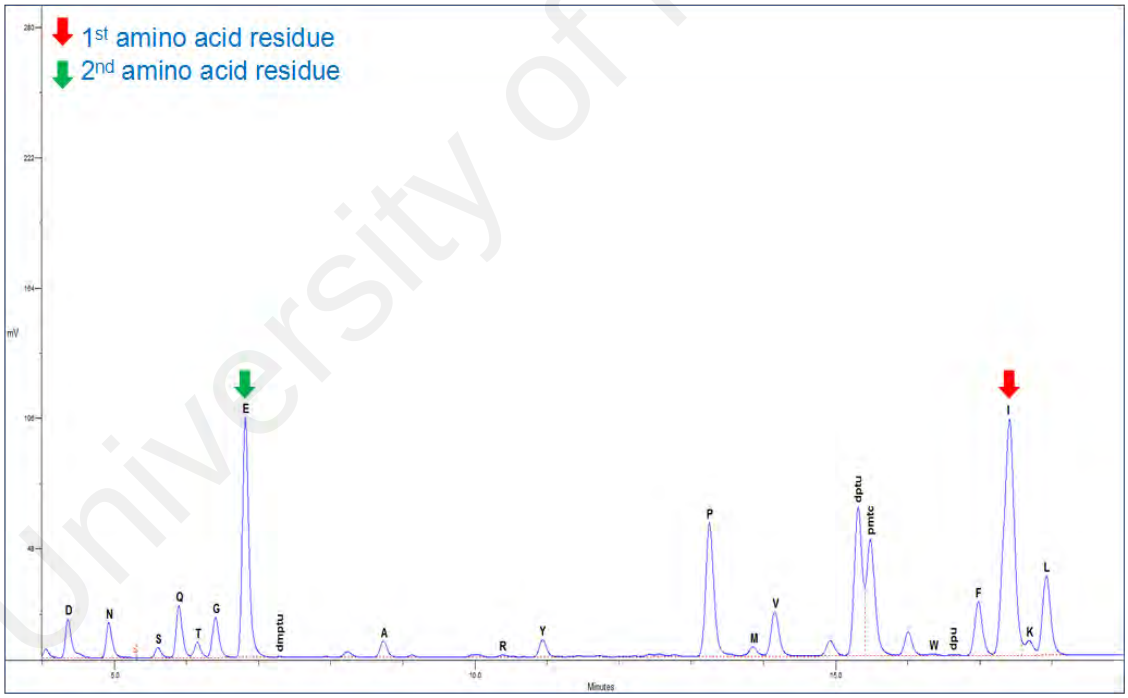
6. 4th residue chromatogram



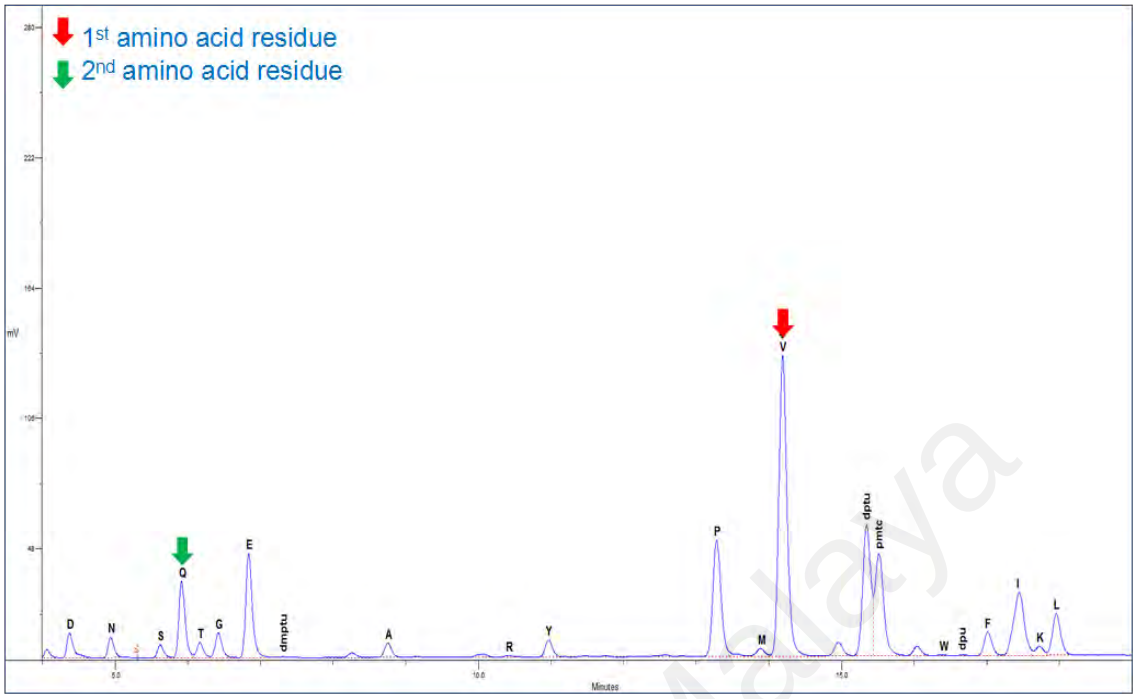
7. 5th residue chromatogram



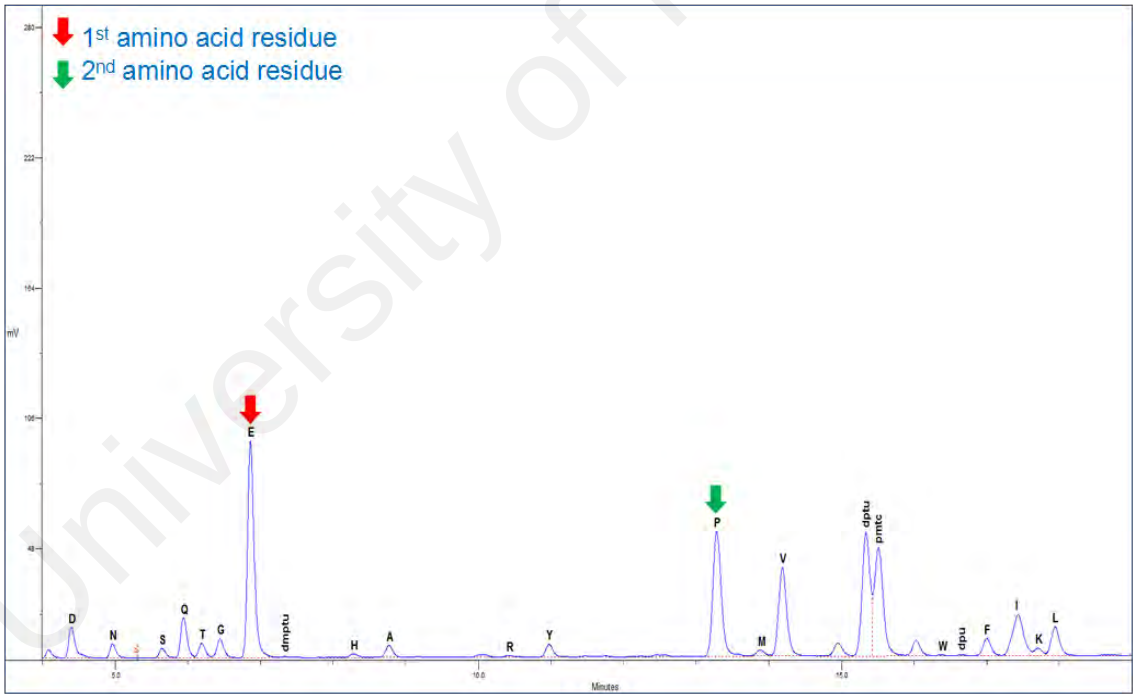
8. 6th residue chromatogram



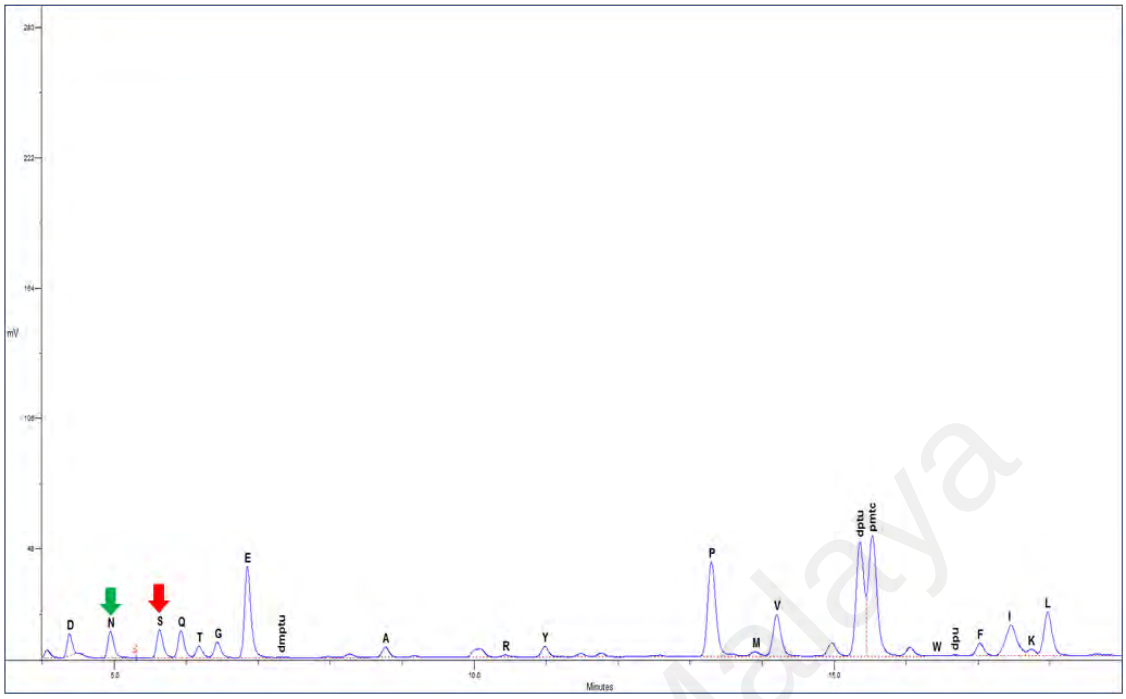
9. 7th residue chromatogram



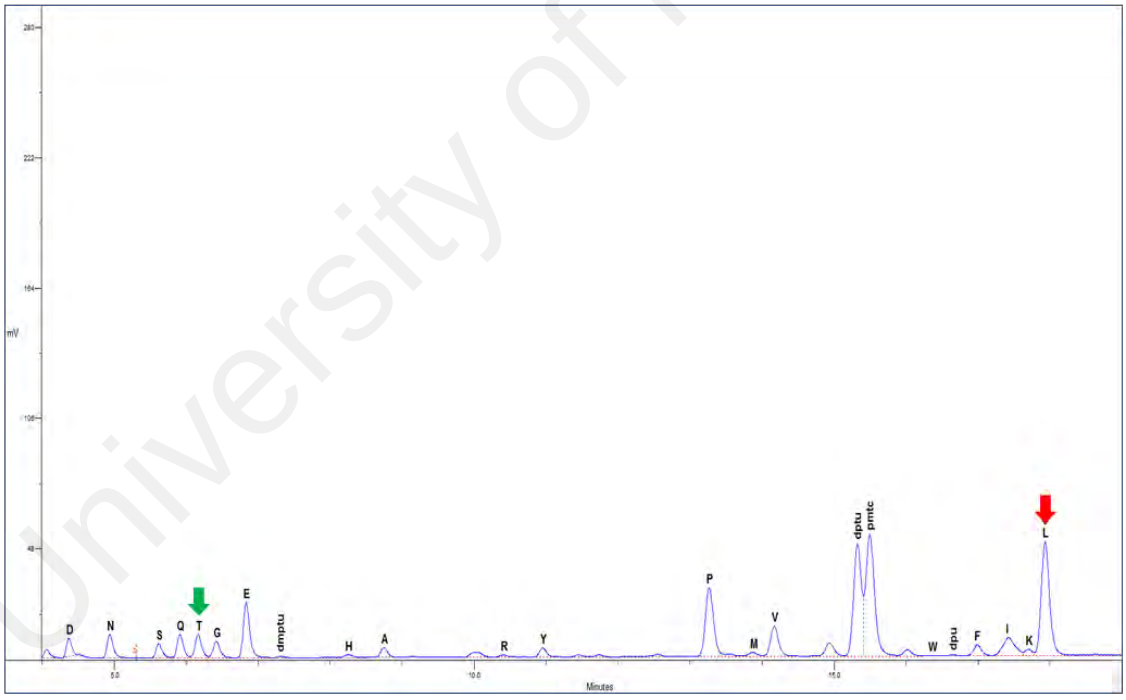
10. 8th residue chromatogram



11. 9th residue chromatogram



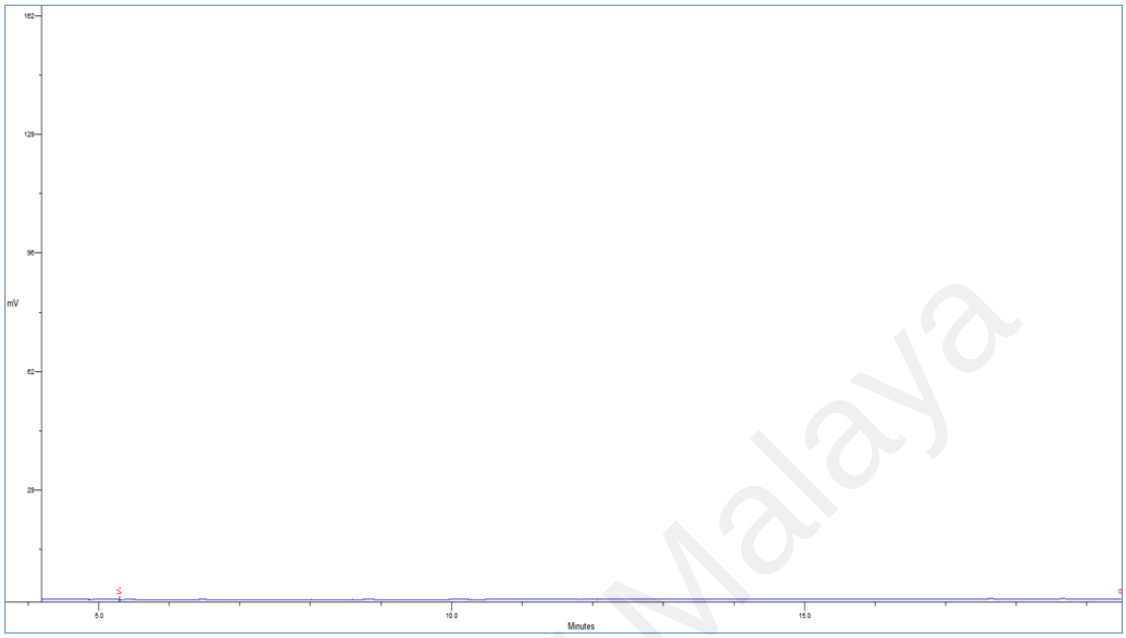
12. 10th residue chromatogram



Appendix D

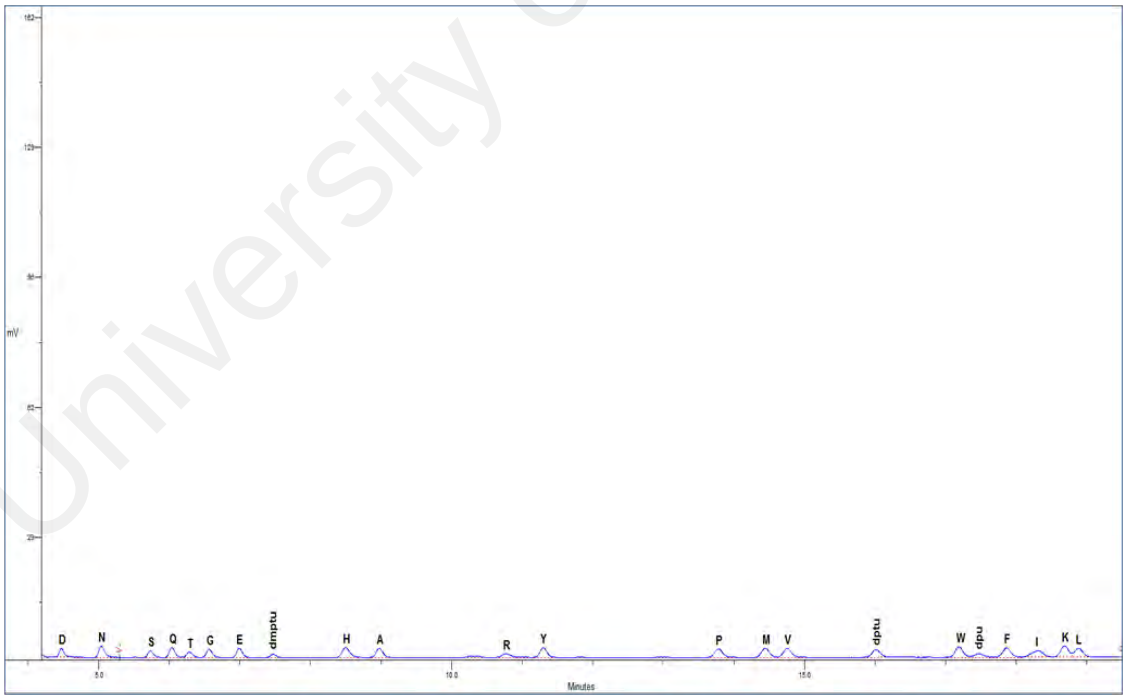
N-terminal sequencing chromatograph of BacC1

1. Blank (-2.0~165mV)



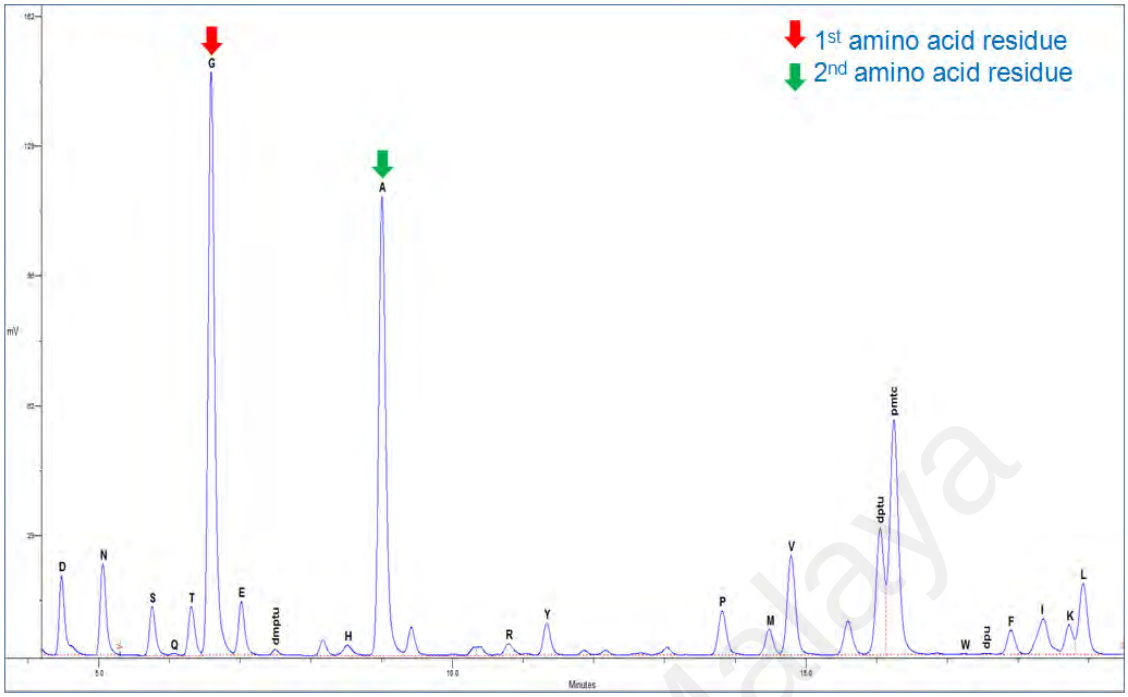
To check column cleaning condition

2. PTH-standard :

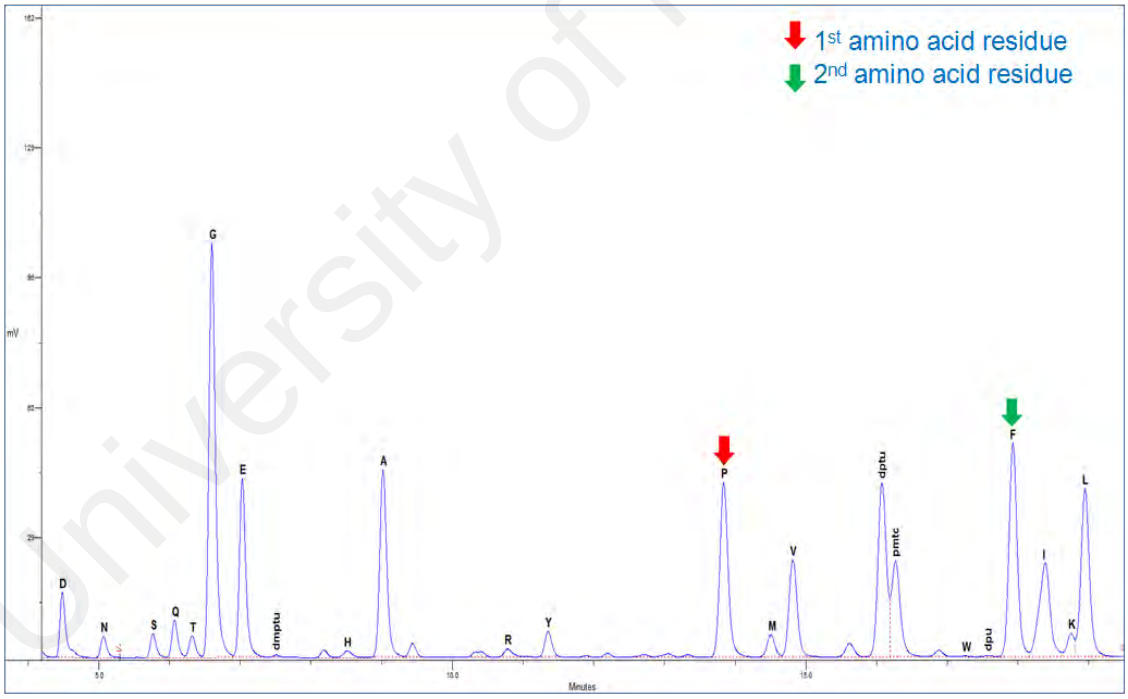


To calculate for retention times about 19 amino acids on PTH column, by loading the standard amino acid mixture.

3. 1st residue chromatogram



4. 2nd residue chromatogram



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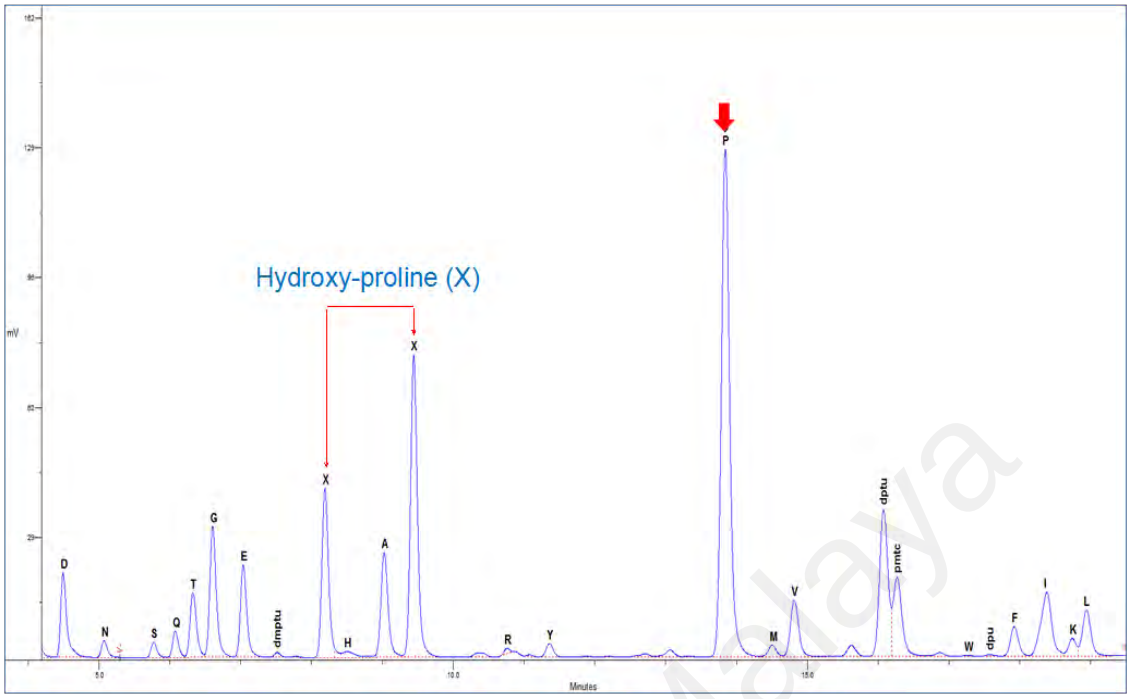
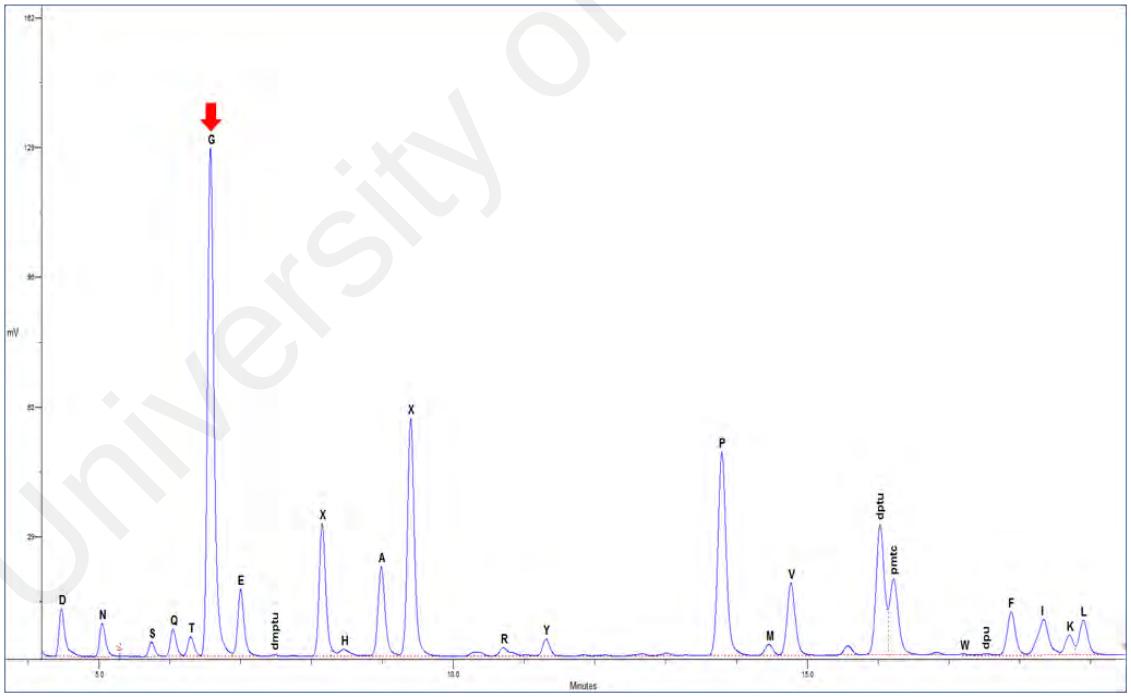
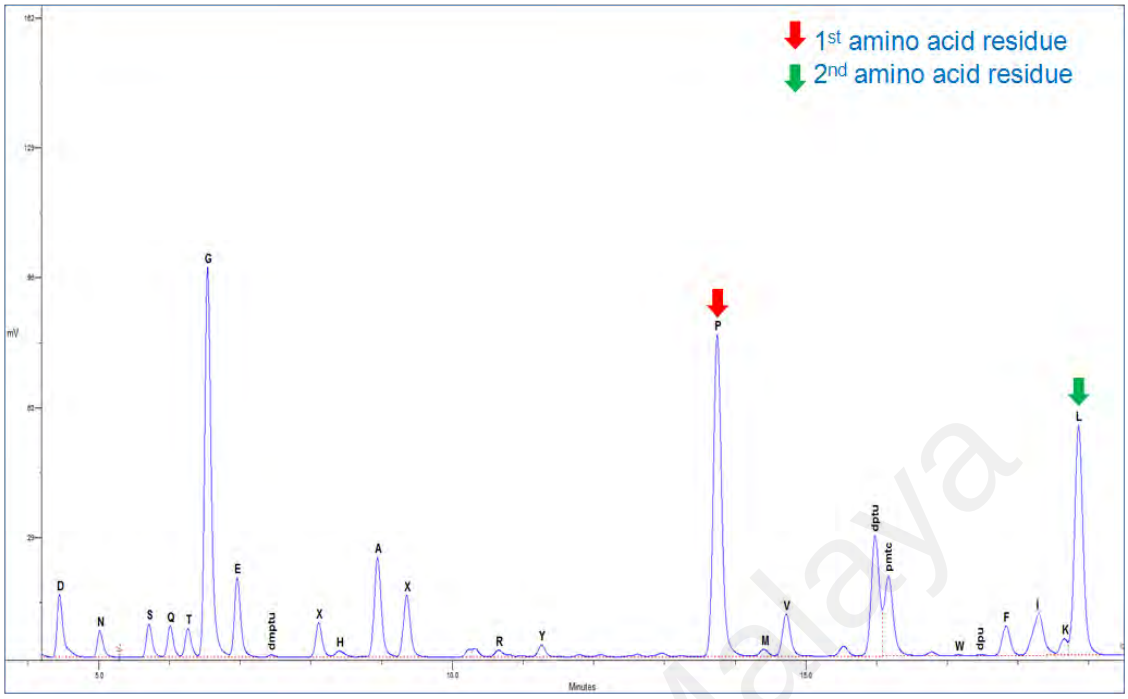


- Occasionally observe hydroxy-proline from the natural peptide / protein

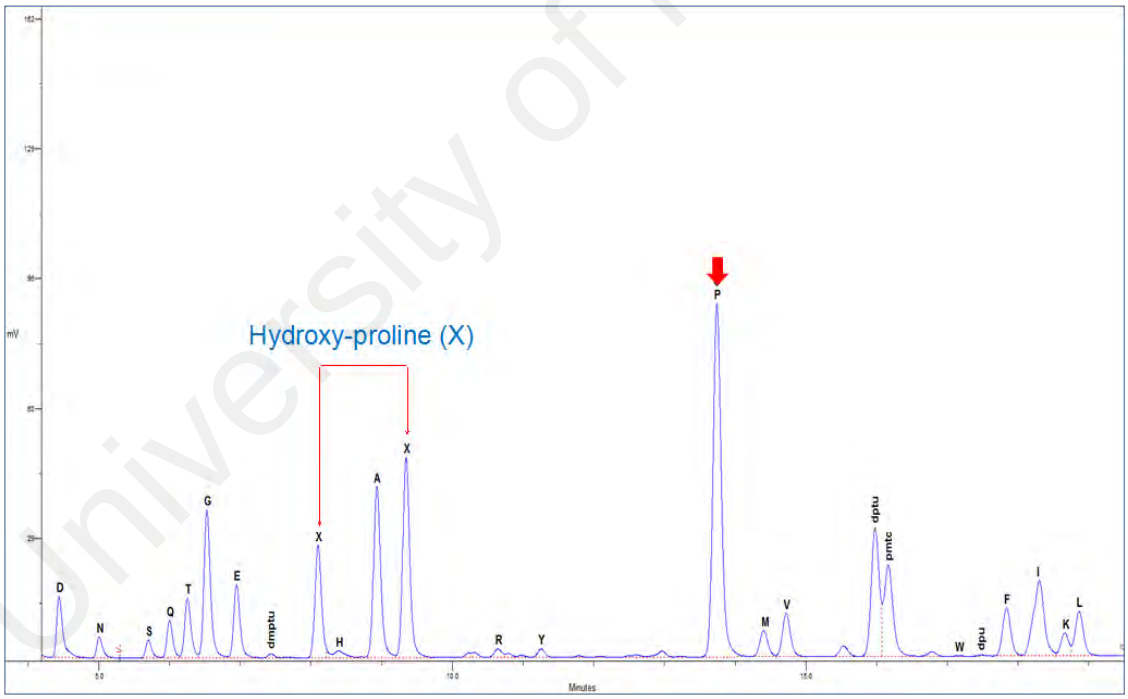
6. 4th residue chromatogram



7. 5th residue chromatogram

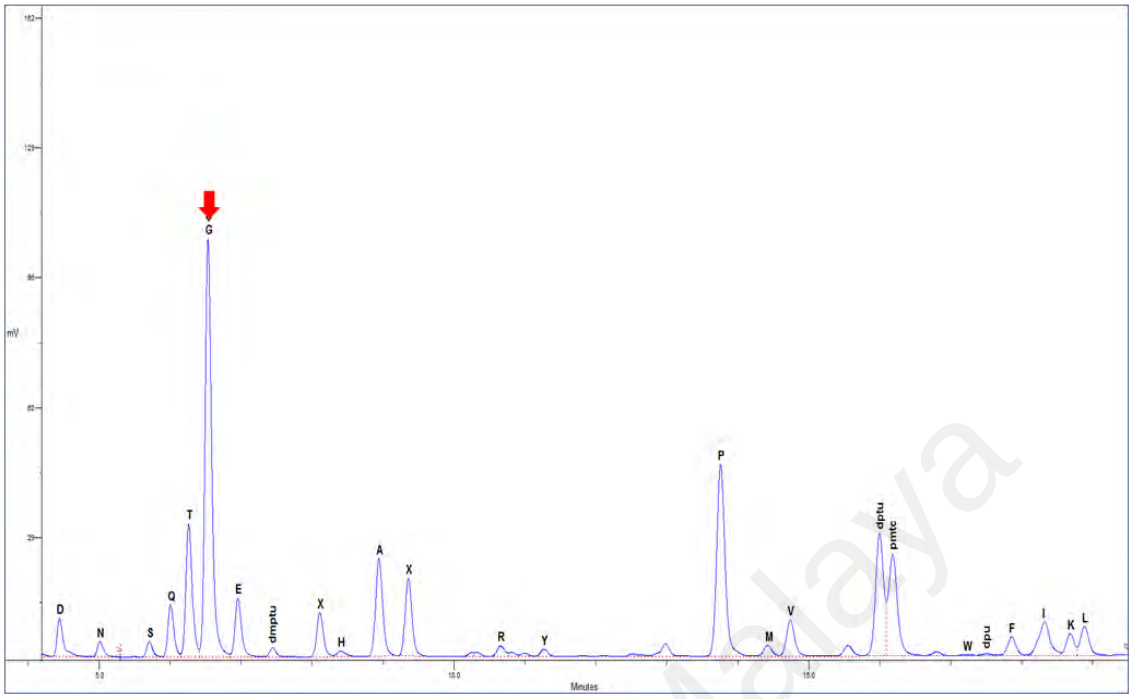


8. 6th residue chromatogram

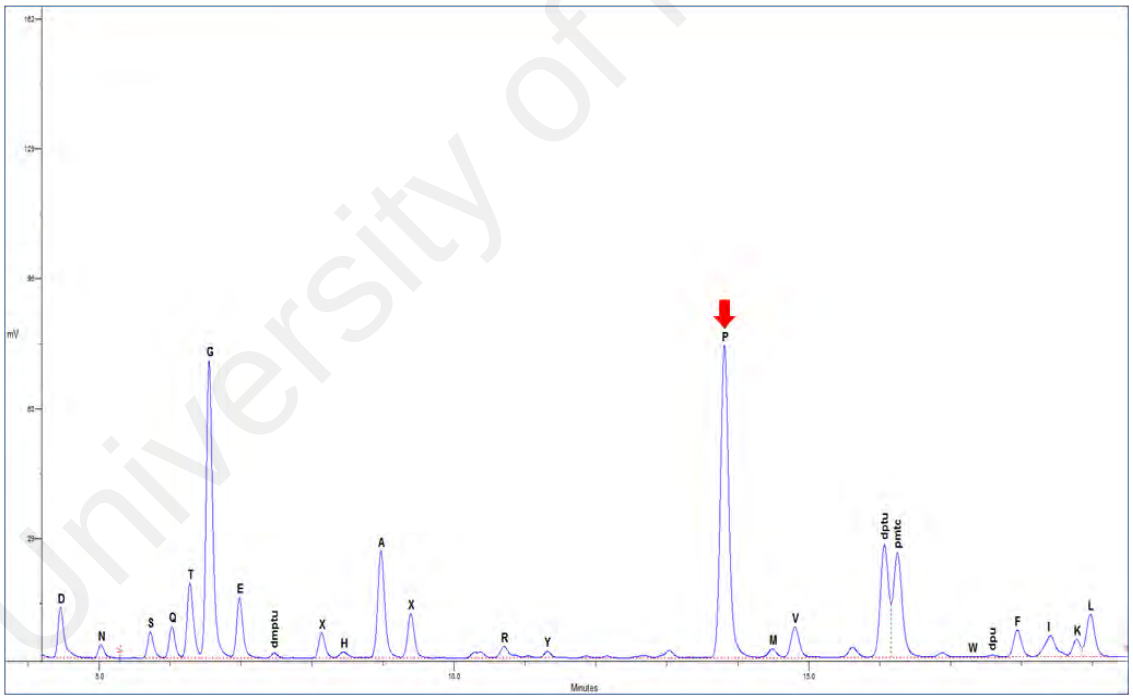


- Occasionally observe hydroxy-proline from the natural peptide / protein

9. 7th residue chromatogram

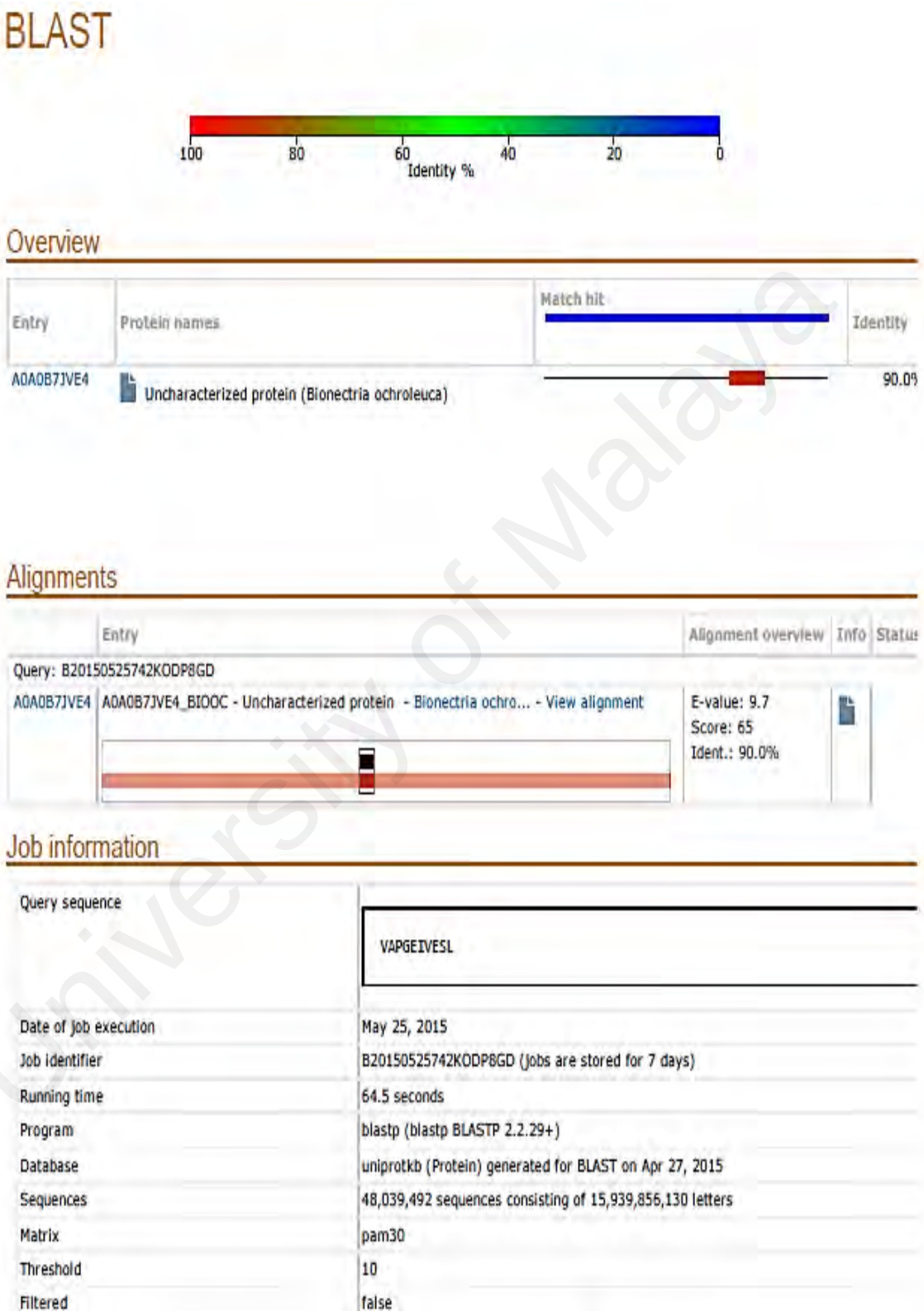


10. 8th residue chromatogram



Appendix E

BLAST search of partial N-terminal sequence of BacA3



Align

Alignment

How to print an alignment in color

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B20150525742KO3FO41          1    ----------------------------------------VAPGEIVESL----------    10
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                                                                        *******:**

B20150525742KO3FO41          11   ------------------------------------------------------------    10
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B20150525742KO3FO41          11   ------------------------------------------------------------    10
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B20150525742KO3FO41          11   ----------------------------------------------------------      10
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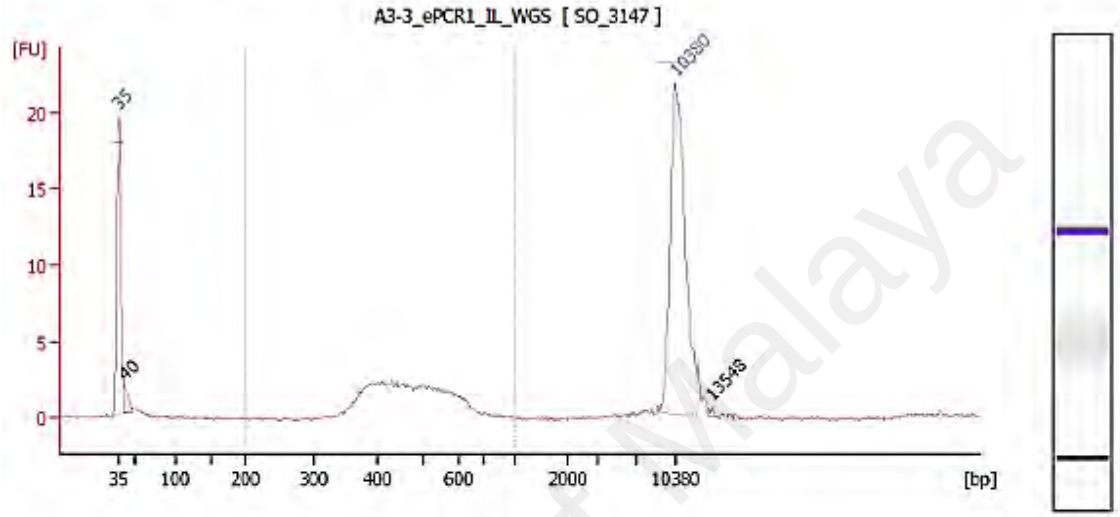
```

Appendix F

Quality validation of the prepared DNA libraries by running an aliquot on High Sensitivity Bioanalyzer Chip

Genotypic Technology **Date:7th July, 2014**
Project Number: SO_3147



Overall Results for sample 4 : A3-3_ePCR1_IL_WGS

Number of peaks found:	2	Corr. Area 1:	32.4
Noise:	0.1		

Peak table for sample 4 : A3-3_ePCR1_IL_WGS

Peak	Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
1	35	125.00	5,411.3	Lower Marker
2	40	8.02	303.0	
3	10,380	75.00	10.9	Upper Marker
4	13,548	0.00	0.0	

Region table for sample 4 : A3-3_ePCR1_IL_WGS

From [bp]	To [bp]	Corr. Area	% of Total	Average Size [bp]	Size distribution in CV [%]	Conc. [pg/µl]	Molarity [pmol/l]	Color
200	1,000	32.4	81	475	16.9	81.80	270.9	

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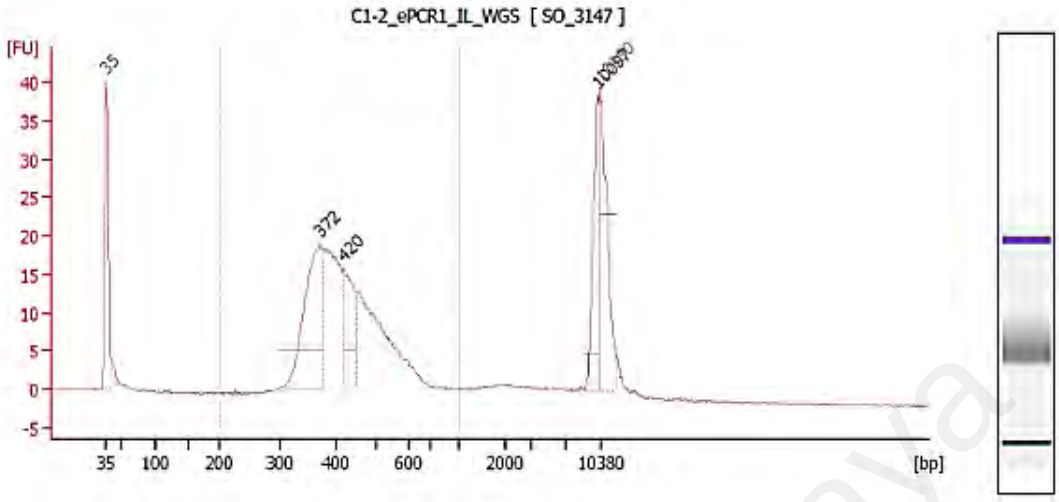


Overall Results for sample 5 : C1-2_ePCR1_IL_WGS

Number of peaks found:	3	Corr. Area 1:	271.7
Noise:	0.1		

Peak table for sample 5 : C1-2_ePCR1_IL_WGS

Peak	Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
1	35	125.00	5,411.3	Lower Marker
2	372	201.10	820.0	
3	420	89.21	322.0	
4	10,097	60.81	9.1	
5	10,380	75.00	10.9	Upper Marker

Region table for sample 5 : C1-2_ePCR1_IL_WGS

From [bp]	To [bp]	Corr. Area	% of Total	Average Size [bp]	Size distribution in CV [%]	Conc. [pg/µl]	Molarity [pmol/l]	Color
200	1,000	271.7	78	440	22.3	717.43	2,597.6	

Appendix G

Summary report of *W. confusa* A3 genome analysis

Sample Sequence Data Information:	
Read length	280-50 bases
Type of sequencing: Single/Paired end sequencing	Paired end
Sequenced using	Illumina MiSeq

Reference Sequence Data Information:	
Organism Name	*Weisella confusa*
Type of Sequence: Genomic/Transcriptomic	Genomic
No. of Chromosomes/Transcripts	NA
Reference Sequence Data QC Information:	
Sequence File Name	NA
Sequence File Size	
Maximum Sequence Length	
Minimum Sequence Length	
Average Sequence Length	
No. of Sequences	
Total Sequences Length	
Total Number of Non-ATGC Characters	
Percentage of Non-ATGC Characters	
QC and Raw data processing *	
QC Program used	SeqQC-V2.0
High Quality Reads filtering	Yes
Vector (Adapter/Primer) contaminated reads filtering: Yes/No	Yes
Adapter Trimming	Yes
B-trimming	Yes
Low-quality end-trimming	Yes
Primary Analysis	
Denovo Assembly Tool used	SPAdes 3.1
Scaffolding tool used	SSPACE v2.0
Minimum Contig Length	100
Minimum Scaffold Length	500

Appendix H

Genome sequence data QC statistics of *W. confusa* A3

No	1	2
Fastq file name	ProcessedReads_SO_3147_R1.fastq	ProcessedReads_SO_3147_R2.fastq
Fastq file size	414.27 MB	414.27 MB
Time taken for Analysis	30.72 Seconds	31.06 Seconds
Maximum Read Length	280	280
Minimum Read Length	32	32
Mean Read Length	255	255
Total Number of Reads	767913	767913
Total Number of HQ Reads 1*	766662	743175
Percentage of HQ Reads	99.837 %	96.779 %
Total Number of Bases	195541798 bases	195541798 bases
Total Number of Bases in Mb	195.5418 Mb	195.5418 Mb
Total Number of HQ Bases 2*	193486437 bases	182843014 bases
Total Number of HQ Bases in Mb	193.4864 Mb	182.8430 Mb
Percentage of HQ Bases	98.949 %	93.506 %
Total Number of Non-ATGC Characters	6696 bases	10635 bases
Total Number of Non-ATGC Characters in Mb	0.007 Mb	0.011 Mb
Percentage of Non-ATGC Characters	0.003 %	0.005 %
Number of Reads with Non-ATGC Characters	488	570
Percentage of Reads with Non-ATGC Characters	0.064 %	0.074 %

Appendix I

Read length distribution of *W. confusa* A3

Fastq file name	ProcessedReads_SO_3147_R1.fastq	ProcessedReads_SO_3147_R2.fastq
Percentage of reads between 271-280bp	58.76	58.76
Percentage of reads between 261-270bp	5.86	5.86
Percentage of reads between 251-260bp	6.34	6.34
Percentage of reads between 241-250bp	6.89	6.89
Percentage of reads between 231-240bp	6.67	6.67
Percentage of reads between 221-230bp	5.21	5.21
Percentage of reads between 211-220bp	3.1	3.1
Percentage of reads between 201-210bp	1.7	1.7
Percentage of reads between 191-200bp	1.03	1.03
Percentage of reads between 181-190bp	0	0
Percentage of reads between 171-180bp	0.66	0.66
Percentage of reads between 161-170bp	0.49	0.49
Percentage of reads between 151-160bp	0.42	0.42
Percentage of reads between 141-150bp	0.37	0.37
Percentage of reads between 131-140bp	0.29	0.29
Percentage of reads between 121-130bp	0.29	0.29
Percentage of reads between 111-120bp	0.25	0.25
Percentage of reads between 101-110bp	0.2	0.2
Percentage of reads between 91-100bp	0.18	0.18
Percentage of reads between 81-90bp	0.15	0.15
Percentage of reads between 71-80bp	0.1	0.1
Percentage of reads between 61-70bp	0.08	0.08
Percentage of reads between 50-60bp	0.06	0.06

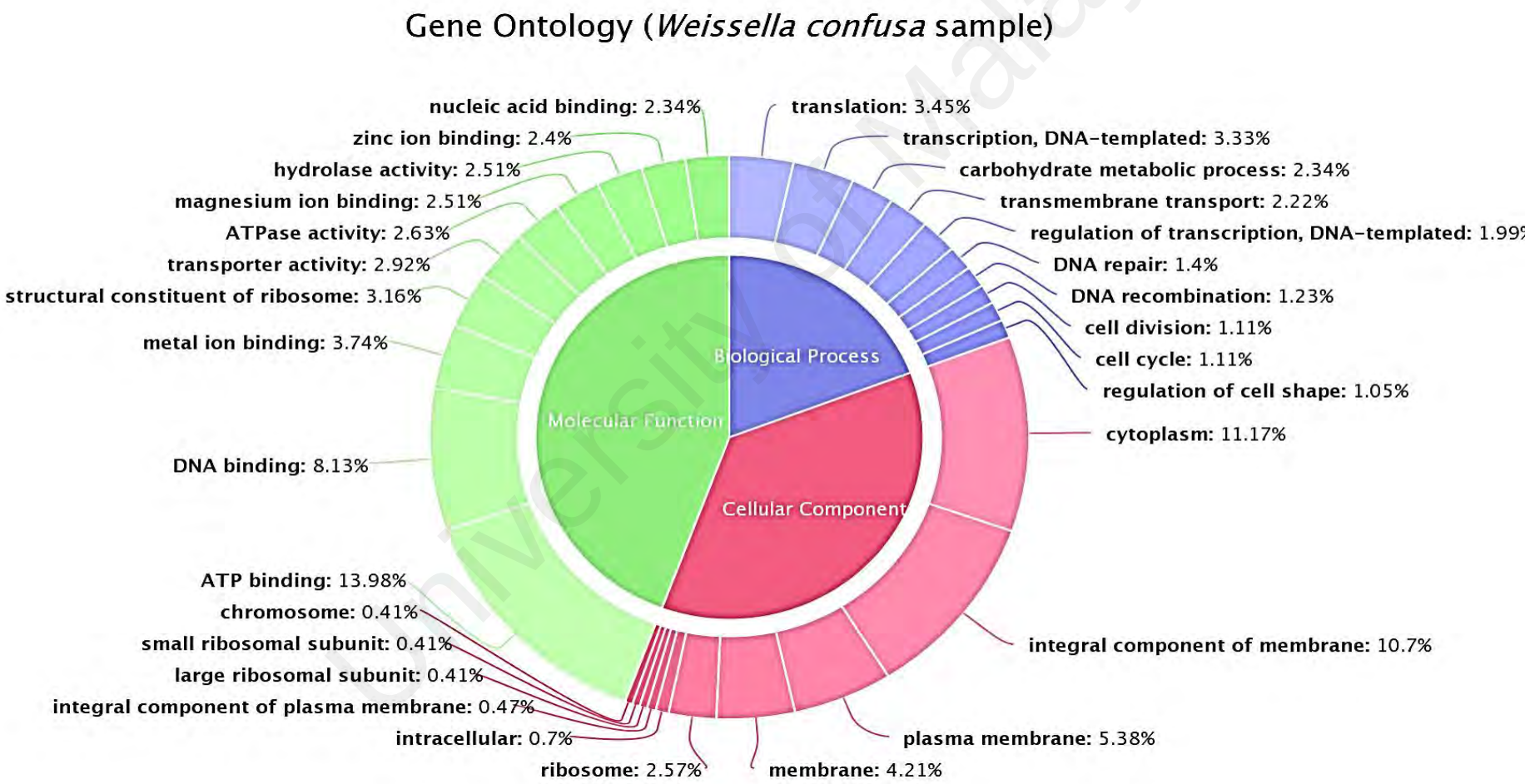
Appendix J

***W. confusa* A3 assembly QC statistics**

Description	Contigs	Scaffolds
Contigs Generated	125	63
Maximum Contig Length	1,86,748	2,37,103
Minimum Contig Length	128	507
Average Contig Length	18,238.4 ± 36,292.0	35,986.7 ± 51,038.4
Median Contig Length	33937	1,531.0
Total Contigs Length	22,79,805	22,67,162
Total Number of Non-ATGC Characters	0	5
Percentage of Non-ATGC Characters	0.000	0.000
Contigs >= 100 bp	125	63
Contigs >= 200 bp	119	63
Contigs >= 500 bp	63	63
Contigs >= 1 Kbp	55	54
Contigs >= 10 Kbp	37	35
Contigs >= 1 Mbp	0	0
N50 value	79,432	79,432

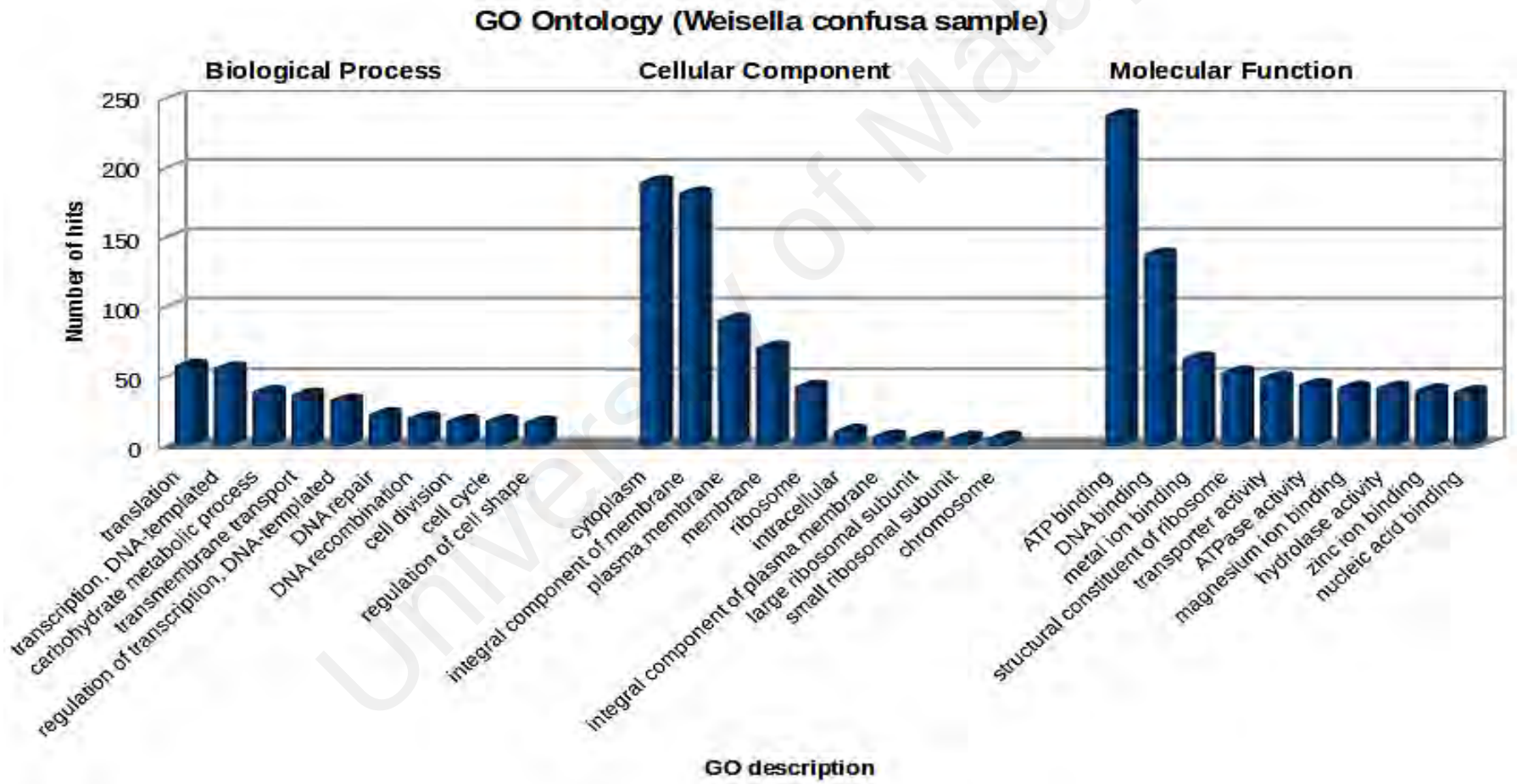
Appendix K

Gene ontology of *W. confusa* A3



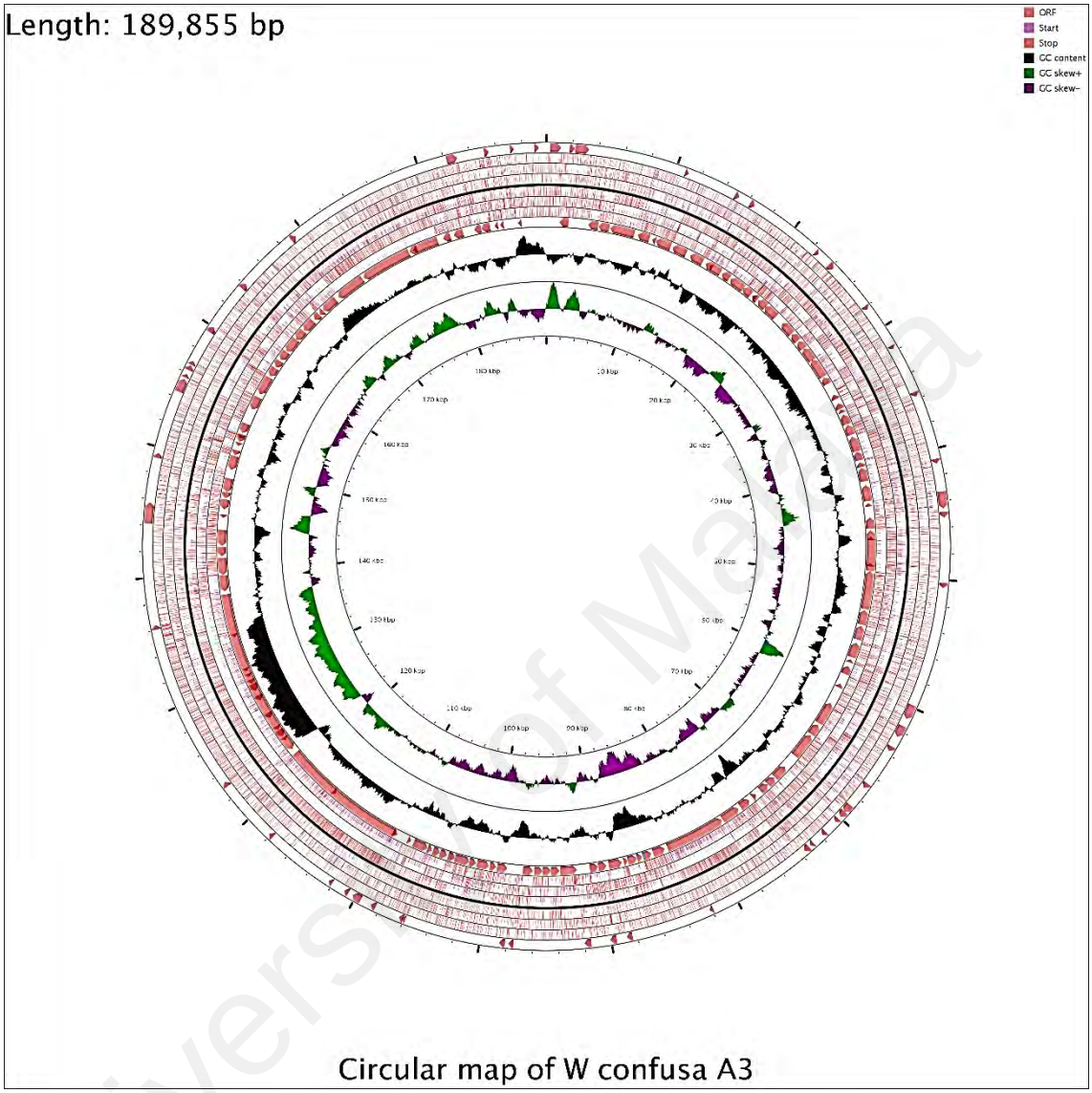
Appendix L

GO ontology of *W. confusa* A3



Appendix M

Circular map of the genome of *W. confusa* A3



Appendix N

Circular map of the genome of *E. faecium* C1