CHARACTERISATION OF QUORUM SENSING AND ANTIBIOTIC RESISTANCE GENES FROM THE GENOMES OF SELECTED CULTIVABLE ORAL BACTERIAL ISOLATES

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CHARACTERISATION OF QUORUM SENSING AND ANTIBIOTIC RESISTANCE GENES FROM THE GENOMES OF SELECTED CULTIVABLE ORAL BACTERIAL ISOLATES

ABSTRACT

Oral cavity is the primary gateway to the human body. It is the desired habitat for microorganism proliferation due to its optimum condition. Oral microorganisms are capable of passing through the circulatory system and spread to different human anatomy. Root caries refers as a significant oral health problem. Dentinal caries occurs at dentine layer which is caused by a supra-gingival microbial biofilm. Whole genome sequencing coupling with systematic bioinformatics analysis offers unprecedented insights into important genomic features and characteristics of genes including those putative quorum sensing genes and profiling of antimicrobial resistance. Quorum sensing is a type of cell-cell communication to control the expression of genes linked to cell density. It could serve as a basis for non-antibiotic based drug discovery to attenuate pathogenic bacterial population by controlling bacterial quorum sensing. Understanding the profile of antibiotic resistance genes is essential to predict antibiotic resistance in oral bacteria and this provides invaluable information for clinical use especially prescription of drugs. The objectives of this study were to analyse the genome sequences of culturable oral bacteria from dental caries, identify the presence of quorum sensing genes as well as to characterise their antimicrobial resistance genes. This work utilised high-resolution technologies such as triple quadrupole liquid chromatography-tandem mass spectrometry for the detection of quorum sensing molecules, MALDI-TOF mass spectrometry for bacterial identification and next generation sequencing on MiSeq platform coupling with numerous bioinformatics resources to understand the genome biology of these isolates. Based on the diverse morphologies of oral microbial, a total of 21 cultivable strains were isolated and identified, namely *Burkholderia cepacia, Citrobacter amalonaticus,*

Elizabethkingia sp., *Enterobacter* spp., *Klebsiella* spp., *Pseudomonas aeruginosa*, *Pluralibacter gergoviae*, *Lacobacillus paracasei*, *Strenotrophomonas maltophilia*, *Citrobacter koseri* and *Proteus mirabilis*. Among these strains, some are uncommon to oral bacteria community that exhibited quorum sensing namely *Citrobacter amalonaticus* and *Enterobacter* spp. To the best of my knowledge, this is the first work of its kind that reports the quorum sensing activity by *Citrobacter amalonaticus*.

Keywords: dentine caries, dental plaque, next generation sequencing, quorum sensing, drug resistance

PENCIRIAN GEN-GEN PENDERIAN KUORUM DAN RINTANGAN ANTIBIOTIK DALAM GENOM BAKTERIA MULUT TERPILIH YANG BOLEH DIKULTUR DALAM MAKMAL

ABSTRAK

Rongga mulut merupakan gerbang utama bagi mikroorganisma untuk masuk ke dalam tubuh badan manusia di mana ia adalah habitat paling sesuai dan optimum bagi proliferasi mikroorganisma. Apabila mikroorganisma ini berada di dalam mulut, ia dapat memasuki ke sistem peredaran dan seterusnya tersebar ke pelbagai bahagian anatomi badan. Selain daripada itu, di dalam masyarakat yang mementingkan kesihatan mulut, masalah karies gigi merupakan masalah utama yang perlu diberi perhatian. Masalah ini menjadi semakin kronik apabila ia berlaku di lapisan dentin atau akar gigi yang berpunca daripada gingival supra biofilm mikrob. Gabungan teknik jujukan seluruh genom dan analisis bioinformatik sistematik menawarkan pemahaman terperinci tentang ciri-ciri genom yang penting termasuklah gen penderiaan kuorum yang boleh diramalkan dan pemprofilan kerintangan antimikrob. Penderiaan kuorum adalah sejenis komunikasi antara sel-sel untuk mengawal ekspresi gen bakteria yang bergantung kepada kepadatan sel. Dengan kebolehan memanipulasi penderiaan kuorum bakteria, ia juga boleh berfungsi sebagai penemuan ubat-ubatan yang bukan dasar antibiotic bagi bakteria patogenik. Selain itu, rintangan antibiotik yang terkandung di dalam bakteria mulut juga dapat diramalkan jika pencirian profil gen kerintangan antibiotik telah dikaji. Oleh itu, maklumat yang bernilai dapat diperolehi untuk kegunaan klinikal terutamanya untuk preskripsi ubat-ubatan, dan kajian ini harus diteruskan supaya memberi manfaat kepada masyarakat. Objektif kajian ini adalah untuk menganalisis genom bakteria mulut yang boleh dikultur dalam makmal dari sampel karies dentin dan plak gigi, mengenal pasti kehadiran gen penderiaan kuorum, dan juga untuk mencirikan profil gen kerintangan anti-mikrob. Kerja ini menggunakan teknologi yang canggih seperti QQQ LCMS/MS (triple quadrupole liquid

chromatography-tandem mass spectrometry) untuk mengesan molekul-molekul penderiaan kuorum, MALDI-TOF MS (Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) untuk mengenalpasti jenis bakteria dan penjujukan generasi seterusnya menggunakan MiSeq platform bergabung dengan pelbagai jenis sumber bioinformatik untuk memahami latar-belakang biologi genom bacteria mulut. Berdasarkan kepelbagaian morfologi mikrob mulut yang dikultur, sebanyak 21 jenis bakteria yang berbeza telah dikenalpastikan iaitu, *Burkholderia cepacia*, *Citrobacter amalonaticus*, *Elizabethkingia* sp., *Enterobacter* spp., *Klebsiella* spp., *Pseudomonas aeruginosa*, *Pluralibacter gergoviae*, *Lacobacillus paracasei*, *Strenotrophomonas maltophilia*, *Citrobacter koseri* dan *Proteus mirabilis*. Antara bakteria-bakteria tersebut, terdapat beberapa jenis bakteria yang jarang ditemui di dalam populasi bakteria mulut yang mempamerkan sifat penderiaan kuorum iaitu *Citrobacter amalonaticus* dan *Enterobacter* spp. Dalam pengetahuan saya, ini adalah kajian pertama yang melaporkan aktiviti penderiaan kuorum pada *Citrobacter amalonaticus*.

Kata Kunci: karies dentin, plak gigi, penjujukan generasi seterusnya, penderiaan kuorum, rintangan dadah

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LIST OF SYMBOLS AND ABBREVIATIONS

β	:	Beta
˚C	:	Degree Celsius
≥	:	Greater than or equal to
–	:	Negative control
%	:	Percentage
+	:	Positive control
3-oxo-C10-HSL	:	*N*-(3-oxodecanoyl)-L-homoserine lactone
3-oxo-C12-HSL	:	*N*-(3-oxododecanoyl)-L-homoserine lactone
3-oxo-C8-HSL	:	*N*-(3-oxooctanoyl)-L-homoserine lactone
ACN	:	Acetonitrile
AES	:	Advanced Expert System
AGE	:	Agarose gel electrophoresis
AHL	:	*N*-acylhomoserine lactone
AIP	:	Autoinducing peptide
ANI	:	Average nucleotide identity
ARG-ANNOT	:	Antibiotic Resistance Gene-ANNOTation
BLAST	:	Basic local alignment search tool
BRIG	:	BLAST Ring Image Generator
C10-HSL	:	*N*-decanoyl-L-homoserine lactone
C12-HSL	:	*N*-dodecanoyl-L-homoserine lactone
C16-HSL	:	*N*-hexadecanoyl-L-homoserine lactone
C4-HSL	:	*N*-butyryl-L-homoserine lactone
C6-HSL	:	*N*-hexanoyl-L-homoserine lactone
C8-HSL	:	*N*-octanoyl-L-homoserine lactone

CDC	:	Centers for Disease Control and Prevention
CF	:	cystic fibrosis
ESI-MS	:	Electron spray ionization mass spectrometry
GO	:	Gene Ontology
GTR	:	General Time Reversible
h	:	Hour
HMP	:	Human Microbiome Project
HOMD	:	Human Oral Microbiome Database
I	:	Intermediate
ICDAS	:	International Caries Detection and Assessment System
LB	:	Lutria-Bertani
LCMS/MS	:	Liquid chromatography mass spectrometry
m/z	:	mass/charge
MALDI-TOF	:	Matrix-associated laser desorption/ionization time-of-flight
MEGA	:	Molecular Evolutionary Genetic Analysis
MIC	:	Minimum inhibitory concentration
Min	:	Minutes
ML	:	Maximum likelihood
mL	:	milliliter
MLSA	:	Multilocus sequence analysis
MOPS	:	3-(*N*-morpholino) propane sulfonic acid
MRSA	:	Methicillin-resistant *Staphylococcus aureus*
MS	:	Mass spectrometry
MSC	:	MiSeq Control Software
NCBI	:	National Center for Biotechnology Information
ND	:	Not determined

NGS	:	Next-generation sequencing
NIH	:	National Institutes of Health
PBS	:	Physiological buffered saline
PCR	:	Polymerase chain reaction
QS	:	Quorum sensing
R	:	Resistant
RAST	:	Rapid Annotation Subsystems Technology
RND	:	Resistance-Nodulation-Division
S	:	Susceptible
SAV	:	Sequencing Analysis Viewer
SRST2	:	Short Read Sequence Typing
TBE	:	Tris borate EDTA
TFA	:	Trifluoroacetic acid
TSA	:	Trypticase soy agar
UniProtKB	:	UniProt Knowledgebase
µL	:	microliter
µm	:	micrometer

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CHAPTER 1: INTRODUCTION

1.1 Research Background

In the past, dental scientists and oral microbiologists, as well as microbial ecologists, have researched on the identification of oral microbes which have led to oral diseases. Dental plaque has been reported as an important microhabitat for oral micro-organisms (Nobbs et al., 2011). Dental plaque forms naturally on the teeth and aids in the colonisation of exogenous species, which disturbs the microbial homoeostasis within the oral cavity and become predisposed sites to the disease (Marsh, 1994). This diseased dental plaque influences the changes in its environmental conditions and plays a vital role in the development of site-specific diseases such as dental caries, gingivitis and periodontitis (Liljemark & Bloomquist, 1996). In addition, a dental plaque acts as a reservoir of Gram-negative bacteria and the periodontium as a reservoir of inflammatory mediators which indirectly play a pivotal role in systemic diseases like bacteremia, cardio-vascular diseases, low birth weight, diabetes mellitus and bacterial pneumonia (Li et al., 2000). Hence, it is of interest to look further at oral inhabitants. It is necessary to characterize as many taxa as possible in order to gain a better understanding of their structure and function in the human oral cavity.

Quorum sensing (QS) is a form of cell-cell communication and it refers to the process where it integrates the stimuli response via small diffusible signaling molecules to regulate bacterial genes expression at cell-density-dependent manner (Swift et al., 1996). This regulatory phenomenon could occur in the human oral cavity bacteria as distinct phenotypes presence including the biofilm formation and virulence factor production. In order to detect the signalling molecules of oral bacteria rapidly and accurately, a variety of bacterial biosensors were utilized to shed a light on this research.

The abundant outbreaks of bacterial infections have led to life-threatening infections such as *Burkholderia cepacia* bloodstream infections (Abe et al., 2007), *Elizabethkingia* infections (Jean et al., 2014) and various species of Enterobacteriaceae infections (Nada et al., 2004; Yan et al., 2002). Of medical importance, antimicrobial agents were used to inhibit certain microorganisms or inhibit their growth. The most widely used types of antibiotics are penicillins, tetracyclines and cephalosporins. In this research, some of the oral isolates were selected for further study through the *in-silico* annotation of the genome, particularly focusing on functional categories that provide insights regarding the cell-cell communication between the bacteria and the drug resistant genes.

1.2 Objectives

The primary objective of this research is to study the human oral bacteria from clinical dental samples using culture-dependent approach coupled with next generation sequencing. The precise objectives of this research are as following:

1. To isolate and identify bacteria from clinical samples using different growth media;
2. To conduct genomic profile of human microbes associated with dentine caries and dental plaque using next generation sequencing and systematic bioinformatics algorithms;
3. To analyse and determine the presence of QS and antibiotics resistant genes in selected oral bacteria;
4. To perform comparative genomics study of bacteria isolated from dentinal caries and dental plaque.

CHAPTER 2: LITERATURE REVIEW

2.1 Human Microbial: An Overview

Human microbiome refers to the enormous community of microorganisms occupying the habitats of the human body. Joshua Lederberg coined the term ‘‘microbiome” and also suggested the concept of the human microbiome, to signify the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space (Lederberg & McCray, 2001). Initial efforts to determine the numbers of microorganisms in a community and their phylogenetic relationships were through the analysing relatively well-conserved 16S ribosomal RNA genes in microbiome (Dymock et al., 1996; Giovannoni et al., 1990; Stahl et al., 1984; Woese & Fox, 1977). Most of our understanding of the human microbiome derived from culture-based approaches using the 16S rRNA phylogenetics analysis. It was estimated that as much as twenty to sixty percent of the human-associated microbiome were uncultivable which also depend on the body sites studied (Aas et al., 2005; Pei et al., 2004; Zhou et al., 2004).

There are approximately ten trillion of human cells constituting our human body which is ten times lesser than microbial cells (hundred trillion) (Proctor, 2014). Scientists believe that a human body is free of any microbes prior to birth. The human microbiome is only established in a newborn from the mother during natural childbirth and developing their dynamic ecosystem.

National Institutes of Health (NIH, USA) initiated the Human Microbiome Project (HMP) in 2008, with the goal of identifying and characterising the microorganisms which were found in association with both healthy and diseased humans (Turnbaugh et al., 2007). This well-established project characterised the microbial community by using culture-independent methods such as metagenomics and extensive whole genome

sequencing. The microbiology of body sites was collected from oral, skin, vaginal, gastrointestinal tract, and nasal cavity. The Demonstration Projects aim to tackle the most important question of the HMP: whether changes in the microbiome can be related to human health and disease (Peterson et al., 2009). The findings have revealed that even healthy individuals differ remarkably in the microbes which might be attributed to variations in diet, environment, host genetics and early microbial exposure (Consortium HMP, 2012). Based on the previous report, wide-range characteristics of microbial communities were being identified in each of the different environment of the human anatomy (Wilson, 2009). Thus, understanding the relationship of the microbiota to human health and disease is one of the primary goals in human microbiome studies.

2.2 Oral Microbiome

In general, human oral cavity housed most of the diverse microorganisms including bacteria, fungi, archaea, protozoa and viruses among the various human anatomies. Human oral microbiome is the most studied human microflora as it is easy to sample and closely related to oral infectious diseases especially dental caries and periodontitis (Chen et al., 2010). Different oral soft tissue surfaces are colonised by distinct microbial communities (Mager et al., 2003). Previous research reported that there was a minimum number of 800 bacterial species (Filoche et al., 2010; Paster et al., 2001). It is expected to be increased into thousands with the advancement of biotyping techniques (Keijser et al., 2008) such as matrix-associated laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) (Croxatto et al., 2012) and next-generation sequencing (NGS) including Illumina sequencing platform (Chan et al., 2013), PacBio, Ion Torrent and Oxford Nanopore sequencing.

The Human Oral Microbiome Database (HOMD) is the first curated library that deciphers human-associated microbiome and provides tools for use in understanding the role of the microbiome in health and disease (Dewhirst et al., 2010). The first aim of the research was to collect 16S rRNA gene sequences into a curated phylogeny-based database and make it online accessible. The second aim was to analyse 36,043 16S rRNA gene clones isolated from studies of the oral microbiota to determine the relative abundance of taxa and identify of the novel candidate taxa. Genome sequences of oral bacteria form part of the HMP and other sequencing projects were being added to the HOMD. The HOMD links sequence data with phenotypic, phylogenetic, clinical, and bibliographic information (Chen et al., 2010). Hence, HOMD could be an ideal database for this study, especially for genomics comparative study.

2.2.1 Common Oral Bacteria

Species common to human oral cavity belonged to the genera *Streptococcus*, *Actinomyces*, *Lactobacillus*, *Veillonella*, *Bacteroides*, *Bifidobacterium*, *Fusobacterium*, *Selenomonas* and *Treponema* (Aas et al., 2005; Rathke et al., 2010; Sutter, 1984). Some of these bacteria have been implicated in the most common oral diseases such as dental caries and periodontitis, which are caused by a mixture of microorganisms and food substances. (Aas et al., 2005).

In the oral microflora of adults, *Streptococcus salivarius* is among the prominent members of the oral microbiota and it has excellent potential for use as a probiotic targeting the oral cavity (Burton et al., 2006). However, different results on the prevalence of *S. salivarius* in the oral cavity of the newborn infants have been obtained (Carlsson et al., 1970a). The existence of *S. salivarius* also depends on the diet of infants, for instance, the absence of this bacteria in the saliva of breastfed infants and become dominant

bacteria of saliva when supplemented with sucrose (Carlsson et al., 1970a). With the eruption of the teeth during the infants' first year, *Streptococcus sanguinis* colonise their dental surface (Carlsson et al., 1970b). Whereas, *Veillonella* are the most numerous anaerobes found in saliva.

2.2.2 Dentine Caries and Dental Plaque

In this research, the oral microbial population diversity was studied on the clinical dental samples collected from the dentinal carious lesion and dental plaque. Dental caries refers to a process of demineralization of the unhealthy tooth surface by bacteria whereas the dental plaque is a layer of biofilm that formed by colonising bacteria and the substances they secrete on the teeth. Development of dentinal caries is due to the gradually localised chemical dissolution at tooth surface resulting from metabolic events of the carious process taking place in dental plaque covering the affected area (Fejerskov & Kidd, 2008). Besides, there are several factors that can affect the dynamic changes in phenotypic and genotypic properties of dental plaque developments. Those factors included the diverse physiological traits in different oral health conditions such as the pH level, amount of oxygen and nutrition available in the oral cavity.

In addition, dentinal caries also can be described as an endogenous disease, which is originated by a shift from mutualistic symbiosis to parasitic symbiosis bacteria within the ecosystem of the oral environment (Takahashi & Nyvad, 2011). These bacteria exhibit a variety of physiological characteristics, produce acid in the presence of carbohydrates and subsequently change the environmental pH resulting in the demineralization of tooth surface (Fejerskov & Kidd, 2008). They also form the bulk of dental plaque that is in close physical contact with the tooth surface, thus increases the probability of these bacteria to modulate the pathogenic traits (Marsh, 2006).

The pathogenicity of these species within the dental plaque towards causing dental caries is explained by three major hypotheses (Marsh, 1994; Theilade, 1986). The specific plaque hypothesis is associated with the onset and progression of dental caries caused by an overgrowth of a specific bacterial species (Loesche, 1992). *Streptococcus mutans* is the most commonly reported specific bacteria involved in the cariogenic process (Loesche, 1986). Whereas the non-specific plaque hypothesis attributes the caries process to the overall activity of the complex indigenous microorganisms (Theilade, 1986) causing gingivitis such as *Lactobacillus* and *Bifidobacterium* (Van Ruyven et al., 2000). The recent ecological plaque hypothesis postulates an environmental dependent shift of resident flora to microorganisms, where acid producing and acid-tolerating bacteria can contribute to caries process (Marsh, 1994). Therefore, dental caries is probably better understood as a polymicrobial disease and specific microbiota that play a vital role in the oral cavity and yet to be elucidated (Kleinberg, 2002; Ling et al., 2010).

2.3 Microbial Activity: Quorum Sensing

Globally infectious diseases are the leading causes of mortality and morbidity and account for more than 13 million deaths annually (World Health Organization, 2010). Over the past century, the public health community has enjoyed periodic major successes in the control and elimination the infectious diseases using vaccination and antibiotics. However, the emergence of multi-drug resistance bacteria and the further increasing routine coverage of immunisation cause the failure of drug discovery programmes over the last ten years to discover new broad spectrum antibiotics. It then becomes a major threat to public health. Thus, novel anti-infective therapy is in compelling need. Indeed, such a magic bullet does occur: Quorum sensing (QS) which can be a targeted to attenuate pathogenic bacteria and served as the basis non-antibiotic drug discovery.

QS has long been appreciated microbial activity that certain group of bacteria express cooperative social behaviour. QS has been well studied where it regulates gene expression by small hormone-like signalling molecules termed autoinducers (AIs). For instance, QS controls the bioluminescence, virulence factor expression, biofilm formation, motility, symbiosis, sporulation, mating and other processes (Bassler, 2002; Surette & Bassler, 1998). With QS system, bacteria can respond to external stimuli by altering their behaviour in response to the cell density (Hooshangi & Bentley, 2008; Vu et al., 2009). AIs are produced, released and detected by bacteria and accumulated at surrounding atmosphere during the growth. AIs accumulate to the threshold concentration which sufficient for activation of luminescence gene at the ambience of high cell density only, AIs remain low concentration at low cell density (Figure 2.1) (Eberhard, 1972; Fuqua et al., 1994).

In a natural habitat, bacteria survive in highly ordered communities such as oral cavity, mosquitoes' gut and soil. These communities are well-managed and tolerated by each species if they carried out their specific role and functions. Successful associations of this conglomerate relationship require effective intra- and interspecies cell-cell communication as well as the interaction between bacteria and the host (Bassler, 2002; Sifri, 2008). Hence, QS is best depicted as a paradigm of communication. Eberl and Tummler (2004) have reported the interspecies QS happened between *Pseudomonas aeruginosa* and *Burkholderia cepacia* via small diffusible AHL molecules in patients with cystic fibrosis.

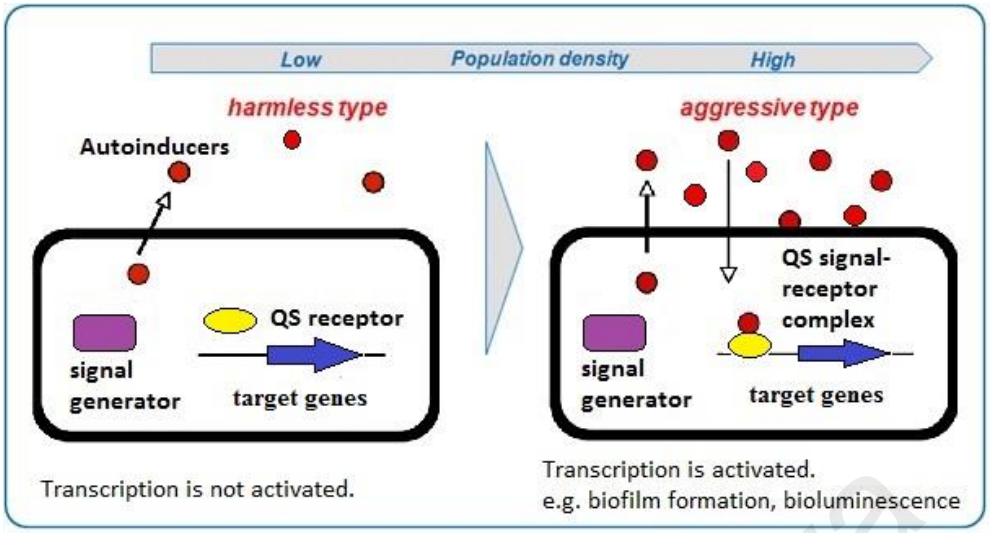

Figure 2.1: QS system. Transcription is activated at high cell density.

2.3.1 Quorum Sensing Signaling Molecules: AHLs

There are several different classes of QS signalling molecules. The archetypal QS communication in bacteria includes Gram-negative bacteria *N*-acylhomoserine lactone (AHL)-based signalling system, the oligopeptide (AIP) signalling system used by Gram-positive bacteria and autoinducer AI-2 furanone-based system that is common to a number of Gram-positive and Gram-negative bacteria (Bassler, 2002; Chong et al., 2012). The AI-1 system is a type intraspecies communication via exogenous small diffusible chemical molecules of acylated homoserine lactones. While the AI-2 system is a furanosyl borate diester which was proposed to serve as 'universal signal' for the interspecies QS circuit (Cao & Meighen, 1989; Jacobi et al., 2012; Xavier & Bassler, 2003).

The structure of AHL is composed of a homoserine lactone ring with an acyl-chain length varies from C4 to C18, presence or absence of carbon-carbon double bond in the fatty acid chain and substitution of C3 (hydrogen, oxo- and a hydroxyl group). In general, AHL-mediated QS system consists of three fundamental components namely LuxI-type autoinducer synthase (signal generator), AHL ligand (the signal itself) and

LuxR-type receptor (cognate receptor) (Galloway et al., 2010; Geske et al., 2008; Rasmussen & Givskov, 2006). LuxI autoinducer of AHL synthase and LuxR autoinducer receptor are typically clustered adjacently in most of the models. There are some articles that reported the functional LuxI/LuxR pairs are located on different bacterial chromosome or plasmids or even function in the absence of LuxI family of autoinducer synthase in bacterial QS paragon (Brameyer et al., 2015; Patankar & Gonzalez, 2009; Subramoni & Venturi, 2009).

With the availability of numbers of AHL bacterial biosensors, it has greatly facilitated the screening of AHL production by using the *lux*, *gfp* or *lacZ* AHL biosensors reporter gene fusions or pigment induction (Lei et al., 2006; Williams, 2007). For example, by using cross-streaked bioassay with *C. violaceum* CV026 and *E. coli* [pSB401] AHL biosensors, purple violacein pigmentation and intensity of bioluminescence were induced if suitable extracellular AHLs were detected. Furthermore, the spent supernatants extracted AHLs' profile from bacterial strain also can be analysed and quantified by high-resolution tandem liquid chromatography-mass spectrometry (MS) system (Lau et al., 2013; Ngeow et al., 2013).

Hitherto, QS is an attractive target for antimicrobial therapy in the treatment of infectious disease (Cegelski et al., 2008) because it does not involve the use of antibiotics.

2.3.2 Bacterial Quorum Sensing Network Architectures in Oral Cavity

Diverse species of bacteria play a vital role in the composition of oral biofilm formation. Several types of research were carried out regarding the production of exogenous autoinducer-like activities that activated the transcription of the luminescence genes in *Vibrio harveyi*. In the earlier QS work, most of the oral bacteria produce extracellular AI-2 type signaling molecules such as *S. mutans* in biofilm formation, *Porphyromonas gingivalis, Fusobacterium nucleatum, Actinobacillus actinomycetemcomitans* and *Prevotella intermedia* in the aetiology of human periodontal disease (Burgess et al., 2002; Fong et al., 2001; Merritt et al., 2003). The first documentation of homologues AHL-producing oral bacteria was reported by Yin and colleagues in 2012, which include *Klebsiella pneumonia*, *Enterobacter* sp. and *Pseudomonas putida* from the posterior dorsal surface of the human tongue (Chen et al., 2013; Yin et al., 2012; Yin et al, 2012).

In general, the biosynthesis of the AI-2 chemical system requires the LuxS protein. For instance, mutation of the LuxS protein of *Streptococcus pneumonia* affects the virulence in infections of the mouse model and mutation in *S. mutans luxS* gene can affect the biofilm formation (Merritt et al., 2003; Stroeher et al., 2003). The AI-2 pathway has been suggested to be useful for chemotherapeutic regulation of bacterial virulence. However, the AHL-based oral bacterial communication is still a mystery and more intense studies should be carried out to provide useful information on QS in the oral bacteria.

2.4 Antibiotics and Toxic Compound Resistance Features

Computational analysis of prokaryotes and eukaryotes genomes is getting popular in the research field, general health and clinical labs. A wide range of research from genetic basis of bacterial, infections outbreak analyses, microbial immunology, pathogenicity to antibiotic resistance has altered the researchers' interests. One of the remarkable scientific accomplishments in the twentieth century was the discovery and use of antimicrobial agents.

Drug resistance and virulence factor of pathogens become an emerging global major climacteric. Hence, unveil the design and developments of new therapeutic strategies are being of great significance by an understanding of the bacterial unique antibiotic resistance mechanisms. Two resistance mechanisms, namely active efflux pump of antibiotic molecules and permeability barriers are suggested to be focused for new drugs discovery as they have been implicated in numbers of outbreaks of antibiotic-resistant microbial (Kumar & Schweizer, 2005).

Community and nosocomial acquired infections by new opportunistic pathogens that are multidrug resistance have become a major topic and risk for human health (Levy & Marshall, 2004; Quinn, 1998; Weinstein et al., 2005). It can raise the jeopardy attributed to intubation, immunosuppression, catheterization and other operation that rely on drugs to cure the infections (World Health Organization, 1999). Hence, determination of acquired antimicrobial resistance genes in the microbial genome is vital through antibiotic susceptibility testing or computational specialised gene prediction.

Moreover, the widespread use of antibiotics for treatment in human medicine and agriculture has likely caused the substantial responsive changes in the community (Sommer et al., 2009) such as immune system and mutation of microorganisms in human. Over the past decades, multidrug resistance gene in human pathogens has increased and

become a critical issue as it poses a challenge for the treatment of bacterial infections (Alekshun & Levy, 2007). For instance, methicillin-resistant *Staphylococcus aureus* (MRSA) led to 18,964 mortalities in the US in 2006 (Sommer et al., 2009). Whole genome sequencing of these bacteria has been annotated and showed that numerous multiple antibiotic resistance genes governed by these strains have not evolved within the genome but were acquired by lateral gene transfer events (Ochman et al., 2000). This was getting complicated as antibiotic resistance proteins were encoded on mobile element and move along diverse bacteria to disseminate resistance genes into a wide variety of interacted microbial communities (Marchall et al., 2009). As a result, a number of interested mobile antibiotic resistance genes that might access to clinical pathogens will be increased (Riesenfeld et al., 2004).

Whole genome sequencing of the multidrug resistance genome coupled with the appropriate computational algorithms and databases are crucial to discovering the genes and to understand the genetics.

2.4.1 Antibiotic Mode-of-action

Antibiotic mechanism classification is based upon drug-target interaction site and whether the consequential inhibition of cellular function is lethal to bacteria. A total of 6 classes of antibiotic can be categorised (Table 2.1) (Schwalbe et al., 2007) and predominantly fall into three classes: inhibition of DNA replication and repair, inhibition of protein synthesis, and inhibition of cell-wall turnover (Walsh, 2000).

Table 2.1: Antibiotic Classification by Mechanism

Inhibitors/ Mechanism	**Antibiotic/ Class**
1. Cell Wall Synthesis Inhibitors	Penicillins Cephalosporins Vancomycin Beta-lactamase Inhibitors Carbapenems Aztreonam Polymycin Bacitracin
2. Protein Synthesis Inhibitors	<u>Inhibit 30S Subunit</u> Aminoglycosides (gentamicin) Tetracyclines <u>Inhibit 50S Subunit</u> Macrolides Chloramphenicol Clindamycin Linezolid Streptogramins
3. DNA Synthesis Inhibitors	Fluoroquinolones Metronidazole
4. RNA Synthesis Inhibitors	Rifampin
5. Mycolic Acid Synthesis Inhibitors	Isoniazid
6. Folic Acid Synthesis Inhibitors	Sulfonamides Trimethoprim

2.4.1.1 DNA Synthesis Inhibitors

Quinolones target DNA replication and repair by binding DNA gyrase complexed with DNA, which drives double-strand DNA break formation and rapid cell death (Drlica & Zhao, 1997). DNA topoisomerases are classified as type I or type II according to whether transient single-strand breaks (type I) or transient double-strand breaks (type II) are made in the DNA substrate to pass the DNA double helical strands through each other and reduce the linking number (Walsh, 2000). Topoisomerases are present in both prokaryotic and eukaryotic cells. Bacterial DNA gyrases are type II topoisomerases which also known as topoisomerase IV. Quinolones or fluoroquinolones are active against both Gram-negative and Gram-positive bacteria.

2.4.1.2 Protein Synthesis Inhibitors

Bacteriostatic drugs of protein synthesis inhibitor mainly inhibit ribosome function that targeting both the 30S ribosome subunit (tetracycline family and aminocyclitol family) and 50S ribosome subunit (macrolide family and chloramphenicol) (Chopra & Roberts, 2001; Poehlsgaard & Douthwaite, 2005; Tenson et al., 2003). Tetracyclines (Rogalski et al., 2012) are the important antibiotic of the aromatic polyketide biosynthetic pathways while the aminoglycosides (Fourmy et al., 1996) of which streptomycin was the founding member of supplanted now by later synthetic variants such as kanamycin.

2.4.1.3 Cell-wall Synthesis Inhibitors

Cell-wall synthesis inhibitors (such as Beta-lactams) interfere with normal cell-wall synthesis, induce lysis and lead to cell death via interaction with penicillin-binding proteins (Tomasz, 1979) and glycopeptides that structure the building blocks for peptidoglycan. (Reynolds, 1989).

The class of β-lactam antimicrobial agents exhibit the most common antibiotic treatment for bacterial infections. However, it also created the primary cause of resistance to β-lactam antibiotics among Gram-negative bacteria worldwide. The persistent exposure of bacterial strains to a multitude of β-lactams has induced dynamic and continuous production and mutation of β-lactamases in these bacteria, as well as expanding their activity against the newly developed β-lactam antibiotics. These enzymes are known as extended-spectrum β-lactamases (ESBLs) (Pitout & Laupland, 2008; Paterson & Bonomo, 2005). Treatment of these multidrug resistant bacteria becomes the deep concern for scientists.

Of the many different β-lactams, carbapenems possess the broadest spectrum of activity and greatest potency against Gram-positive and Gram-negative bacteria. As a result, they are often used as “last-line agents” or “last resort of antibiotics” when the bacterial infected patient becomes gravely ill or is suspected of harbouring resistant bacteria (Bradley et al., 1999; Torres et al., 2007). Unfortunately, the emergence of multidrug-resistant (MDR) pathogens seriously threatens this class of lifesaving drugs (Queenan & Bush, 2007). Several recent studies clearly show that resistance to carbapenems is increasing throughout the world (Chouchani et al, 2011; Gaibani et al, 2011; Patel & Bonomo, 2011; Pathmanathan et al, 2009; Rossi, 2011).

2.4.2 Application of Antimicrobial Susceptibility Testing: Vitek 2 System

The objectives of antimicrobial sensitivity testing are to determine the possible antibiotic resistance and then assure the selection of susceptibility drugs to a pathogen of a particular infection. Antibiotics susceptibility testing can be performed via manually or automated instrument methods. The most common approaches used by laboratory technicians are manually broth microdilution and automated instrument methods. These testing included broth dilution tests, disk diffusion test, gradient diffusion test and automated system such as Vitek 2 system. Some of them provide quantitative minimum inhibitory concentration (MIC) result while some with qualitative results using the categories susceptible (S), intermediate (I), or resistant (R).

The bacterial identification antimicrobial susceptibility Vitek 2 automated system gives rapid, reliable, and highly reproducible results by using a new fluorescence-based principle (Ling et al., 2001). It provides automated inoculation, reading and interpretation through a very compact reagents test card which contains test media in a 64-well format and little volume of antibiotics. The susceptibility test cards allow testing for most of the Gram-positive and Gram-negative anaerobe, and also included slowly growing *S. pneumoniae* in a period of 4–10 h which does not available for Vitek 1 system (Reller et al., 2009). The components of Vitek 2 system as illustrated in Figure 2.2.

Figure 2.2: The components of automated Vitek 2 system.

2.5 Generation of Sequencing Technologies

In 1977, the technology of dideoxynucleotide sequencing of DNA was first described by Sanger and colleagues (Sanger et al., 1977). This technique has gone through a rapidly changing from a small-scale industry into an enormous production enterprise that requires a devoted and specialised robotics infrastructure, bioinformatics, computation databases and high-end instrument (Mardis, 2008).

The recent introduction of next generation instruments is able to produce millions of nucleotides sequence reads in one single run and steady changing the genetics landscape and provide a better understanding of genome with heretofore unimaginable speed.

Illumina MiSeq, HiSeq platform, PacBio RS SMRT and other sequencer technologies able to provide cost-effective genome-wide or whole genome sequence readout as an endpoint for chromatin immunoprecipitation, mutation mapping, polymorphism discovery, non-coding RNA discovery and other suitable applications (Fox et al., 2009). The principle of whole genome sequencing is depicted as Figure 2.3.

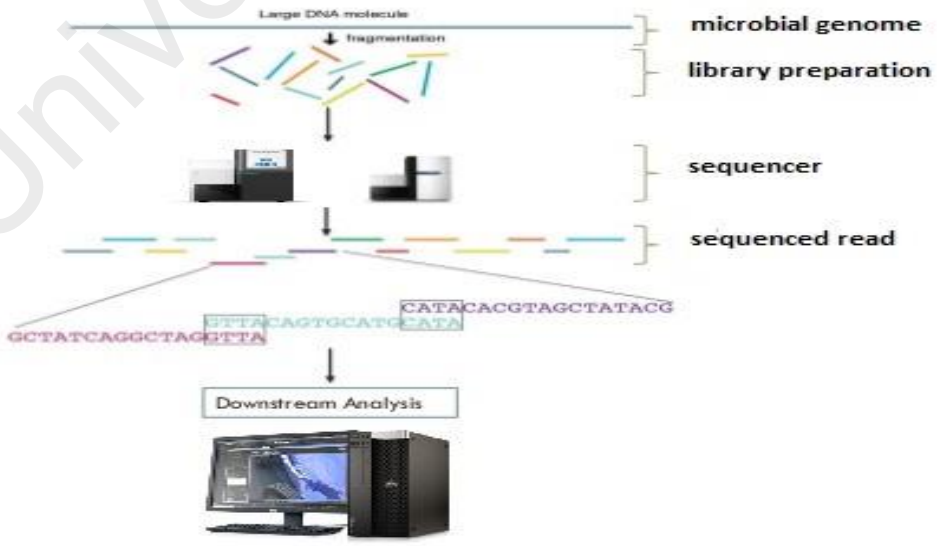

Figure 2.3: Principle of whole genome sequencing. The large microbial genome was fragmented for library template preparation prior to NGS.

2.5.1 DNA Sequencer

Whole genome sequencing or complete genome sequencing is an automated experimental process of defining the entire genome of an organism by DNA sequencer. Hitherto, sequencing has been progressively developed to more recent third-generation sequencing. The first automated DNA sequencer or first generation sequencing was introduced by Applied Biosystems which is an electrophoresis system. Second generation sequencing or NGS has increased the sequencing rate and produce more accurate high throughput results. Third generation sequencing provides real-time sequencing and the precise order of nucleotide in longer DNA fragments. The comparison of various sequencing platform was tabulated in Table 2.2 (Rhoads & Au, 2015).

Table 2.2: Comparison on different DNA sequencer

		Sequencer	**Manufacturer**	**Read length (bp)**	**Cost / million bases (USD)**	**Duration (h)**
Generation	**First**	Sanger ABI PRISM	Applied Biosystems	600- 1000	500	0.5 - 3
	Second	454 GS FLX	Life Sciences, Roche	700	8.57	24
		HiSeq/ MiSeq	Illumina	50-300	0.03 – 0.04	4 – 144
		Ion Torrent PGM	Life Technologies	200-400	0.1	2 - 4
	Third	PacBio RS II	Pacific Biosciences	exceeding 10,000	0.4 – 0.8	0.5 – 4
		GridION and MinION	Oxford Nanopore Technologies (ONT)	2000 - 5000	6.44 – 17.9	50

2.5.2 Comparison Genomics Microbial

In early of the 1990s, the genomics revolution targeted the study of whole genomes of microorganisms, animals and plants (Huson et al., 2007). A large number of the complete genome of clinical pathogens were sequenced and whole genome comparison studied along with reference genomes in order to identify polymorphic sequences with potential relevance to immunity, disease pathogenesis and virulence factors (Aparicio et al., 2002). The presence of a significant divergent region in the sequence represents the available of polymorphic genes in particular genome. These genes products would be essential targets against the virulence determinants (Fleischmann et al., 2002). Hence, comparisons between the complete genomes of different microorganisms will guide future approaches to reveal their genes functionality and regulation.

2.6 Identification of Clinical Species by MALDI-TOF

Besides the traditional 16S ribosomal DNA-based PCR and whole genome sequencing approaches for strain identification, Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) was an alternative tool used for microbiologists. This technology was employed for the evaluation of strain differentiation among a vast number of microbial via the mass spectral analysis or proteomic profiling, biochemical and genome-based identification schemes. The analysis was fast with minimum consumable expenses, reproducible and reliable result, simple protocol and mass spectral patterns of targeted species are independent of the age of culture, growth conditions, or medium selection (Hsieh et al., 2008; Saenz et al., 1999; Stevenson et al., 2010).

Figure 2.4 illustrated the schematic structure of MALDI-TOF MS. In details, the complete drying of a co-crystallize mixture of sample and matrix was laser beam shot in sample ionization chamber. Then, the generated energy was transferred from the matrix to the non-volatile analytes, with desorption of analytes into the gas phase during the ionization process. The ionised molecules were extracted, focused and accelerated by electrical potentials through a time of flight tube to the mass spectrometer, with separation of the biomarkers. The separated biomarkers were determined by their mass/charge (*m/z*) ratio where z typically is 1. The unique profiles of biomarkers or known as "peptide mass fingerprint" were then compared to a reference in a database of well-characterized organisms (Cobo, 2013).

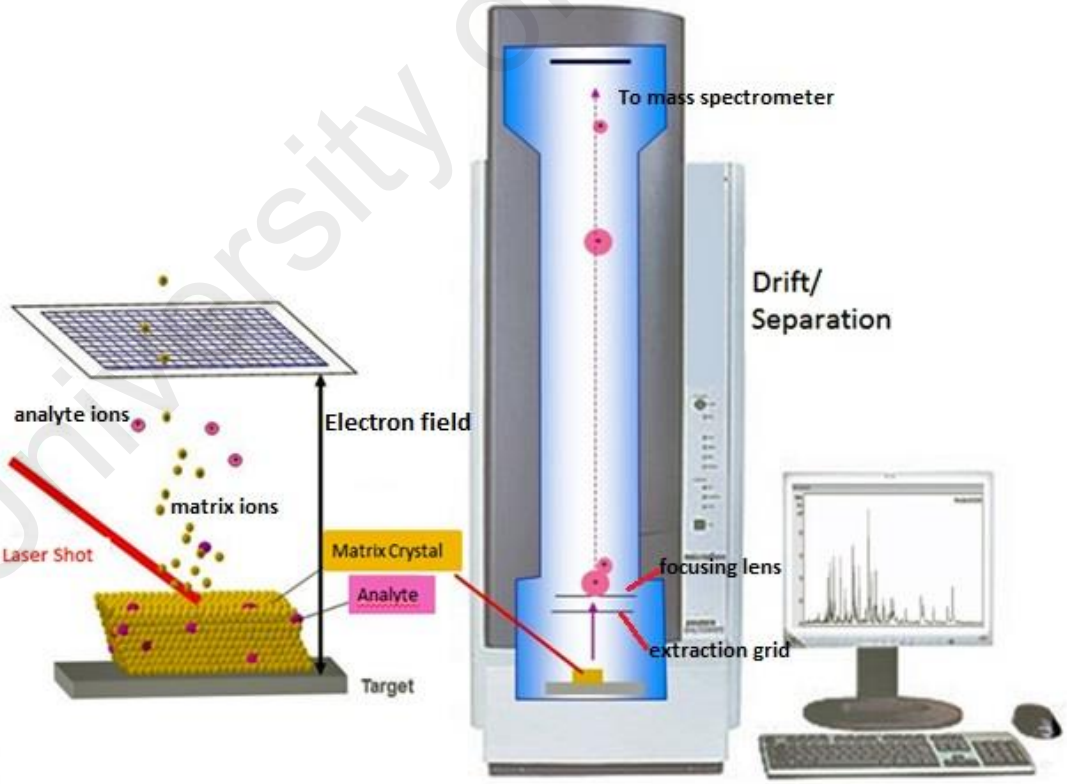

Figure 2.4: Mechanism of MALDI-TOF MS.

CHAPTER 3: MATERIALS AND METHODOLOGY

3.1 Materials

3.1.1 Instruments and Equipment

4 ˚C chiller (Thermo Scientific, USA), -20 ˚C freezer (Liebherr, UK), -80 ˚C freezer (Gaia Science, Singapore), Milli-Q® integral water purification system (Merck, Germany), autoclave machine (Hirayama, USA), laboratory hood (Labconco, Missouri), fume hood (Esco Technologies, USA), class II biosafety cabinet (Thermo Scientific, USA), ice maker (Nuove Tecnologie Del Freddo, Italy), laminar flow cabinet (Esco Technologies, USA), incubator (Memmert, Germany), shaking incubator (N-Biotek, Korea), shaking incubator (Sartorius, Germany), centrifuge machine (Eppendorf, North America), eco-spin microcentrifuge (Elmi, Latvia), belly dancer orbital mixer (IBI Scientific, USA), vortex mixer (Core Life Sciences, CA), thermomixer (Eppendorf, North America), weighing machine (Sartorius, Germany), water bath (Benchmark, USA), pH meter (Sartorius, Germany), agarose gel electrophoresis (AGE) (Biorad, USA), GenePulser Xcell™ electroporation system (Biorad, USA), high performance UV transilluminator (UVP, USA), gel documentary image analyzer (UVP, USA), Hamamatsu Photonics photon camera (Hamamatsu, Japan), spectrophotometer (Biochrom, USA), infinite M200 luminometer-spectrophotometer (Tecan, Switzerland), nanodrop spectrophotometer (Thermo Scientific, USA), CFX96 Touch™ real-time PCR detection system (Biorad, USA), polymerase chain reaction (PCR) T100 thermal cycler (Biorad, USA), Applied Biosystems Veriti 96 Fast Thermal Cycler (Thermo Fisher Scientific, USA), eco real-time PCR system (Illumina, USA), 2100 Bioanalyzer (Agilent Technologies, USA), Qubit® 2.0 fluorometer (Invitrogen, USA), high-resolution triple quadrupole liquid-chromatography mass spectrometry (LCMS/MS) (Agilent Technologies, USA), MiSeq personal sequencer (Illumina, USA), HiSeq 2000 next generation DNA platform

(Illumina, USA), matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) (Bruker, Germany), Dell Precision T7600 Workstation (Intel® Xeon(R) CPU E5-2620 0 @ 2.00GHz × 18), dryer, pipettes and pipette tips (Eppendorf, North America), microcentrifuge tubes, polypropylene tubes (15 mL and 50 mL), syringe (Terumo, USA), syringe filter (pore size of 0.22 μm) (Sartorius, Germany), disposable Petri dishes, hockey stick spreader, inoculating loop and laboratory glassware (Schott's bottles, universal bottles, conical flasks, volumetric flasks, beaker, measuring cylinder) were used in this study.

3.1.2 Commercial Kits

Masterpure™ DNA Purification Kit (Epicentre Biotechnologies, USA), MasterPure™ Gram Positive DNA Purification Kit (Epicentre Biotechnologies, USA), Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, USA), Agilent 2100 High Sensitivity DNA Kit (Agilent Technologies, USA), Nextera Index Kit (Illumina, USA), Kapa SYBR Fast qPCR Master Mix (Kapa Biosystems, USA), KAPA Library Quantification Kit (Kapa Biosystems, USA), Nextera DNA Sample Preparation Kit (Illumina, USA), AMPure XP beads (Beckman Coulter, USA), MiSeq Reagent Kits v3 (Illumina, USA), PhiX Control v3 (Illumina, USA) and AST-GN66 Test Kit (bioMérieux, USA) were used in this study.

3.1.3 Chemicals and Reagents

All the chemicals and reagents used in this study are of analytical grade purchased from Merck, Germany; Sigma-Aldrich© Chemical Corp., U.S.A. and BDH Laboratory Supplies, England. Sterilisation of chemical solutions was prepared via filter sterilisation with syringe filter at a pore size of 0.22 μm.

3.1.4 Synthetic *N*-Acyl-Homoserine Lactones

Synthetic AHL molecules (Sigma-Aldrich© and Cayman Chemicals) were dissolved using acetonitrile (ACN) to the desired concentration. Standards (1g/L) of stock solutions were stored at -20 ˚C freezer.

3.1.5 Phosphate Buffer Saline (PBS)

To prepare PBS, 8.0 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 and 0.24 g of KH_2PO_4 were dissolved in 800 mL of distilled water. The pH was adjusted to 7.4 with HCl prior to the volume adjusted to 1 L with distilled water. The final solution was autoclaved for sterilisation at 121 ˚C for 15 min and stored at room temperature.

3.1.6 Antibiotics

Antibiotics stock was obtained from Sigma-Aldrich®, USA and Amresco, USA. Appropriate antibiotics concentration, tetracycline (20 µg/mL) was prepared in this study.

3.1.7 DNA Ladder and Reference Markers

GeneRuler™ 1 kb DNA ladder (Fermentas International Inc., Thermo Fisher Scientific, USA) was used in this study.

3.1.8 Agarose Gel Electrophoresis (AGE)

For AGE, 1 × TBE buffer was prepared by dissolving 10.8 g of Tris and 5.5 g Boric acid in 900 mL distilled water. Four millilitres of 0.5 M Na_2EDTA (pH 8.0) was added to the solution prior to adjusting the final volume to 1 Litre. TBE buffer was autoclaved and kept at room temperature.

Agarose solution was prepared by dissolving 0.5 g of agarose powder in 50 mL of 1 × Tris-borate-EDTA (TBE) buffer. The mixture was heated using the microwave for 1 min until agarose powder completely dissolved. The agarose solution was added 1 µL of 0.5 × GelStar™ Nucleic Acid Gel Stain (Lonza, USA) prior to gel casting and 2 µL of 6 × bromophenol blue loading dye (Fermentas International Inc., Thermo Fisher Scientific, USA) was added to 12 µL of each genomic DNA sample prior to loading into the well of agarose gel.

3.1.9 Bacterial Strains, Biosensors and Cultivate Conditions

Bacterial strains for cross-streak bioassay and biosensors with their culturing conditions were summarised in Table 3.1.

Table 3.1: Bacterial strains and biosensors used in this study

Strain	cultivate conditions	Description	Reference
Chromobacterium violaceum CV026	aerobically overnight culture on Luria-Bertani agar at 28 ˚C	a biosensor, violacein-negative, mini-Tn*5* mutant of *C. violaceum* ATCC 31532 in which purple violacein pigment production can be restored if extracellular short chain AHL signalling molecule is detected.	McClean et al., 1997
Escherichia coli [pSB401]	aerobically overnight culture in Luria-Bertani broth supplemented with tetracycline (20 μg/mL) and shaking incubator with 220 rpm at 37 ˚C	a short chain AHL biosensor with LuxR receptor cognate AHL = 3-oxo-C6-HSL, Tet^R	Winson et al., 1998
Erwinia carotovora GS101	aerobically overnight culture on Luria-Bertani agar at 28 ˚C	a positive control for biosensors in QS cross-steak assay by producing small diffusible AHL molecules	McGowan et al., 1995
Erwinia carotovora PNP22	aerobically overnight culture on Luria-Bertani agar at 28 ˚C	a negative control for biosensors in QS cross-steak assay	McGowan et al., 1995

3.2 Growth Media

Growth media prepared in this study included Luria Columbia Agar (Isolac, Isolab, Malaysia) supplemented with 5% v/v sheep blood, Trypticase soy agar (Scharlau, Scharlab, S.L., European Union), Difco™ R2A agar (Difco, BD, USA), Difco™ Czapek-Dox broth (Difco, BD, USA) with addition of 1.5% w/v Bacto-agar and Luria-Bertani agar (in grams per 100 mL: tryptone, 1; yeast extract, 0.5; NaCl, 0.5; Bacto agar, 1.5) (Scharlau, Scharlab, S.L., European Union). Unless otherwise stated, preparation of the growth media and solutions stated in this study required sterilisation by autoclaving at 121 ˚C, 15 psi for 15 min.

3.3 Sample Collection

3.3.1 Teeth Selection, Inclusion and Exclusion Criteria

This study was conducted with medical ethics approval from Faculty of Dentistry (University of Malaya), Ethics and Research Committee (DFRD-1302/0033-L). Express consent was obtained from patients prior to tooth extraction at the Department of Oral and Maxillofacial Surgery, University of Malaya. Based on the inclusion and exclusion criteria, four healthy adults (19-62 years) were selected as participants in this study. Carious teeth were selected based on International Caries Detection and Assessment System (ICDAS) Codes 5 and 6, assuming there are enamel breakdown and dentin cavitation that has involved half of the tooth structure (Ekstrand et al., 2007). Teeth with restorations, clinical signs of remineralization and pulp exposure were excluded. All extracted teeth were stored in physiological buffered saline (PBS) at 4 °C and dentinal caries was excavated within 24 hours of post extraction.

3.3.2 Caries Excavation and Microbiological Sampling

Soft loose debris present over the cavity was removed and the cariogenic biomass from the dentinal lesion was manually excavated with individual sterile sharp spoon excavators (Ash, G5-Claudius Ash Ltd, Potters Bar, Herts, UK) by one operator. For some of the collected teeth, minimum cutting of unsupported enamel was done from the peripheries using sterile water-cooled diamond bur operated in an air-turbine hand-piece to create an easy access for caries excavation. While unsupported enamel was removed, precautions were taken to ensure dental caries not to be disturbed. A standardized scale of measurements was used for identification of infected dentine (Kidd et al., 1993) by justifying the color of the lesion was light brown, with soft consistency (probe penetrating dentine with no resistance when the probe is removed) and lesion was damp in nature, which was confirmed after establishing the presence of humidity after mild drying of the cavity for 3 seconds. The plaque samples were collected prior to extraction of the tooth. Soft and loose dental plaque surrounded the buccal, proximal surfaces or subgingival pockets of the carious tooth were carefully picked using sterile Gracey curettes. The samples obtained from dentine caries and plaque were placed in a sterile 1.5 mL microcentrifuge tube in saline solution and transported to the laboratory for further analysis.

3.4 Laboratory Preparation and Analysis

3.4.1 Bacterial Strains Isolation and Culture Conditions

The collected samples were kept in 4 °C sterile physiological buffered saline (PBS, 0.01 M, pH 7.4). Further laboratory analysis was performed within 24 hours. Isolation of bacterial strains was performed on various selective culture media, namely Columbia Agar, Trypticase soy agar, R2A agar, Czapek-Dox agar and Luria-Bertani agar. Firstly, saline solutions from each collected dentine caries and plaques sample were vortex-homogenized for 1 min prior to 100 µ L in aliquots spread on the various selected media. All the plates were aerobically cultured at 37 ˚C overnight in the incubator. A single colony was picked based on their diverse morphology, colour, shape and size. Repeated streaking on the media was performed to obtain pure colonies for further strain identification and preliminary AHLs screening.

3.4.2 Rapid and Basic Strain Identification

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) was used to classify and identify the isolates through direct transfer sample preparation method. The sample preparation for MALDI-TOF MS analysis was carried out as reported previously by Mellmann and colleagues (2008). The MALDI-TOF α-cyano-4-hydroxycinnamic acid matrix (10 mg/mL) was prepared as a saturated solution in 50% (v/v) acetonitrile (ACN), 47.5% (v/v) highly purified water and 2.5% (v/v) trifluoroacetic acid (TFA). Firstly, the fresh culture of each targeted isolate on their growth media was directly smeared on MSP 96 target polished steel BC plate (Bruker, Germany) as a thin film and overlaid with 1 µL of prepared Bruker MALDI HCCA matrix. To ensure the correct strain identity, quadruplicate of each sample was assayed. The mixture of sample spot was air-dried until a homogeneous preparation was observed

before further analysis using the Microflex MALDI-TOF bench-top MS apparatus included the Microflex MALDI Biotyper (Bruker Daltonik GmbH, Leipzig, Germany) bench-top mass spectrometer (equipped with UV laser at 337 nm and 355 nm wavelength) and Bruker FlexControl software version 3.3. The method for analysis of the sample was conducted as previously described (Ngeow et al., 2013) and the identity of the sample was evaluated based on a dedicated scoring system and colour coded where the spectra information of the sample was compared to the best match with the Bruker database. The scoring value was described as user manual (Table 3.2) and a dendrogram was constructed from the standard MALDI Biotyper MSP creation method. It was generated by similarity scoring of a set of mass spectra illustrating the graphical distance values between species constructed from their MALDI-TOF reference spectra. The distance values of the dendrogram are normalised to a maximum value of 1,000 and are always relative. The created distance dendrogram of MALDI-TOF analysis showed the percentage spectrum identity and the position for all of the investigated isolates.

Table 3.2: Scoring value system in MALDI Biotyper

Score value	Evaluation
2.300 – 3.000	highly probable species identification
2.000 – 2.299	secure genus identification and probable species identification
1.700 – 1.999	probable genus level identification
Less than 1.700	Not reliable identification

3.4.3 AHLs Preliminary Screening

Chromobacterium violaceum CV026 and *Escherichia coli* [pSB401] were used as two different short chains AHLs bacterial biosensors in this study to detect microbial quorum sensing activity via cross-streak bioassay. In the screening, *Erwinia carotovora* GS101 and *E. carotovora* PNP22 were used as a positive and negative control, respectively (Chen et al., 2013; Chong et al., 2012). All bacterial biosensors, positive and negative controls strains were cultured routinely in Luria-Bertani (LB) medium with 24 h incubation at 28^{o}C. In the presence of short chain AHLs, *C. violaceum* CV026 responded by producing purple violacein pigmentation (McClean et al., 1997; Winson et al., 1998) whereas *E. coli* [pSB401] showed the intensity of bioluminescence towards detection of exogenous short chain AHL. The image of bacterial bioluminescence at the meeting point between two strains was recorded by Hamamatsu Photonics photon camera.

3.4.4 AHLs Extraction

All the QS positive strains were subcultured in LB broth buffered with 50 mM 3-(*N*-morpholino) propane sulfonic acid (MOPS) at pH 5.5 in a shaking incubator (200 rpm, 37^{o}C, 18 h). The organic solvent, ethyl acetate, was acidified with 0.1 % v/v glacial acetic acid prior to performing the extraction of the subculture's AHLs twice with a ratio of 1:1, as described previously (Ortori et al., 2011). The extracted supernatant was air-dried in a fume hood. The completely dried extracts were resuspended in 200 µL of acetonitrile (HPLC grade) and vortex-homogenized to dissolve the dried extracts. The mixture was centrifuged at high speed for 5 min to filter any insoluble residue. The dissolved sample in 100 µL was aliquot from the upper layer and inserted into sample vials for mass spectrometry analysis.

3.4.5 AHLs Profiling by Mass Spectrometry (MS) Analysis

High-resolution MS was performed as previously described by Ortori and coworkers (2011). The 100 μL extracted AHLs mixture was analysed using an Agilent 1290 Infinity LC system coupled with Agilent 6490 Triple Quadrupole LCMS/MS system and Agilent ZORBAX Rapid Resolution High Definition SB-C18 threaded column. The AHLs standard of *N*-butyryl-L-homoserine lactone (C4-HSL), *N*-hexanoyl-L-homoserine lactone (C6-HSL), *N*-(3-oxohexanoyl)-L-homoserine lactone (3-oxo-C6-HSL), *N*-octanoyl-L-homoserine lactone (C8-HSL), *N*-(3-oxooctanoyl)-L-homoserine lactone (3-oxo-C8-HSL), *N*-decanoyl-L-homoserine lactone (C10-HSL), *N*-(3-oxodecanoyl)-L-homoserine lactone (3-oxo-C10-HSL), *N*-dodecanoyl-L-homoserine lactone (C12-HSL), *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) and *N*-hexadecanoyl-L-homoserine lactone (C16-HSL) were used in this study for comparison. The parameter settings such as its temperature, flow rate and injection volume were adjusted as described previously (Lau et al., 2013). The mobile phases A and B used in this study were water and acetonitrile (both mobile phases added with 0.1 % v/v formic acid), respectively and set to a ratio of 80:20. The parameter for high-resolution electron spray ionization mass spectrometry (ESI-MS) set for the run was as described previously (Lau et al., 2013). The precursor ion scan mode targeting the *m/z* 102 product ion that represented the $[M + H]^+$ ion of the lactone ring moiety and indicated the presence of AHL. The *m/z* value range to detect the precursor ions was set at 80–400 and the MS data analysis was done using Agilent MassHunter Qualitative Analysis B.05.00 software with electrospray ionization (ESI) positive mode.

3.4.6 Antibiotic Susceptibility Testing with Vitek 2 System

A pure colony of the bacterial isolate was subcultured on tryptic soy agar (TSA) and grown at 37°C for 18 to 24 hours. A single colony of the cultures was picked and made into a suspension of 0.5 MacFarland standards in 0.45% w/v saline according to manufacturer's recommendations. The adjusted suspension was used as inoculums for test cards of VITEK 2 system (bioMérieux, USA). Test card AST-GN66 (bioMérieux cat. no. 413398) was selected for Gram-negative oral bacteria antibiotic susceptibility inspection and the antimicrobial agents contained in the card were listed in Table 4.1. With the results from VITEK 2 system, the minimal inhibitory concentration (MIC) of each antibiotic was estimated and interpreted by Advanced Expert System (AES) based on approximate 20,000 MIC distributions from 2,000 phenotypes (Leverstein-van Hall et al., 2002).

3.4.7 DNA Extraction

The single colony of each sample was picked and kept in a sterile 1.5 mL microcentrifuge tube at -20 ˚C. Total genomic DNA extraction was performed using MasterPure^{TM} Gram Positive DNA Purification Kit or MasterPure^{TM} DNA Purification Kit for each interested oral isolate according to the manufacturer's instructions with minor modifications. Briefly, the bacterial cell was lysed with Cell Lysis solution prior to DNA precipitation in 35 µl of TE Buffer. The extracted genomic DNA was subjected to agarose gel electrophoresis (AGE) to detect its molecular size and integrity and to roughly estimate the DNA content. The quality and quantity of DNA were examined using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) and Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA) by using dsDNA High-Sensitivity Assay Kit, respectively.

For NanoDrop 2000 spectrophotometer application, the acceptable ratio of absorbance for extracted genomic DNA was listed in Table 3.3.

Table 3.3: Acceptable quality of genomic DNA

Ratio of absorbance	**range**
A260/A280	~ 1.8
A260/A230	1.8 – 2.2

A260 displays absorbance at 260 nm normalised to a 10 mm path length; similarly for A280 and A230.

3.4.8 Library Preparation for Next Generation Sequencing (NGS)

The extracted DNA with acceptable quality was subjected to NGS library preparation using Nextera Sample Prep Kit (Illumina, USA) according to the manufacturer's protocol prior to DNA library quantification and qualification. DNA quality control was carried out on the 2100 Bioanalyzer using High Sensitivity DNA Analysis Kit (Agilent Technologies) and quantitative real-time PCR was conducted in the Eco Real-Time PCR System using KAPA Library Quantification Kit for Illumina sequencing platforms (KAPA Biosystems, Boston, MA, USA). This assay is important to create optimum cluster densities to ensure the highest quality of data is obtained by the Illumina sequencing platform. Each of the DNA libraries was normalised to 2nM in 10 mM Tris-CL, pH 8.5 with 0.1% v/v Tween 20. The template DNA was then pooled, denatured and diluted to a concentration of 20 pM with freshly prepared 0.1N NaOH and Hybridization Buffer HT1. The 20 pM library stock was further diluted to 10 pM with Hybridization Buffer HT1 in order to obtain desired optimum raw density and a low concentration PhiX control spike-in at 1% to improve the sequencing quality control.

3.4.9 Whole Genome Sequencing

In this study, the whole genome sequencing was performed on Illumina MiSeq platform. A total of 600 μL final library templates was loaded into the cartridge in the designated reservoir and proceed to MiSeq sequencing using MiSeq Reagent Kits v3 (Illumina, USA) coupling with MiSeq Control Software (MSC) interface. In brief, select 'Sequence' to start the run setup steps as guided in the software interface. The flow cell was washed carefully and dried thoroughly prior to loading into MiSeq platform and followed by loading reagent cartridge. The sequencing was initiated after the run parameters were reviewed with positive pre-run check results. During the sequencing, the run was monitored from MSC interface via Sequencing Analysis Viewer (SAV). This step is performed by recording the synthesis of DNA strands in clusters of library templates attached to the flow cell through bridge amplification. A post-run wash with Tween 20 was performed after completing a sequencing run to flush any remaining reagents from the sippers and fluidics line, prevent cross-contamination from the previous run and salt accumulation or crystallisation on MiSeq platform.

3.4.10 Raw Data Processing

3.4.10.1 Genome assembly

The raw reads generated by MiSeq were first quality evaluated using FastQC analyzer prior to trimming the bad reads based on their base quality and filtering according to read length using CLC Genomics Workbench version 7.0.4 (Qiagen, Germany). In this genome data analysis, raw reads with an average quality value lower than Q20 and ambiguous nucleotides were excluded from further analysis. One 5' terminal nucleotide of all the reads from MiSeq data was removed and read length of trimmed reads was further filtered by discarding those reads shorter than 15 nucleotides. In order to obtain the best assembly result, *de novo* genome assemblies were performed multiple times using a range of word size parameter from post-filtered reads.

3.4.10.2 Genome annotation

A rapid prokaryotic genome annotation system (Prokka) (Seemann, 2014) was used to annotate the whole genome sequences of oral isolates. All predicted genes by Prodigal version 2.60 (Hyatt et al., 2010) were searched against the updated databases of known sequences (NCBI-NT/NR databases) using BLAST tool in order to further assign the accurate functional annotation. In addition, all the whole genome sequences were subject to an automated SEED-based annotation using Rapid Annotation Subsystems Technology (RAST) server (Aziz et al., 2008).

3.4.11 Downstream Analyses

3.4.11.1 Phylogenetic Analysis

The predicted DNA coding sequences of 16S rRNA and QS synthase gene were extracted from whole genome sequences of related oral isolates in order to study their phylogenetic relationships. Molecular Evolutionary Genetic Analysis version 5.1 (MEGA) tool (Tamura et al., 2011) was used to construct the maximum likelihood (ML) phylogenetic trees with General Time Reversible (GTR) nucleotide substitution model and the percentage of robustness of the associated taxa clustered together is indicated by the bootstrap test (1000 replicates), as shown by the side of the branch. The extracted sequence was compared and aligned with the related gene sequences from public available NCBI databases.

3.4.11.2 HOMD 16S rRNA Sequence Identification

The extracted 16S ribosomal RNA sequence of each sample was mapped against the Human Oral Microbiome Database (HOMD) for 16S rRNA sequence identification by submitting the sequence to their server and using their default run parameter. The results were returned with top four 16S rRNA sequences best match to query sequence.

3.4.11.3 Synteny analysis of QS related genes

The QS synthase and regulator genes were determined from the annotated whole genome sequences. Easyfig version 2.2 (Sullivan et al., 2011) was implemented for downstream synteny analysis of QS synthase and regulator genes with the aim of identifying and displaying genome synteny alignments.

3.4.11.4 *In silico* Discovery QS System

The *in silico* discovery QS system was performed on the whole genome sequence of strain L8A. Gene Ontology terms or physical interaction network associated with amino acid-regulated genes based on their involvement in three aspects, namely cellular component, biological process and molecular function was focused in this analysis. The targeted protein such as *N*-acyl-homoserine lactone synthase of strain L8A was searched against UniProt Knowledgebase (UniProtKB) to get the accession number. The pathway was determined by fast web-based GO browser, QuickGO (Binns et al., 2009) with the accession number (A0A083ZD14) in this study. Five AHL synthase related GO terms coupled with three GO child terms from the GO term of 'quorum sensing' were added to Term Basket prior to generating the ancestor comparison chart.

3.4.11.5 Antibiotics Resistance Genes

Firstly, the predicted coding DNA sequences from the whole genome sequences of interested oral isolates were studied and divided into subsystems by RAST. The abundance of each categorised subsystem was shown in the pie chart. To focus on antibiotics resistance genes of the oral isolates, the features of antibiotics and toxic compound resistance under the subsystem of 'Virulence, Disease and Defense' was then analysed by addition computational algorithms. For instance, the putative genes conferring drug resistance and virulence were further BLAST searched using PSI-BLAST against NCBI database.

In addition to this, a program of Short Read Sequence Typing (SRST2) (Inouye et al., 2014) for Bacterial Pathogens coupled with the database from ARG-ANNOT (Antibiotic Resistance Gene-ANNOTation) (Gupta et al., 2014) were implemented to directly detect the acquired antibiotic resistance genes from the short-read whole genome

sequence data or also known as raw data of oral isolate. The whole genome sequences of the oral strain and its predicted β-lactamase relatedness genes were illustrated using DNA plotter in Artemis.

3.4.11.6 Comparative Genome Studies

In order to determine genotypic differences between closely related prokaryotes, comparative genome analysis was established. To perform multiple genomes comparison for draft genome data, the assembled contigs were ordered and re-oriented by aligning them to a closely related reference genome using Mauve aligner (Rissman et al., 2009). The reordered contigs of each draft genome were then concatenated to engineer a pseudo-molecule. The comparison between the query pseudo-molecule genome sequences and reference genome sequence were created using the local BLASTn program to show the percentage of match hits between the sequences. The structural similarities and differences between the genomes sequences were then visualised using BLAST Ring Image Generator (BRIG) software (Alikhan et al., 2011).

CHAPTER 4: RESULTS

4.1 Oral Bacteria Isolates and Identification by MALDI-TOF

In this study, a total of 21 different culturable bacterial species with distinctive morphology were isolated and identified by MALDI-TOF MS. From these 21 isolates, eight of them were solely found in dentine caries which included *Burkholderia cepacia*, *Candida albicans*, *Enterococcus faecalis*, *Enterobacter gergoviae*, *Lactobacillus paracasei*, *Lactobacillus pentosus*, *Proteus mirabilis* and *Stenotrophomonas maltophilia*. Of these isolates, five were solely found in dental plaques namely *Acinetobacter pitti*, *Citrobacter amalonaticus*, *Klebsiella oxytoca*, *Streptococcus pneumonia* and *Streptococcus salivarius*; and the remaining eight of them can either be isolated from dentine caries or dental plaques (Figure 4.1). However, the most common strains found in both dentine caries and plaques were *Klebsiella pneumonia*, followed by *Citrobacter koseri* and *Elizabethkingia meningoseptica*.

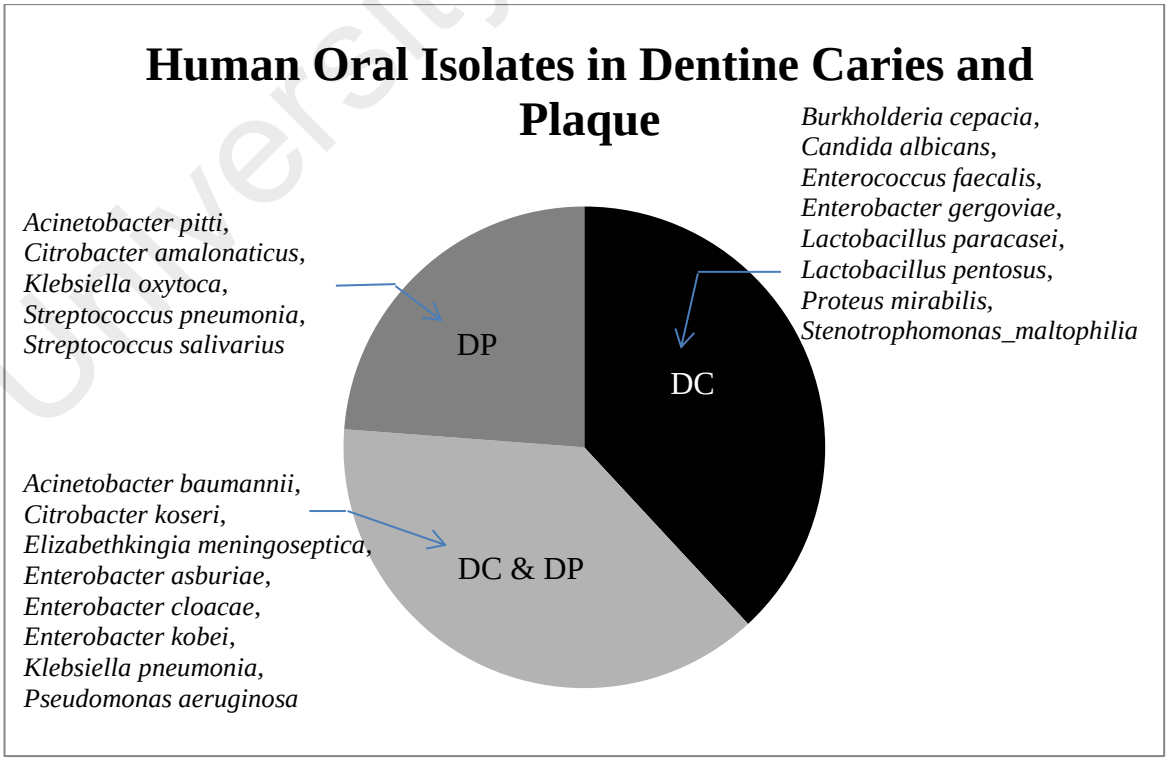

Figure 4.1: Human oral isolates in dentine caries and dental plaque. DC denotes dentine caries and DP denotes dental plaque.

Each of the species was randomly selected for further study. The MALDI-TOF mass spectra from the dentine caries and dental plaque isolates were generated and analyzed by standard pattern matching to Bruker database through MALDI-TOF Biotyper software which led to the identification of strains as listed in Table 4.1.

Table 4.1: Summary of strains identification using MALDI-TOF MS

Strain	Identification	Figure
R1C	*Acinetobacter baumannii*	4.2
B8E	*Acinetobacter pitti*	4.2
C10B	*Burkholderia cepacia*	4.3
L10D	*Candida albicans*	4.4
L8A	*Citrobacter amalonaticus*	4.5
B1B	*Citrobacter koseri*	4.5
B2D	*Elizabethkingia meningoseptica*	4.6
R8E	*Enterobacter asburiae*	4.7
R2A	*Enterobacter cloacae*	4.8
C7B	*Enterobacter gergoviae*	4.9
B2F	*Enterobacter kobei*	4.10
L10E	*Enterococcus faecalis*	4.11
R8A	*Klebsiella oxytoca*	4.12
B1A	*Klebsiella pneumonia*	4.12
L9D	*Lactobacillus paracasei*	4.13
C9C	*Lactobacillus pentosus*	4.13
T1C	*Proteus mirabilis*	4.14
L10A	*Pseudomonas aeruginosa*	4.15
R5G	*Stenotrophomonas_maltophilia*	4.16
R6B	*Streptococcus pneumonia*	4.17
R6A	*Streptococcus salivarius*	4.17

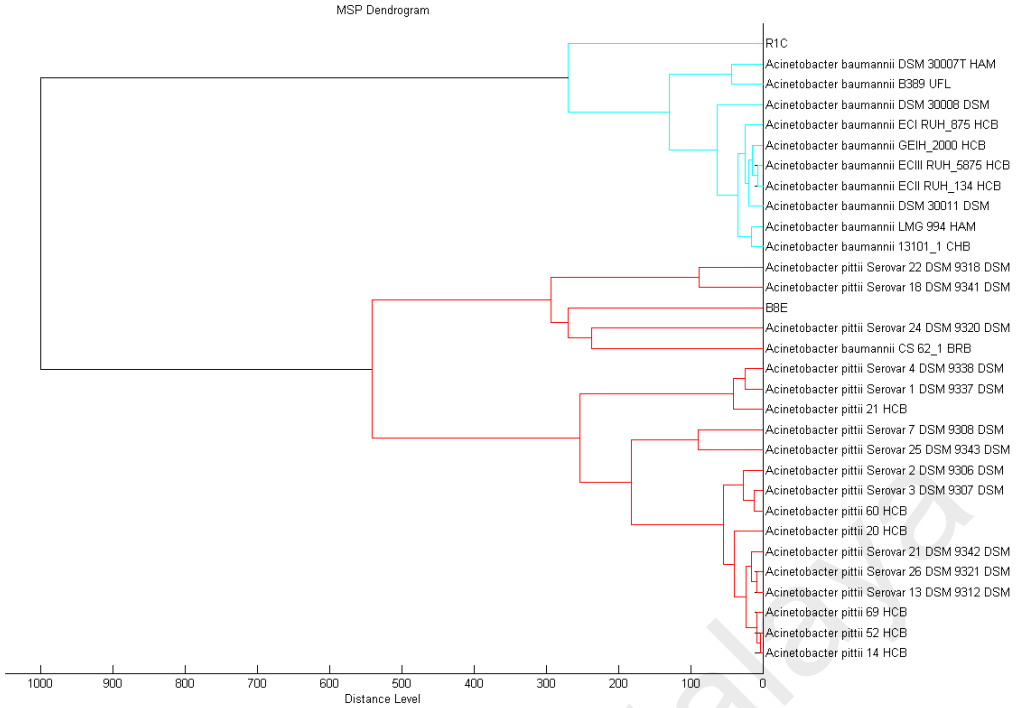

Figure 4.2: Score-oriented dendrogram of strains RC1 and B8E. Oral isolates *Acinetobacter baumannii* strain RC1 from dentine caries sample (score value: 2.310) and *Acinetobacter pitti* strain B8E from dental plaque sample (score value 2.245)

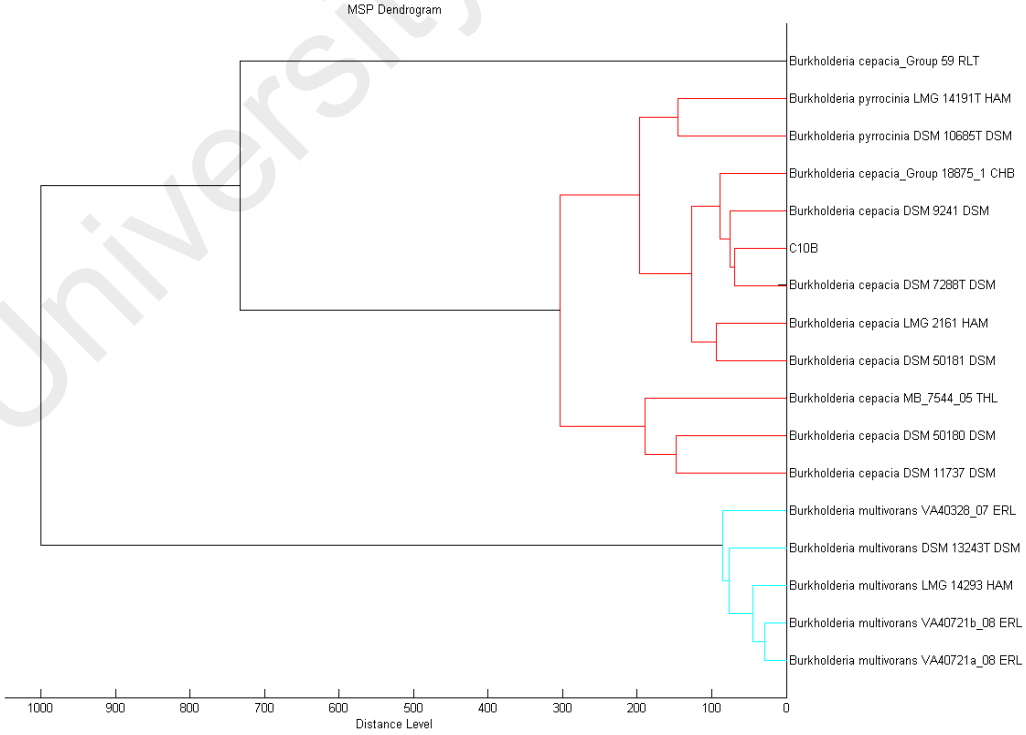

Figure 4.3: Score-oriented dendrogram of strain C10B. Oral isolate *Burkholderia cepacia* strain C10B from dentine caries (score value: 2.335).

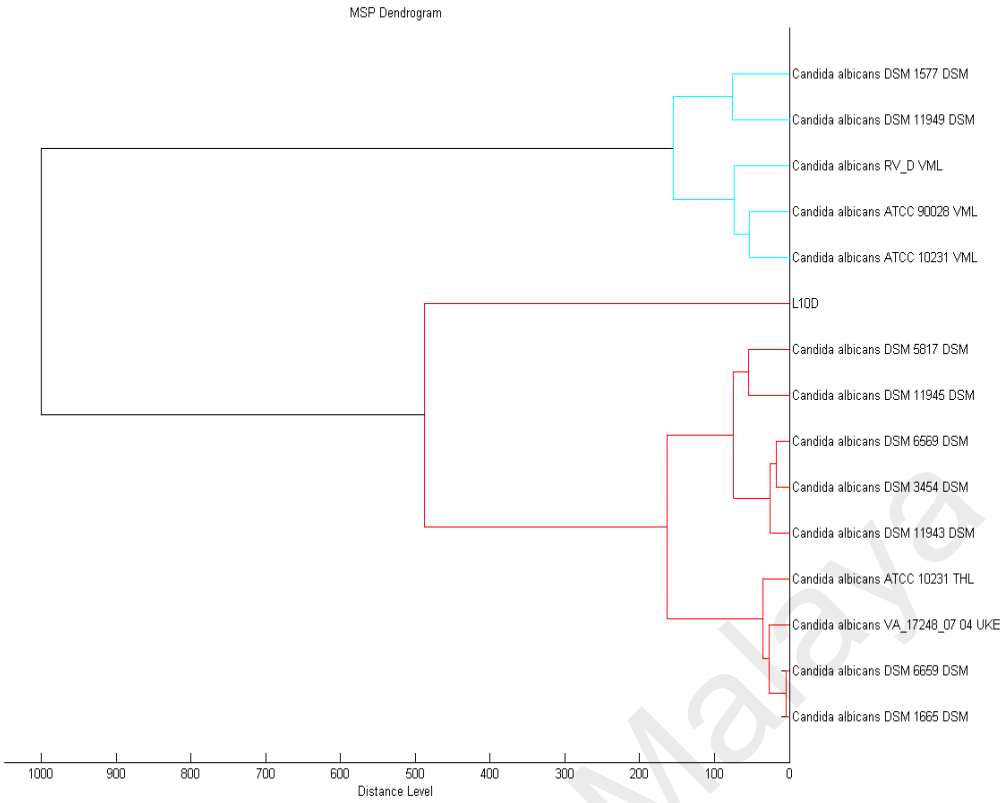

Figure 4.4: Score-oriented dendrogram of strain L10D. Oral isolate *Candida albicans* strain L10D from dentine caries (score value: 2.108)

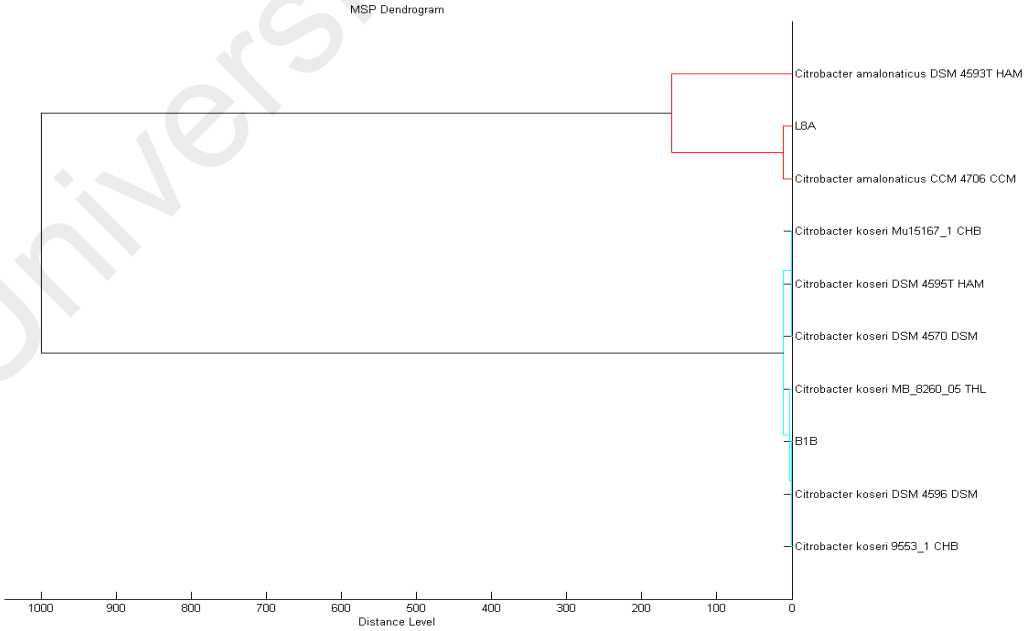

Figure 4.5: Score-oriented dendrogram of strains L8A and B1B. Oral isolates *Citrobacter amalonaticus* strain L8A from dental plaque (score value: 2.390) and *Citrobacter koseri* strain B1B from dentine caries (score value 2.245)

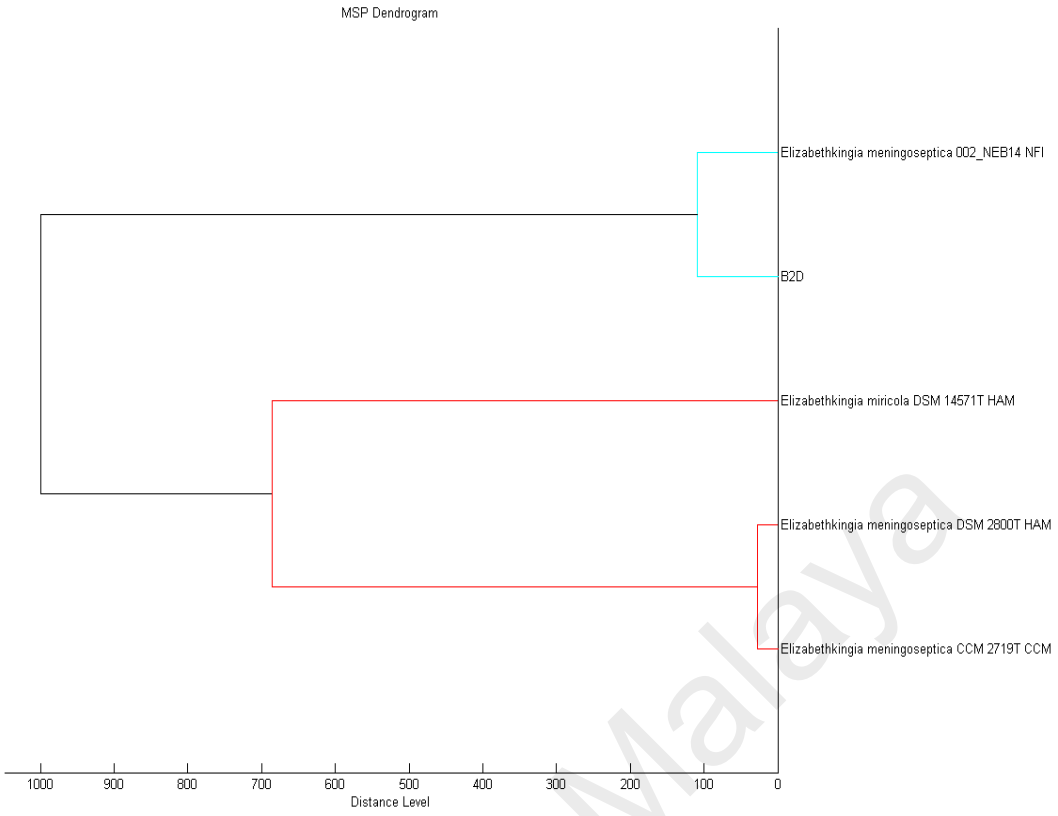

Figure 4.6: Score-oriented dendrogram of strain B2D. Oral isolate *Elizabethkingia meningoseptica* strain B2D from dental plaque (score value: 2.375)

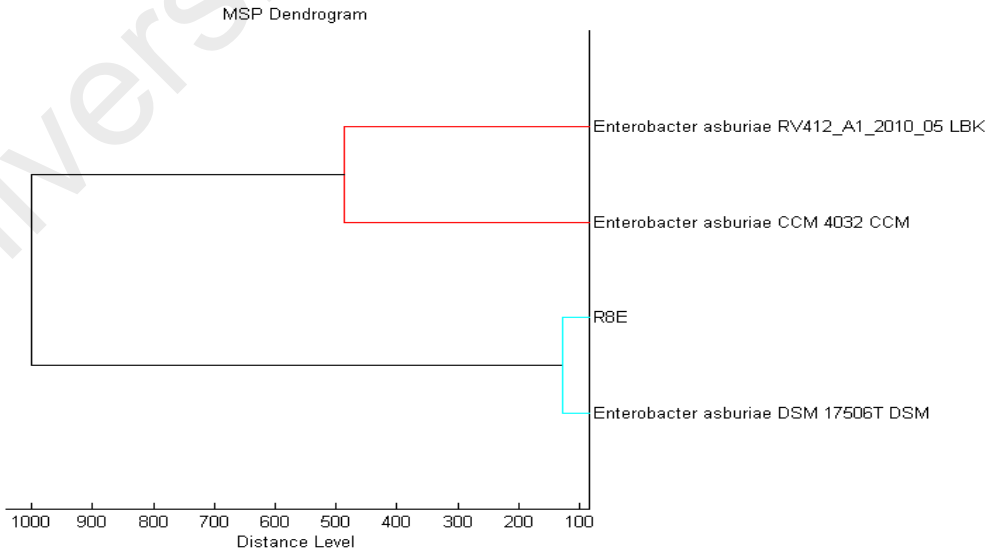

Figure 4.7: Score-oriented dendrogram of strain R8E. Oral isolate *Enterobacter asburiae* strain R8E from dental plaque (score value: 2.446)

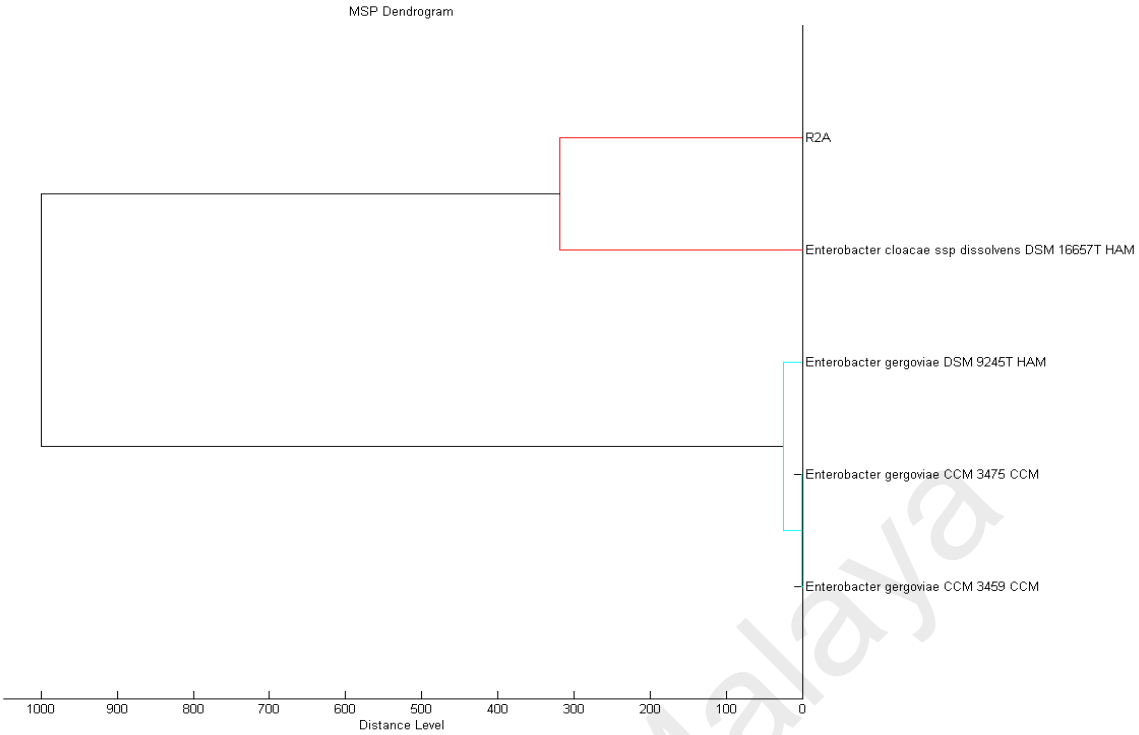

Figure 4.8: Score-oriented dendrogram of strain R2A. Oral isolate *Enterobacter cloacae* strain R2A from dental plaque (score value: 2.210)

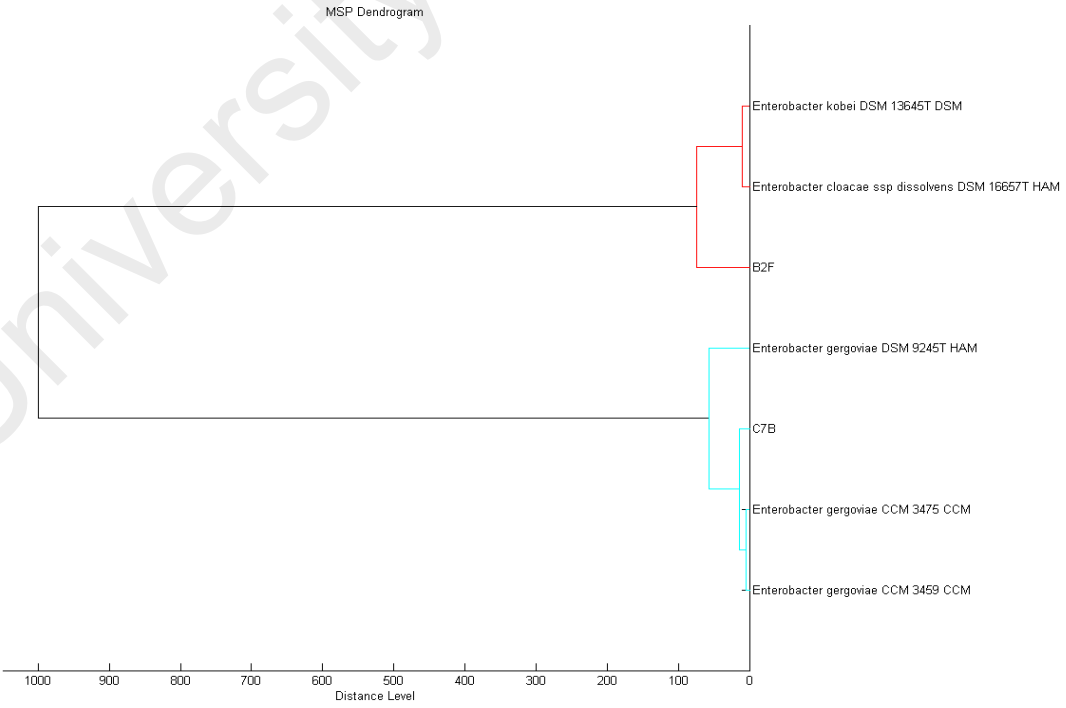

Figure 4.9: Score-oriented dendrogram of strain C7B. Oral isolate *Enterobacter gergoviae* strain C7B from dentine caries (score value 2.218)

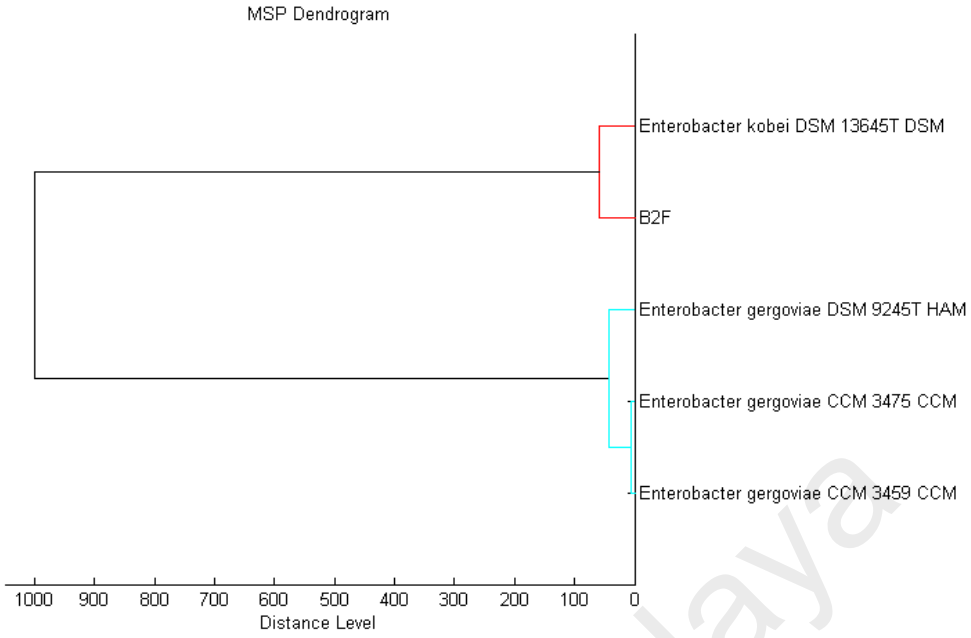

Figure 4.10: Score-oriented dendrogram of strain B2F. Oral isolate *Enterobacter kobei* strain B2F from dental plaque (score value: 2.306)

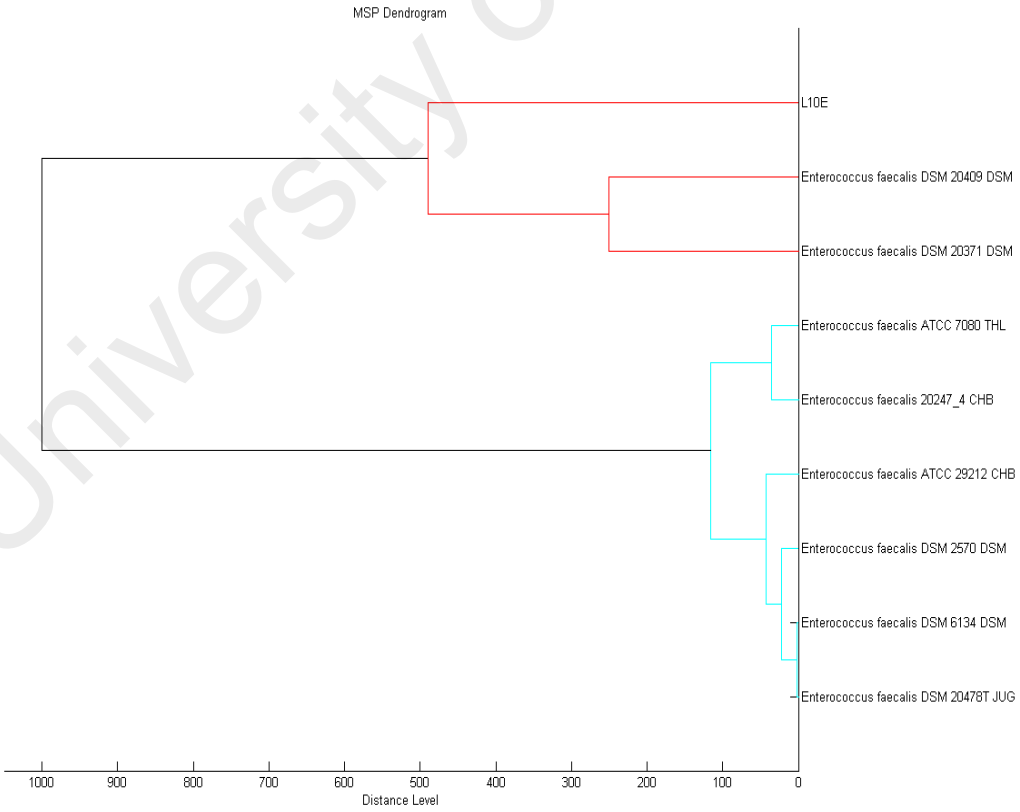

Figure 4.11: Score-oriented dendrogram of strain L10E. Oral isolate *Enterococcus faecalis* strain L10E from dentine caries (score value: 2.454)

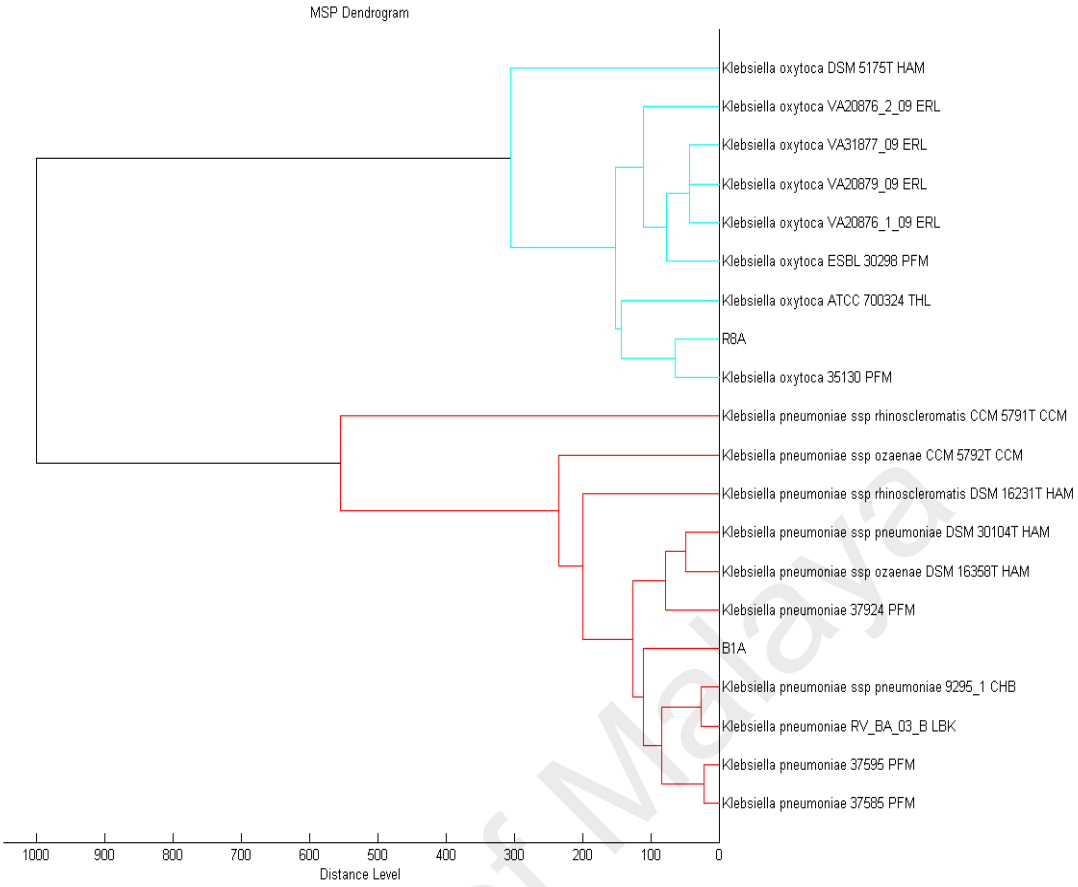

Figure 4.12: Score-oriented dendrogram of strains R8A and B1A. Oral isolates *Klebsiella oxytoca* strain R8A from dental plaque (score value: 2.298) and *Klebsiella pneumonia* strain B1A from dentine caries (score value 2.528)

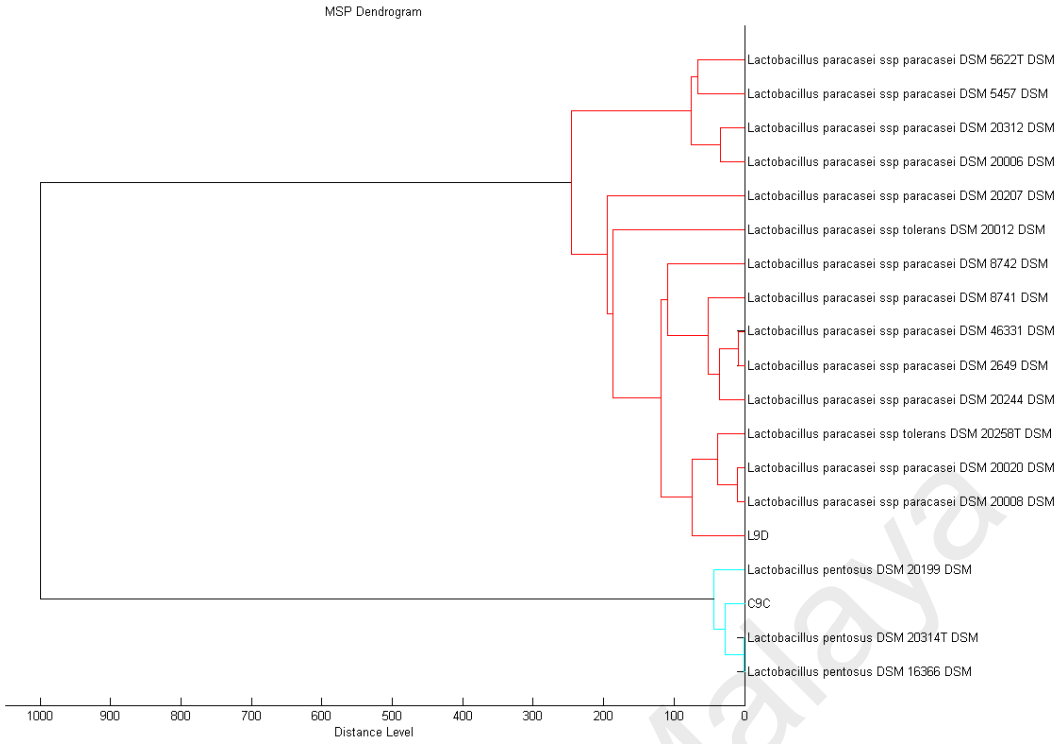

Figure 4.13: Score-oriented dendrogram of strains L9D and C9C. Both oral isolates *Lactobacillus paracasei* strain L9D (score value: 2.403) and *Lactobacillus pentosus* strain C9C (score value 2.395) from dentine caries.

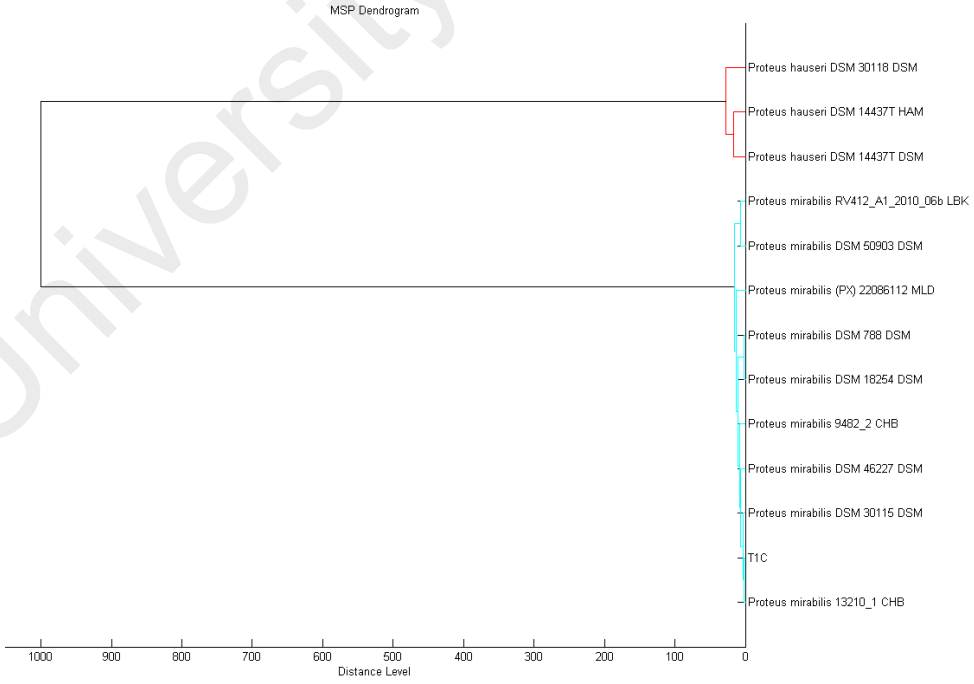

Figure 4.14: Score-oriented dendrogram of strain T1C. Oral isolate *Proteus mirabilis* strain T1C from dentine caries (score value: 2.610)

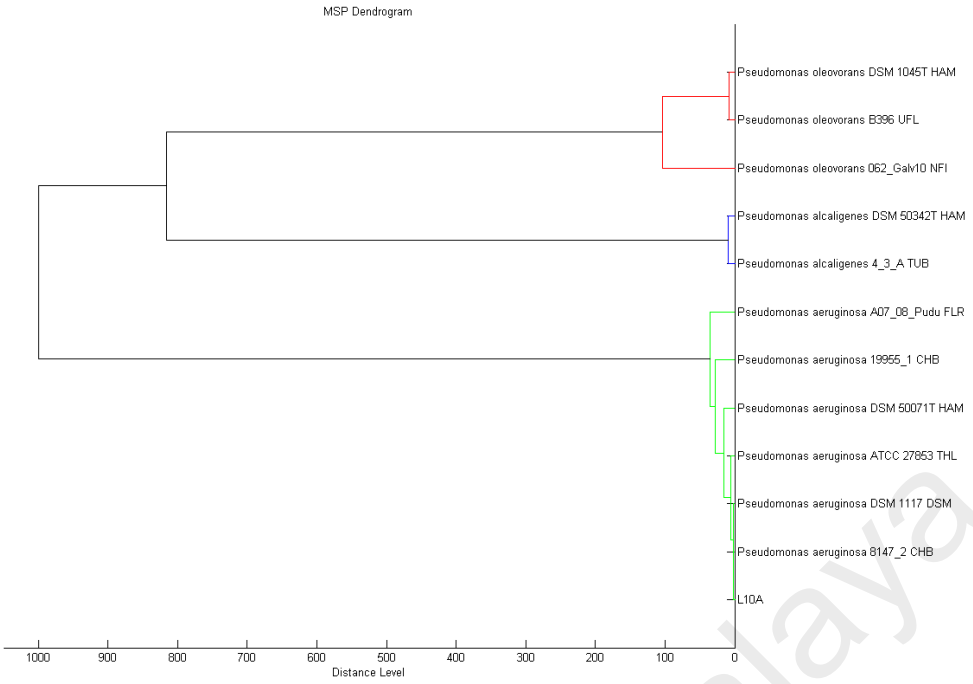

Figure 4.15: Score-oriented dendrogram of strain L10A. Oral isolate *Pseudomonas aeruginosa* strain L10A from dentine caries (score value: 2.445)

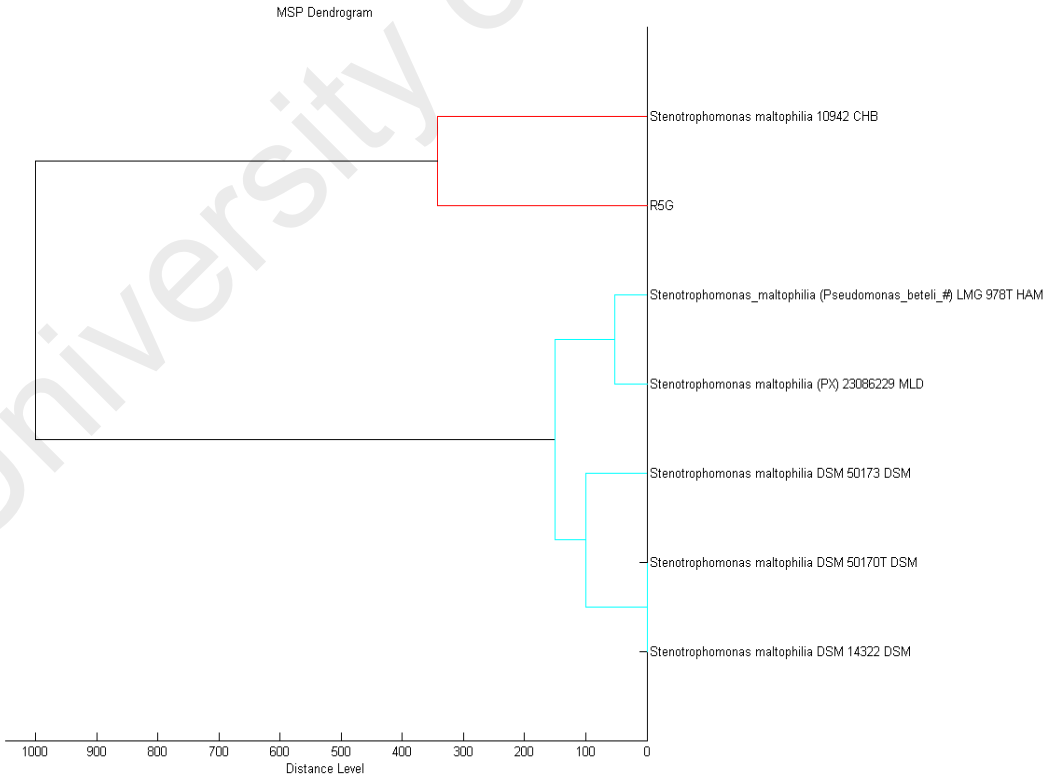

Figure 4.16: Score-oriented dendrogram of strain R5G. Oral isolate *Stenotrophomonas maltophilia* strain R5G dentine caries (score value: 2.381)

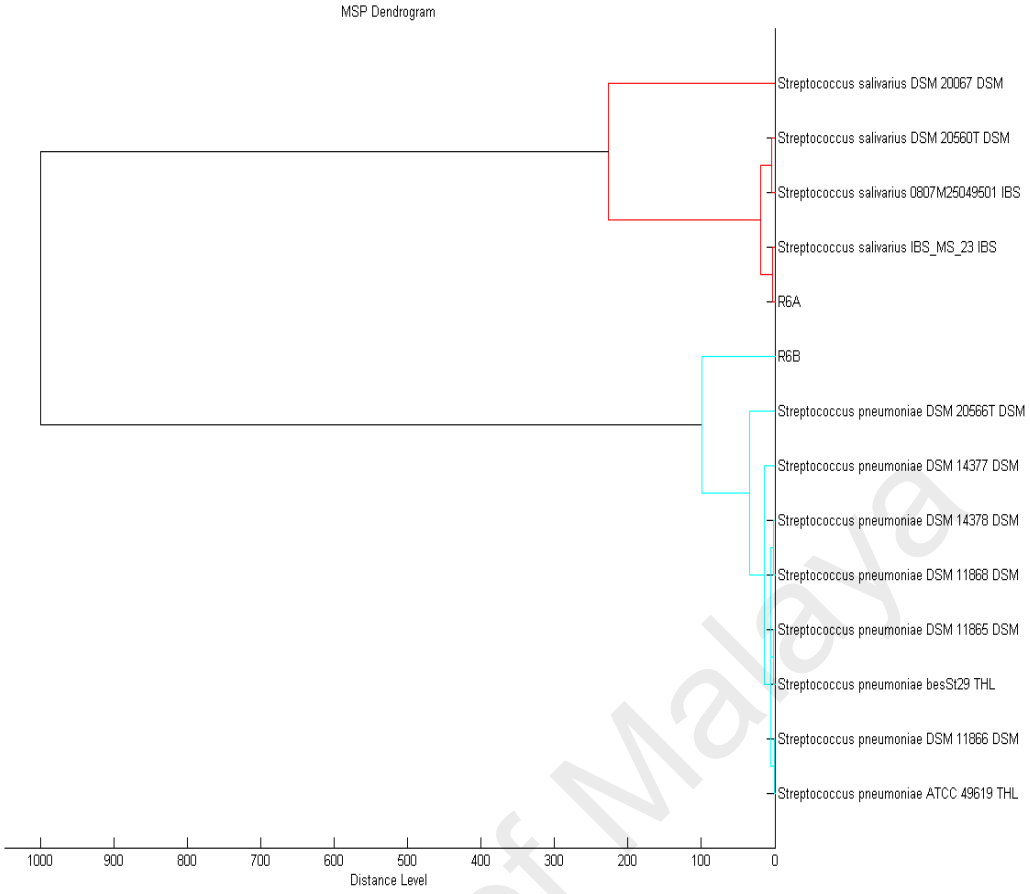

Figure 4.17: Score-oriented dendrogram of strain R6A. Both oral isolates *Streptococcus pneumonia* strain R6B (score value: 2.075) and *Streptococcus salivarius* strain R6A (score value 2.221) from dental plaque.

4.2 Detection of Quorum Sensing Activity in Oral Bacteria

4.2.1 AHLs Preliminary Screening

QS activity in oral bacteria was determined via cross-streaked with two types of AHL biosensors namely *C. violaceum* CV026 and *E. coli* [pSB401]. The purple violacein pigmentation or intensive bioluminescence were induced by *Burkholderia cepacia* strain C10B (Figure 4.18), *Citrobacter amalonaticus* strain L8A (Figure 4.19), *Pseudomonas aeruginosa* strain L10A (Figure 4.20), *Enterobacter* sp. strains R8E (Figure 4.21) and R2A (Figure 4.22) which indicate the presence of AHL molecules.

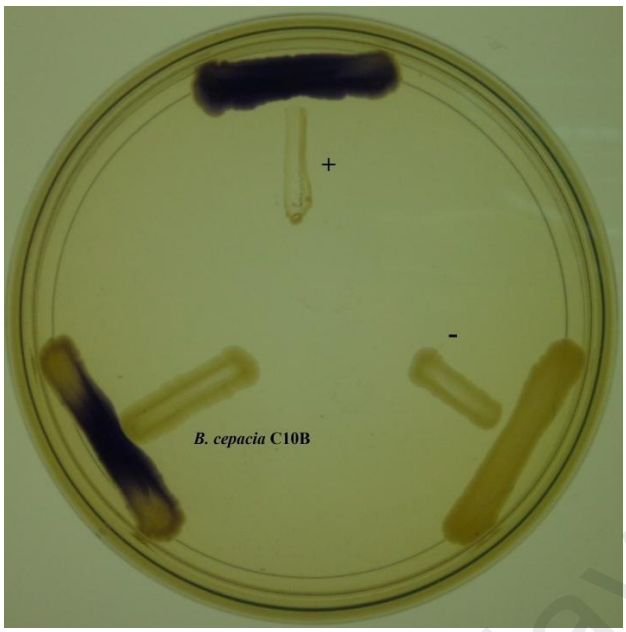

Figure 4.18: AHL screening of *B. cepacia* strain C10B with CV026. *E. carotovora* PNP22 (negative control "–") and *E. carotovora* GS101 (positive control "+") that can activate CV026 was included for comparison. Purple violacein pigmentation was induced by *B. cepacia* strain C10B.

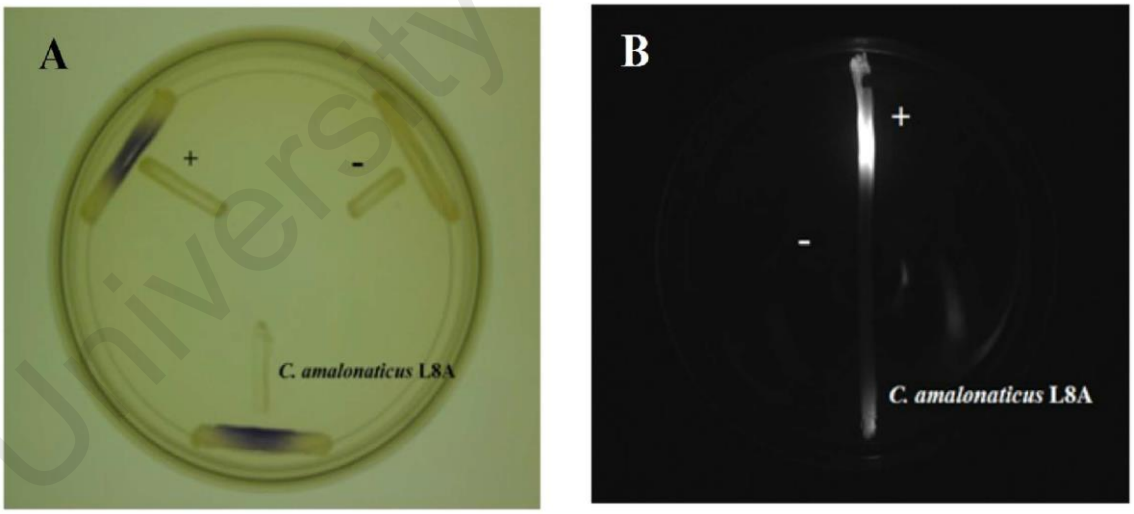

Figure 4.19: AHL screening of *C. amalonaticus* strain L8A with cross streak bioassay. **(A)** Short chain AHL screening of *C. amalonaticus* strain L8A with CV026. **(B)** AHL screening of *C. amalonaticus* strain L8A with *E. coli* [pSB401]. *E. carotovora* PNP22 (negative control "–") and *E. carotovora* GS101 (positive control "+") were included for comparison.

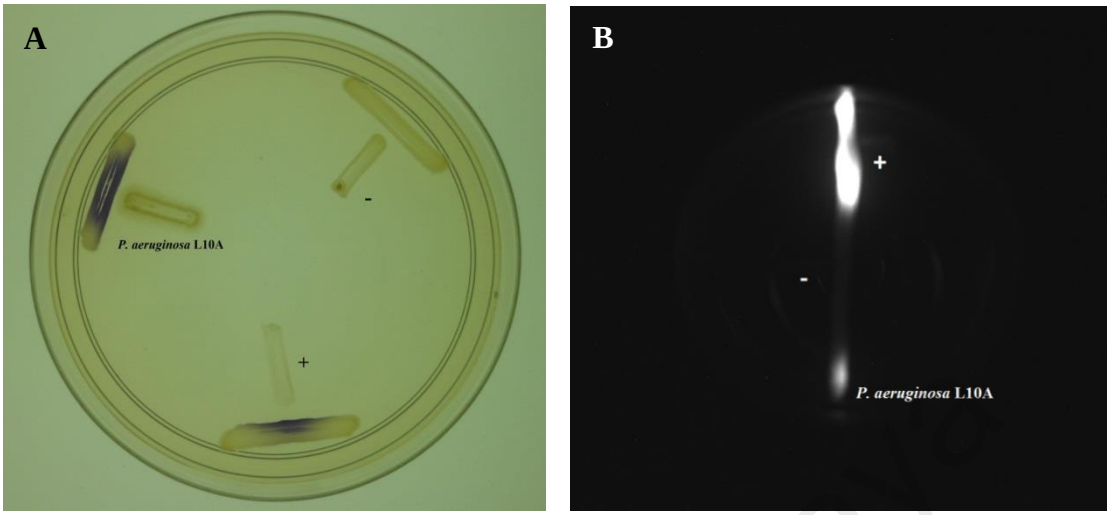

Figure 4.20: AHL screening of *P. aeruginosa* strain L10A with CV026 and *E. coli* [pSB401]. *E. carotovora* PNP22 (negative control "-") devoid of QS activity and *E. carotovora* GS101 (positive control "+") that can activate CV026 were included for comparison. **(A)** AHL screening with CV026. **(B)** AHL screening with *E. coli* [pSB401].

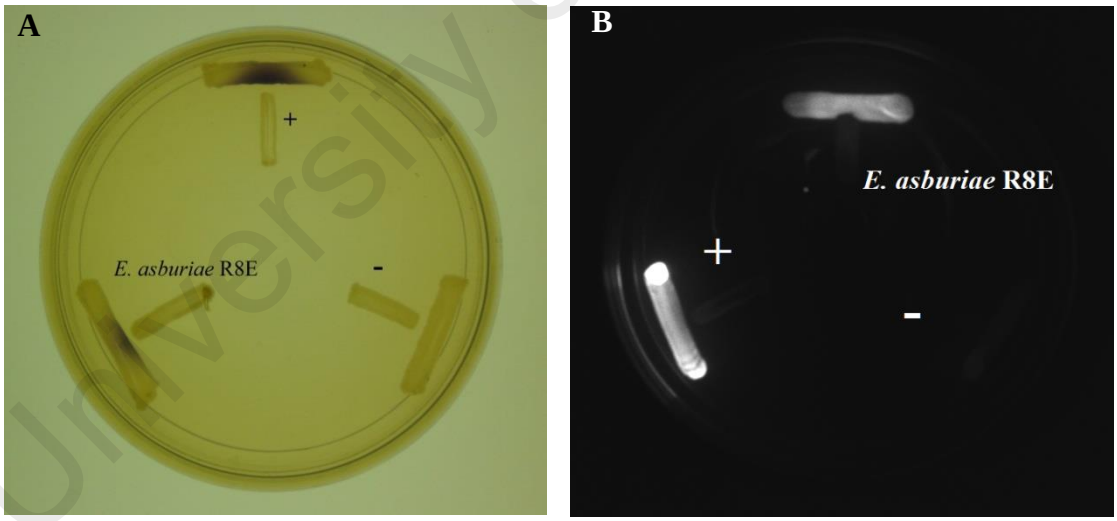

Figure 4.21: AHL screening of *Enterobacter* sp. strain R8E with cross-streak bioassay. **(A)** Screening with the CV026 bacterial biosensor. **(B)** Screening with *E. coli* [pSB401] AHL biosensor. *E. carotovora* PNP22 (negative control "–") and *E.* carotovora GS101 (positive control "+") were included for comparison**.**

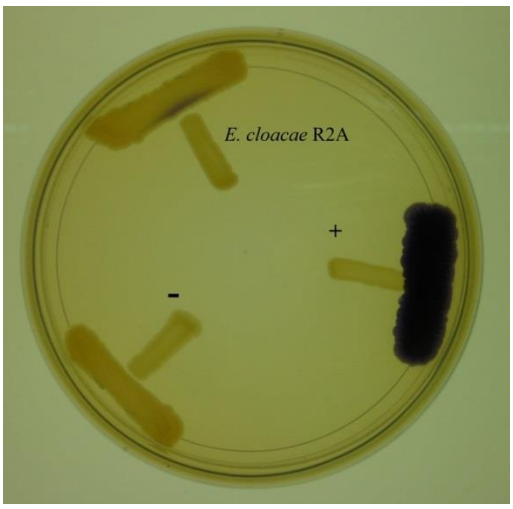

Figure 4.22: AHL screening of *Enterobacter* sp. strain R2A with CV026. *E. carotovora* PNP22 (negative control "-") devoid of QS activity and *E. carotovora* GS101 (positive control "+") that can activate CV026 were included for comparison.

4.2.2 AHLs Molecules Profiling by High-Resolution Triple Quadrupole Mass Spectrometry (LCMS/MS)

All the five strains with AHLs production were further investigated. The extracted AHL from spent supernatants of each triplicate samples were analysed by using the LCMS/MS technology. The extracted AHLs mass spectra were compared to the standard AHLs' mass-to-charge ratio (*m/z*) value and the corresponding retention time (Appendix 1). Using this approach, a number of different types of AHL signalling molecules were detected in each strain as summarised in Table 4.2.

Table 4.2: The detected AHL signalling molecules in each QS strain

Strain	AHLs (Relative abundance)	Figure (mass spectra)
B. cepacia strain C10B	C6-HSL (56.98), C8-HSL (100), C10-HSL (94.26), C12-HSL (100)	4.23
C. amalonaticus strain L8A	C4-HSL (29.17), C6-HSL (17.93), C8-HSL (14.84), C16-HSL (53.6)	4.24
P. aeruginosa strain L10A	C4-HSL (15), C6-HSL (57.98), C8-HSL (100), 3-oxo-C8-HSL (100), 3-oxo-C12-HSL (100)	4.25
Enterobacter sp. strain R8E	C4-HSL (37.7), C6-HSL (28.11), C8-HSL (31.57), 3-oxo-C12-HSL (35.65)	4.26
Enterobacter sp. strain R2A	C4-HSL (11.34), C12-HSL (100)	4.27

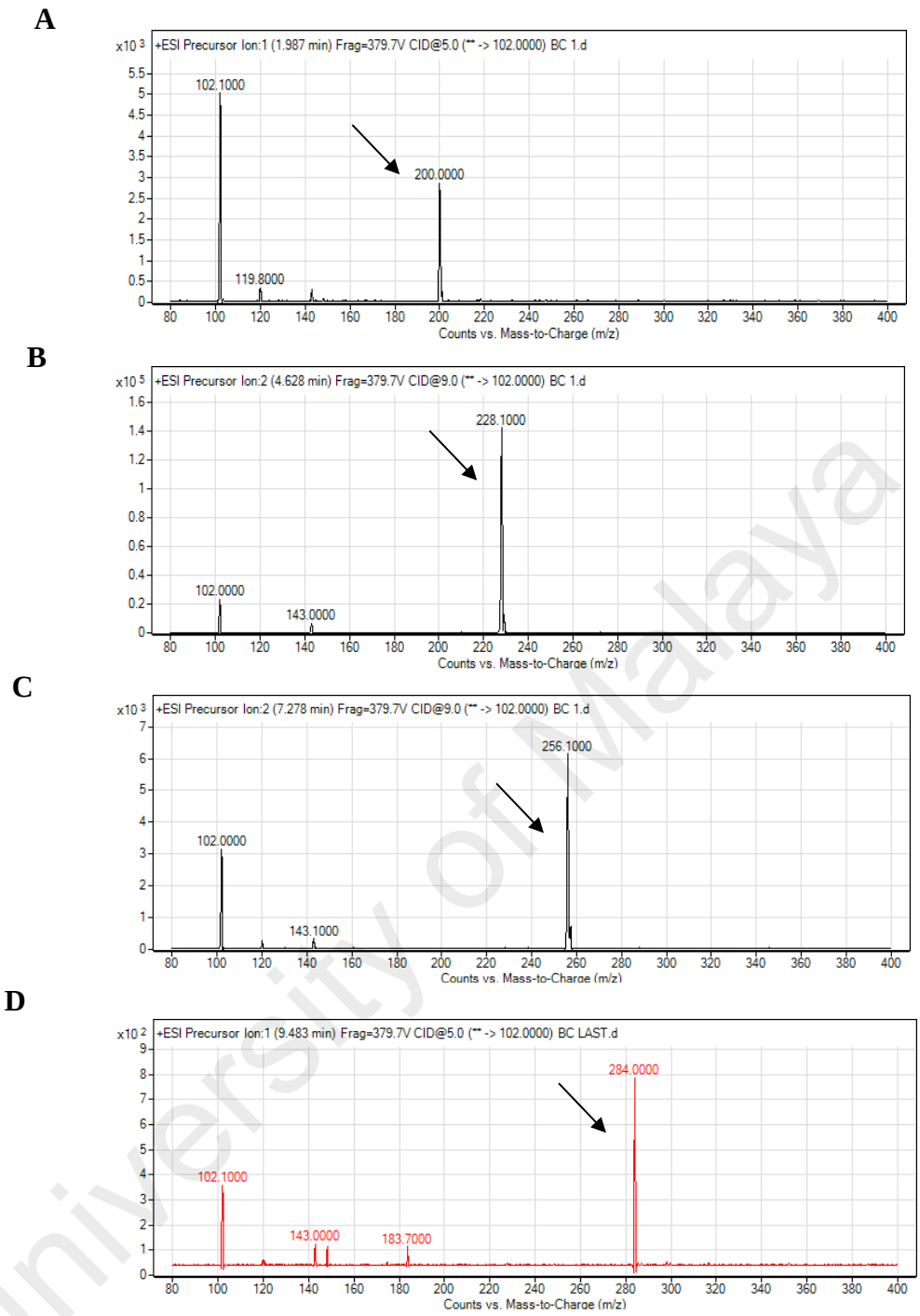

Figure 4.23: Mass spectrometry analysis of spent supernatant extract of *B. cepacia* strain C10B. All corresponding *m/z* for respective AHLs are marked by arrows; **(A):** mass spectrum of C6-HSL (*m/z* 200.0000), **(B):** mass spectrum of C8-HSL (*m/z* 228.1000), **(C):** mass spectrum of C10-HSL (*m/z* 256.1000), **(D):** mass spectrum of C12-HSL (*m/z* 284.1000).

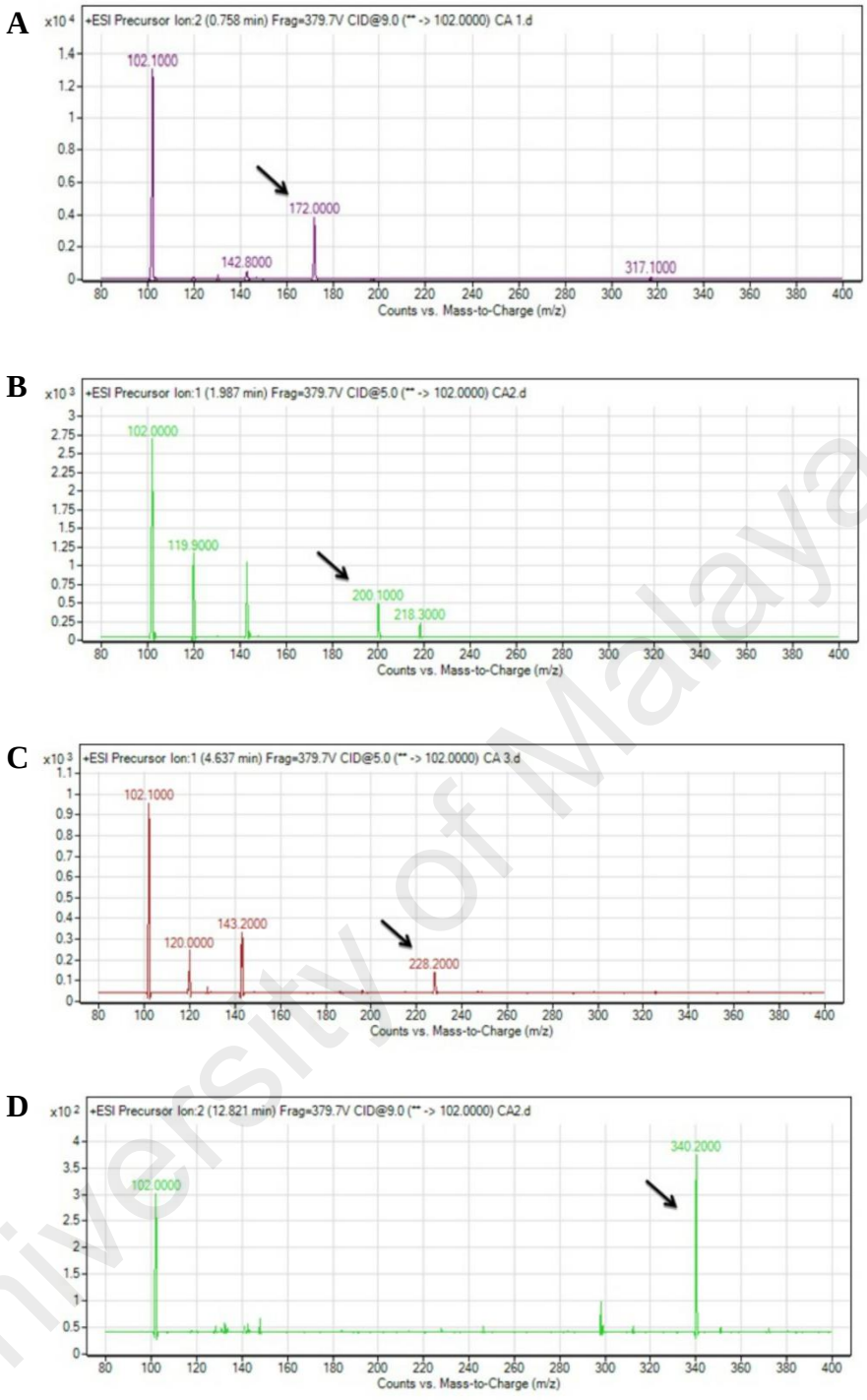

Figure 4.24: Mass spectrometry analysis of spent supernatants extract *C. amalonaticus* strain L8A. **(A)** Mass spectra of C4-HSL (*m/z* 172.0000). **(B)** Mass spectra of C6-HSL (*m/z* 200.3000). **(C)** Mass spectra of C8-HSL (*m/z* 228.2000). **(D)** Mass spectra of C16-HSL (*m/z* 340.0000).

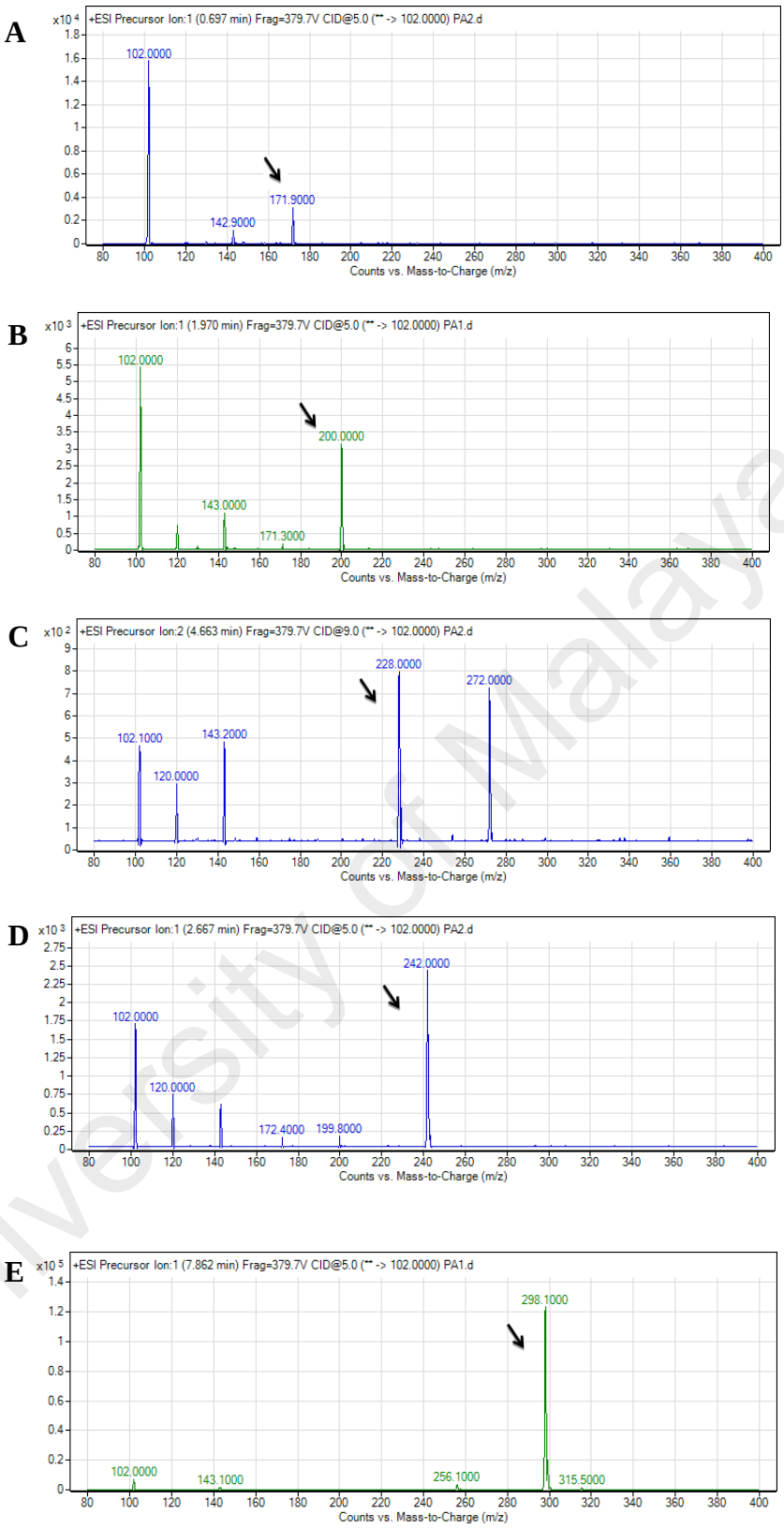

Figure 4.25: Mass spectrometry analysis of spent supernatants extracts *P. aeruginosa* strain L10A. **(A)** Mass spectra of C4-HSL (*m/z* 171.9000). **(B)** Mass spectra of C6-HSL (*m/z* 200.0000). **(C)** Mass spectra of C8-HSL (*m/z* 228.0000). **(D)** Mass spectra of 3-oxo-C8-HSL (*m/z* 242.0000). **(E)** Mass spectra of 3-oxo-C12-HSL (*m/z* 298.1000).

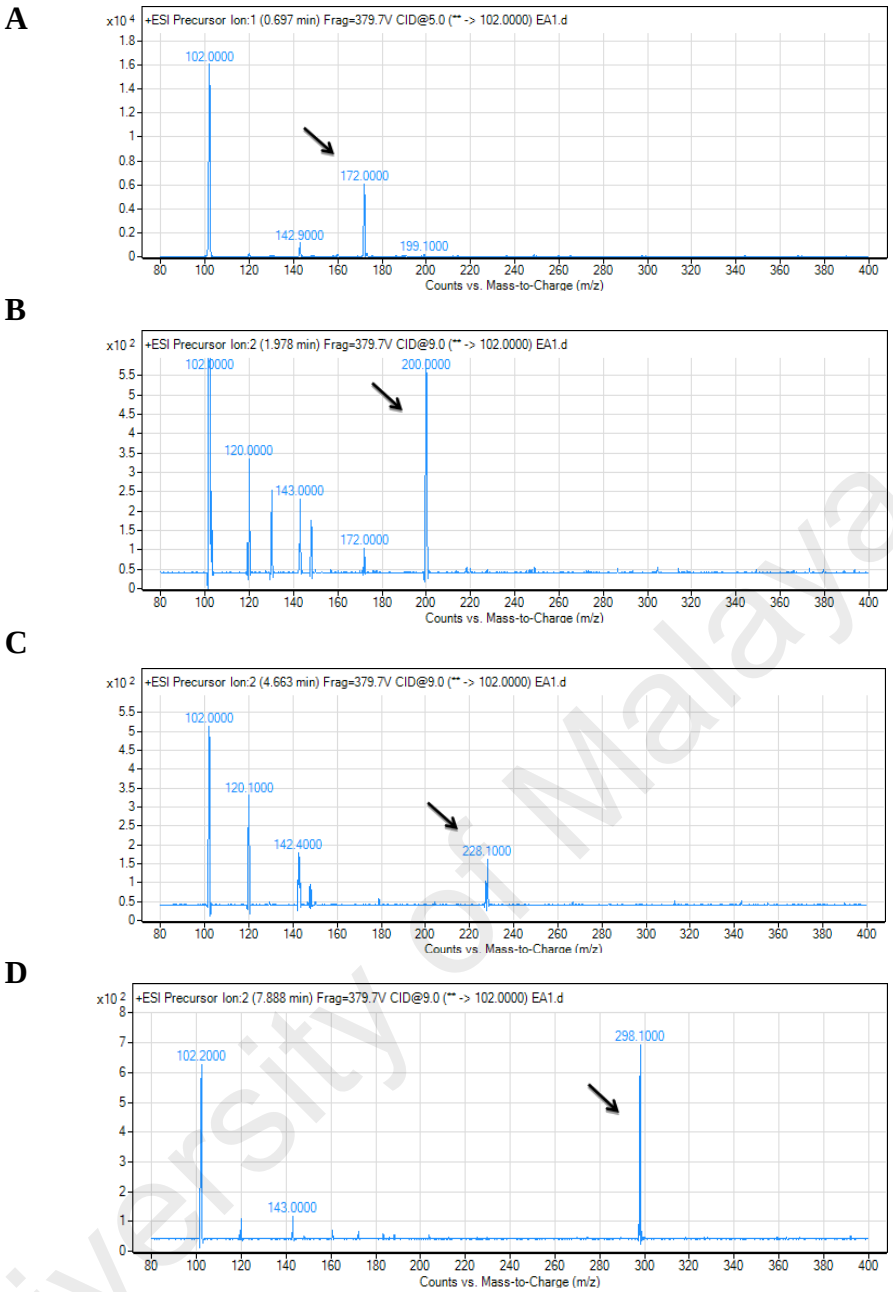

Figure 4.26: Mass spectrometry analysis of spent supernatants extracts *Enterobacter* sp. strain R8E. **(A)** Mass spectra of C4-HSL (*m/z* 171.9000). **(B)** Mass spectra of C6-HSL (*m/z* 200.0000). **(C)** Mass spectra of C8-HSL (*m/z* 228.0000). **(D)** Mass spectra of 3-oxo-C12-HSL (*m/z* 298.1000).

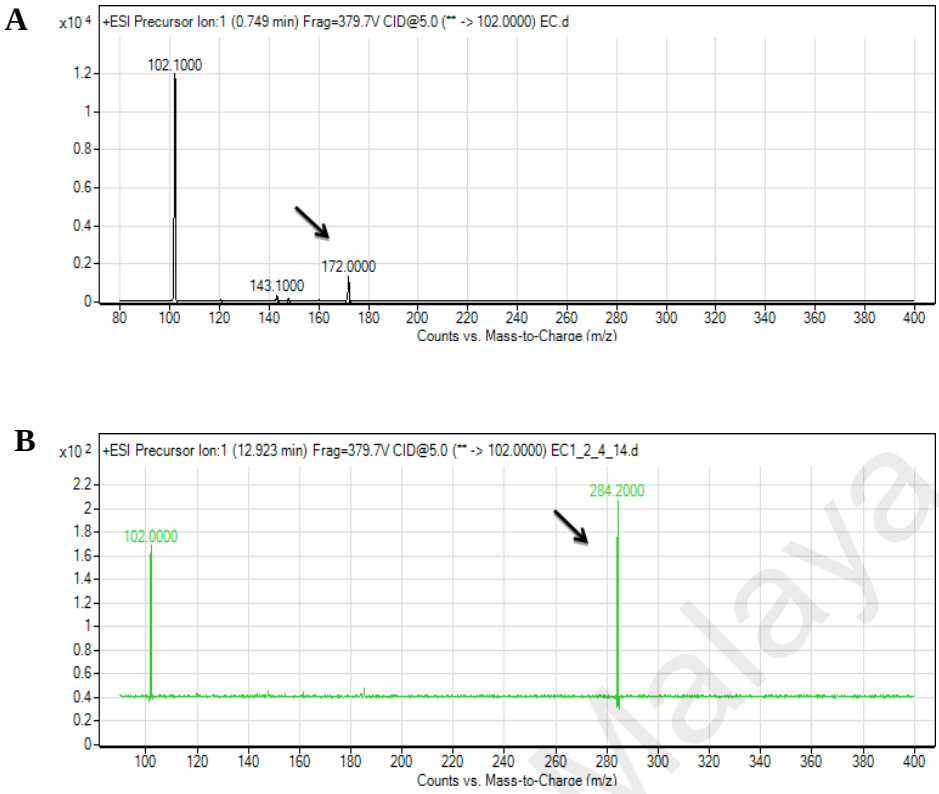

Figure 4.27: Mass spectrometry analysis of spent supernatants extracts *Enterobacter* sp. strain R2A. **(A)** Mass spectra of C4-HSL (*m/z* 172.0000). **(B)** Mass spectra of C12-HSL (*m/z* 284.2000).

4.3 Antibiotic Susceptibility Test

Oral isolate *Elizabethkingia* sp. strain B2D was selected for further study of the antimicrobial susceptibility. The Advanced Expert System (AES) proposed the quantitative method of antibiotic susceptibility of *Elizabethkingia* sp. strain B2D using VITEK 2 system (Table 4.3). The result from VITEK 2 system analysis depicted the *Elizabethkingia* sp. strain B2D showed resistance towards all beta-lactam antibiotics in the card tested. This strain also resistant to antibiotics that acted as folate antagonist namely trimethoprim/sulfamethoxazole (MIC ≥ 16). Besides, *Elizabethkingia* sp. strain B2D also displayed an antibiogram with resistance or intermediate susceptibility to

antibiotics that inhibit protein syntheses such as gentamicin (MIC ≥ 16) and tobramycin (MIC 2). However, the strain exhibited sensitivity to fluoroquinolones ciprofloxacin (MIC 0.5) but not levofloxacin (MIC ≥ 512).

Table 4.3: Antibiotic susceptibility of *Elizabethkingia* sp. strain B2D tested by VITEK 2 system and interpreted by AES.

VITEK 2 test cards	Antimicrobials	MIC (μg/ml)	AES Interpretation
AST-GN66	Ampicillin	≥ 32	R
	Ampicillin/Sulbactam	≥ 32	R
	Piperacillin/Tazobactam	≥ 128	R
	Cefazolin	≥ 64	R
	Cefoxitin	≥ 64	R
	Ceftazidime	≥ 64	R
	Ceftriaxone	≥ 64	R
	Cefepime	≥ 64	R
	Ertapenem	≥ 16	R
	Imipenem	≥ 16	R
	Gentamicin	≥ 16	R
	Tobramycin	2	I
	Ciprofloxacin	0.5	S
	Levofloxacin	≥ 512	R
	Nitrofurantoin	80	R
	Trimethoprim/Sulfamethoxazole	≥ 16	R

ND: not determined; R: resistant; I: intermediate; S: sensitive; ≥: greater than or equal to; <: less than.

4.4 Whole Genome Sequencing

Among the 21 oral isolates, ten of them were chosen for further genetic basic study of their whole genome sequences such as genome identification based on the 16S ribosomal RNA phylogenetic analyses, QS-related gene analyses and drugs resistant features.

4.4.1 Genome Analysis of Oral Bacterial Isolates

All the raw reads which had passed the quality control as stated in section 3.4.10.1 were filtered and processed prior to *de novo* genome assembly using CLC Genomics Workbench. The assembled draft genomes were submitted to an automated Rapid Annotation Subsystems Technology (RAST) server for genome annotation, using an E-value cutoff of $1e^{-10}$. Table 4.4 illustrated the genome overview of the oral isolates.

Table 4.4: Genome overview of oral isolates

Strain	Genome size (bp)	Average coverage (×)	GC content (%)	Number of contigs	Coding sequences	Number of RNA
C. amalonaticus strain L8A	5,273,145	40	54.4	110	4972	73
Enterobacter sp. strain R8E	5,604,168	83.5	55.1	146	5281	76
Enterobacter sp. strain R2A	5,639,670	47	55.1	168	5326	75
Elizabethkingia sp. strain B2D	3,936,249	121.8	35.5	50	3609	44
P. mirabilis strain T1C	4,060,112	50.8	38.5	60	3764	74
L. paracasei strain L9D	3,157,711	208	46.2	138	3147	56
P. gergoviae strain C7B	5,890,255	112.5	58.4	87	5497	85
Klebsiella sp. strain R8A	6,300,368	99	55.5	97	5906	76
C. koseri strain B1B	4,635,645	92	53.8	21	4340	79
S. maltophilia strain R5G	4,992,669	200.8	65.9	79	4446	77

4.4.2 16S ribosomal RNA (16S rRNA) Phylogenetic Analysis

The extracted 16S ribosomal RNA gene from whole genome sequences of each strain was subjected to phylogenetic analysis. The constructed maximum likelihood phylogenetic trees depicted the evolutionary relationship and closest organism to that strain. The 16S rRNA gene phylogram of strain L8A was closely related to *Citrobacter amalonaticus* and strain B1B related to *C. Koseri* (Figure 4.28), strain R8E and R2A related to *Enterobacter* sp. (Figure 4.29), strain B2D related to *Elizabethkingia anopheles* (Figure 4.30), strain T1C related to *Proteus mirabilis* (Figure 4.31), strain L9D related to *Lactobacillus paracasei* (Figure 4.32), strain C7B related to *Pluralibacter gergoviae* (Figure 4.33), strain R8A related to *Klebsiella oxytoca* (Figure 4.34) and strain R5G related to *Stenotrophomonas maltophilia* (Figure 4.35).

When the 16S rRNA gene of each oral isolate was searched (BLASTn) against the HOMD 16S rRNA reference sequences, the top three results returned the highest percentage identical were summarised in Table 4.5. Compared to the Human Oral Microbiome Database (HOMD) as part of Human Microbiome Project (HMP), most of the strains have not been reported to be associated with oral cavity, namely *C. amalonaticus* strain L8A, *C. koseri* strain B1B, *Elizabethkingia* sp. strain B2D, *P. gergoviae* strain C7B, *Klebsiella* sp. strain R8A, *Enterobacter* sp. strains R8E and R2A.

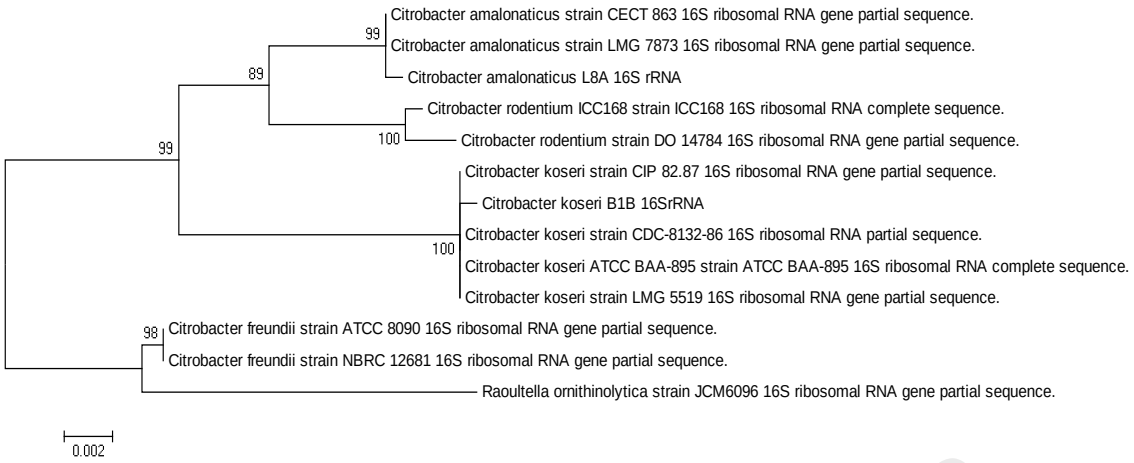

Figure 4.28: Maximum likelihood phylogenetic tree of strain L8A and strain B1B 16S rRNA sequence. The phylogenetic analysis of 16S rRNA gene of the strain L8A was hierarchically clustered under *C. amalonaticus* and strain B1B as *C. koseri* using MEGA 5.1.

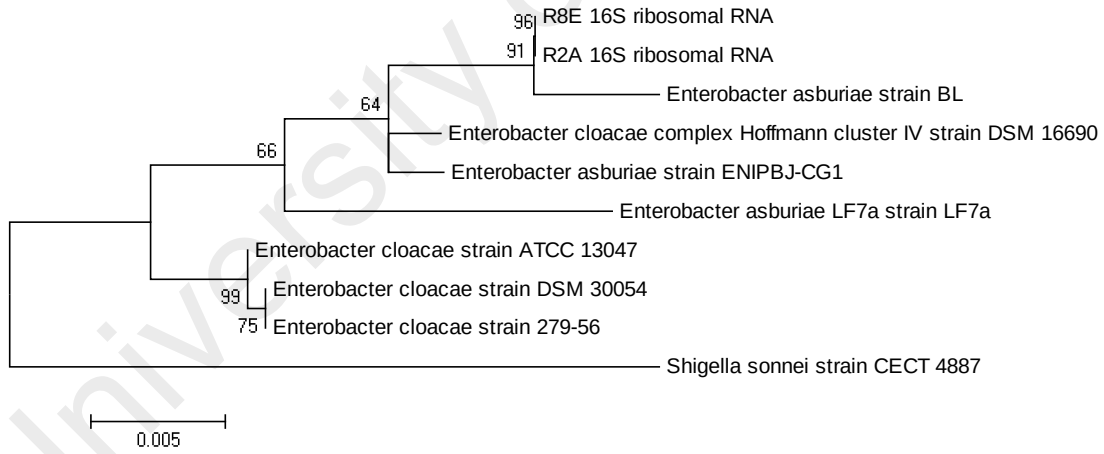

Figure 4.29: Maximum likelihood phylogenetic tree of strain R8E and strain R2A 16S rRNA sequences. The phylogenetic analysis of 16S rRNA gene of the strain R8E and strain R2A was hierarchically clustered under *Enterobacter* sp. using MEGA 5.1.

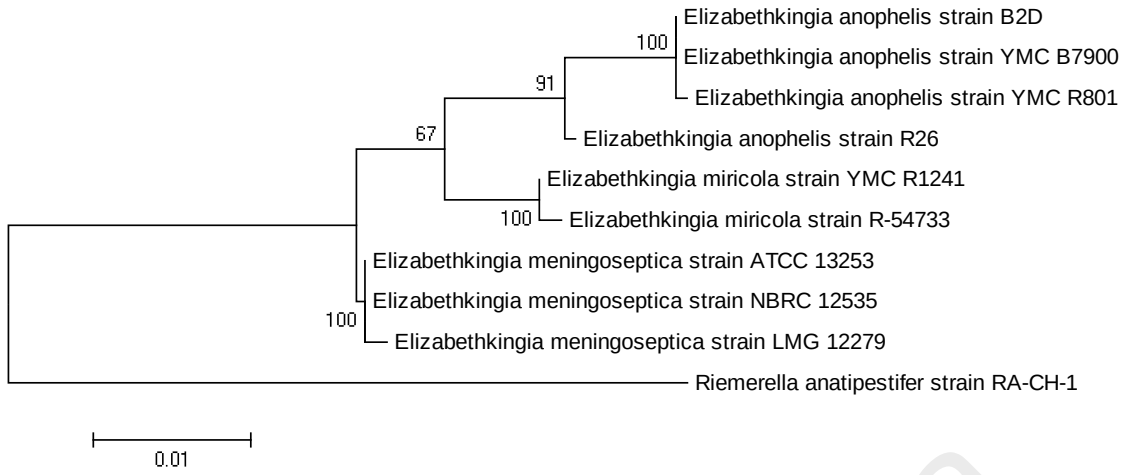

Figure 4.30: Maximum likelihood phylogenetic tree of strain B2D 16S rRNA sequence. The phylogenetic analysis of 16S rRNA gene of the strain B2D was hierarchically clustered under *E. anophelis* using MEGA 5.1.

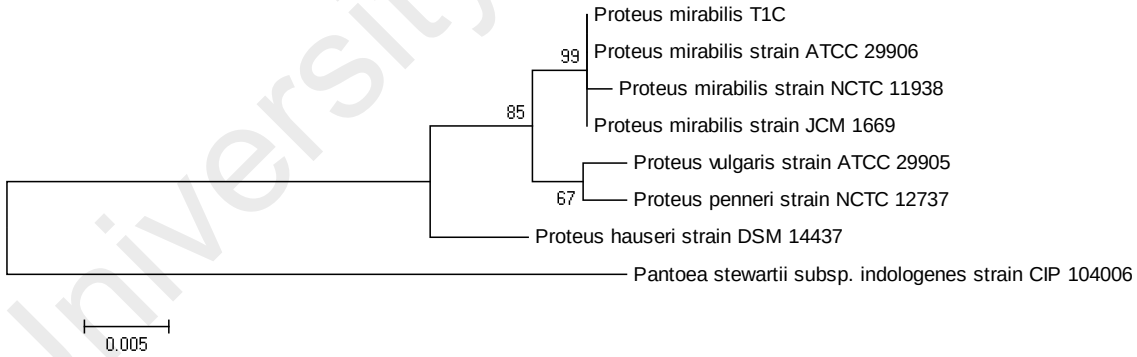

Figure 4.31: Maximum likelihood phylogenetic tree of strain T1C 16S rRNA sequence. The phylogenetic analysis of 16S rRNA gene of the strain T1C was hierarchically clustered under *P. mirabilis* using MEGA 5.1.

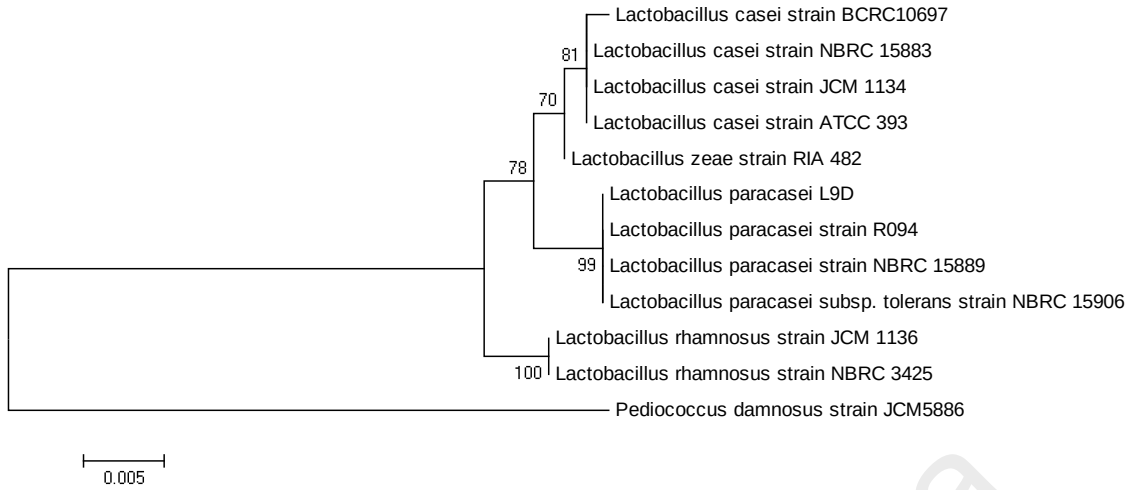

Figure 4.32: Maximum likelihood phylogenetic tree of strain L9D 16S rRNA sequence. The phylogenetic analysis of 16S rRNA gene of the strain L9D was hierarchically clustered under *L. paracasei* using MEGA 5.1.

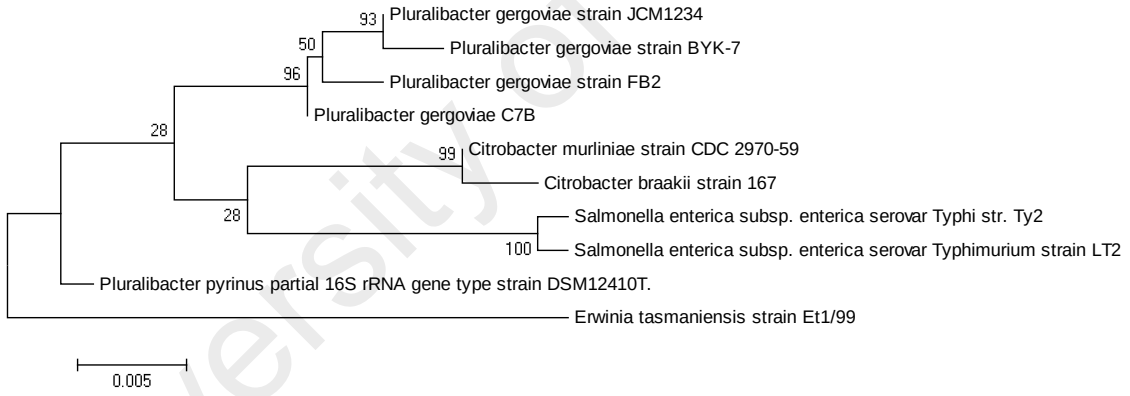

Figure 4.33: Maximum likelihood phylogenetic tree of strain C7B 16S rRNA sequence. The phylogenetic analysis of 16S rRNA gene of the strain C7B was hierarchically clustered under *P. gergoviae* using MEGA 5.1.

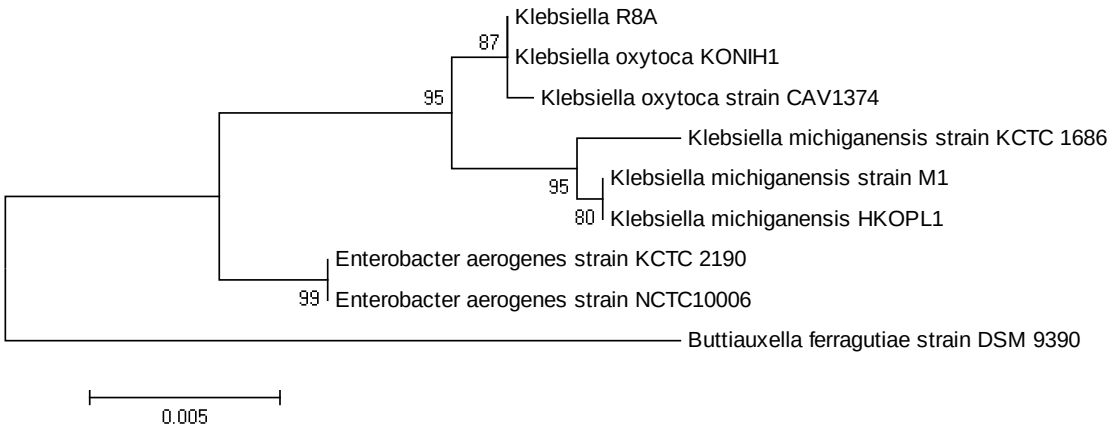

Figure 4.34: Maximum likelihood phylogenetic tree of strain R8A 16S rRNA sequence. The phylogenetic analysis of 16S rRNA gene of the strain R5G was hierarchically clustered under *K. oxytoca* using MEGA 5.1.

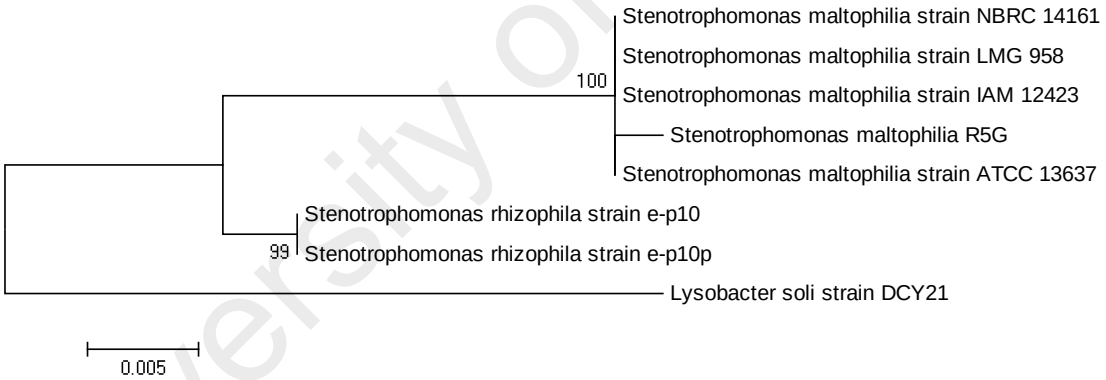

Figure 4.35: Maximum likelihood phylogenetic tree of strain R5G 16S rRNA sequence. The phylogenetic analysis of 16S rRNA gene of the strain R5G was hierarchically clustered under *S. maltophilia* using MEGA 5.1.

Table 4.5: HOMD 16S rRNA sequence identification of oral isolates

Oral Isolate	HOMD Identification	Accession	Identities (%)
L8A	*Enterobacter hormaechei* DSMZ 16691	GB_AJ853890	97.7
	Kluyvera ascorbata F0526	GB_tbd	97.5
	Escherichia coli	GB_M25588	97.4
B1B	*Escherichia coli*	GB_M25588	98.2
	Cronobacter sakazakii JCM1233	GB_AB004746	97.3
	Enterobacter hormaechei DSMZ 16691	GB_AJ853890	97.2
B2D	*Bergeyella* sp. clone C4MKM119	GB_AY278614	93.2
	Bergeyella sp. clone ncd369e02c1	GB_HM320564	92.9
	Bergeyella sp. clone AK152	GB_AY008691	92
R8E	*Enterobacter cancerogenus* LMG 2693	GB_Z96078	98.5
	Enterobacter hormaechei DSMZ 16691	GB_AJ853890	98.3
	Klebsiella pneumonia ATCC 13884	GB_Y17657	97.6
R2A	*Enterobacter cancerogenus* LMG 2693	GB_Z96078	98.5
	Enterobacter hormaechei DSMZ 16691	GB_AJ853890	98.3
	Klebsiella pneumonia ATCC 13884	GB_Y17657	97.6
R8A	*Enterobacter cancerogenus* LMG 2693	GB_Z96078	99
	Klebsiella pneumonia ATCC 13884	GB_Y17657	98.6
	Enterobacter hormaechei DSMZ 16691	GB_AJ853890	98.4
C7B	*Enterobacter hormaechei* DSMZ 16691	GB_AJ853890	98.2
	Enterobacter cancerogenus LMG 2693	GB_Z96078	97.7
	Escherichia coli	GB_M25588	97.4
T1C	*Proteus mirabilis* ATCC 29906	GB_AF008582	100
	Enterobacter hormaechei DSMZ 16691	GB_AJ853890	93.8
	Cronobacter sakazakii JCM1233	GB_AB004746	93.8
L9D	*Lactobacillus paracasei* JCM 8130	GB_D79212	100
	Lactobacillus casei JCM 1136	GB_D16552	98.8
	Lactobacillus rhamnosus F11	GB_AF243146	98.6
R5G	*Stenotrophomonas maltophilia* LMG 958	GB_X95923	99.7
	Stenotrophomonas nitritireducens clone AY088	GB_AF385546	97.1
	Pseudomonas pseudoalcaligenes LMG 1225	GB_Z76666	87.4

4.4.3 Nucleotide Sequence Accession Number

The whole genome sequences of oral isolates were deposited at DDBJ/EMBL/GenBank as shown in Table 4.3. A new quality control test for submitted bacterial genomes was performed by NCBI using average nucleotide identity (ANI) (Federhen et al., 2016). The suggested and assigned strain identification is listed in Table 4.6. Compared to NCBI ANI analysis, the identification of strains R8E, R2A and R8A were different from the MALDI-TOF MS and 16S ribosomal RNA sequence analyses.

Table 4.6: Assigned nucleotide sequence accession number of oral isolates

Strain	Identification	Accession number
L8A	*Citrobacter amalonaticus*	JMQQ00000000
R8E	*Enterobacter cloacae* complex 'Hoffmann cluster IV'	MBTX00000000
R2A	*Enterobacter cloacae* complex 'Hoffmann cluster IV'	MBMT00000000
B2D	*Elizabethkingia anophelis*	JNCG00000000
T1C	*Proteus mirabilis*	MJAS00000000
L9D	*Lactobacillus paracasei*	MBTZ00000000
C7B	*Pluralibacter gergoviae*	MBGD00000000
R8A	*Klebsiella michiganensis*	JNCH00000000
B1B	*Citrobacter koseri*	MBTW00000000
R5G	*Stenotrophomonas maltophilia*	MJAS00000000

4.4.4 QS Gene Relatedness Analysis

The QS relatedness genes of *Citrobacter amalonaticus* strain L8A, *Enterobacter* sp. strains R8E and R2A were further studied through the phylogenetic analysis. The putative *N*-acyl homoserine lactone synthase gene of each strain was determined and matched against the nr database prior to computing the relationship phylogram. Figure 4.36 indicates that the putative genes of strain R8E and strain R2A which were grouped with *N*-acyl homoserine lactone synthase gene of other *Enterobacter* spp. The maximum likelihood phylogenetic tree in Figure 4.37 represented the *in silico* study of QS synthase gene extracted from whole genome sequences of *C. amalonaticus* strain L8A. The 212 bp of the putative protein-coding gene of *C. amalonaticus* strain L8A *N*-acyl homoserine lactone synthase was grouped with *C. farmeri* GTC1319 QS synthase gene.

In order to understand the principle of AHL-mediated QS system in *C. amalonaticus* strain L8A, the synteny analysis of its annotated QS features were depicted in Figure 4.38 using Easyfig. The genome feature of QS *N*-acyl homoserine lactone synthase gene and QS transcriptional activator gene were found in contig 12. LuxI/LuxR functional pair of *C. amalonaticus* strain L8A was paired adjacently.

In addition to QS homologous gene analysis in *C. amalonaticus* strain L8A, the association between the AHL synthase related terms (signal transduction, cell-cell signaling involved in quorum sensing, interspecies quorum sensing, quorum sensing involved in interaction with host, transferase activity and *N*-acyl homoserine lactone synthase activity) and gene products were represented in the comparison chart (Figure 4.39). Under the biological process, the implication of cell-cell signalling and interspecies cell-cell communication, as well as the interaction between bacteria and the host were predicted to occur in the subclasses of QS activity in *C. amalonaticus* strain L8A which was a type of homeostasis utilised by a number of free-living cells within a population

such as oral microbiome. Under the subclasses of molecular function, *N*-acyl homoserine lactone synthase activity in *C. amalonaticus* strain L8A was simulated and found to be related to transferase activity. The occurrence of this transferase activity in oral isolate *C. amalonaticus* strain L8A involved in catalysis of transferring acyl group to the nitrogen atom in AHL.

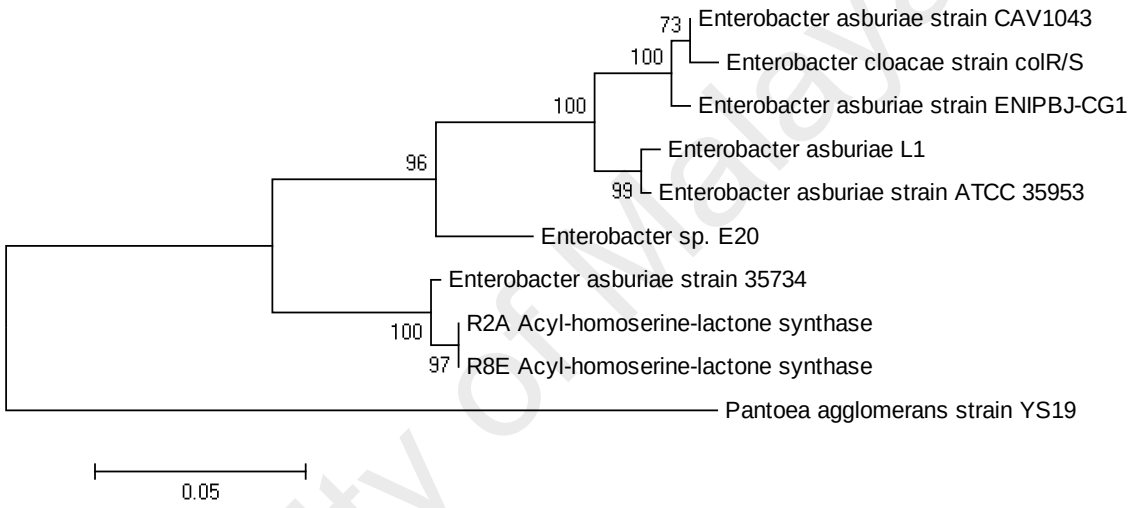

Figure 4.36: Phylogenetic tree based on QS synthase gene in *Enterobacter* sp. strains R8E and R2A. *In silico* study of QS synthase gene of genome strain, *Enterobacter* sp. strains R8E and R2A were determined and represented in maximum likelihood phylogenetic tree.

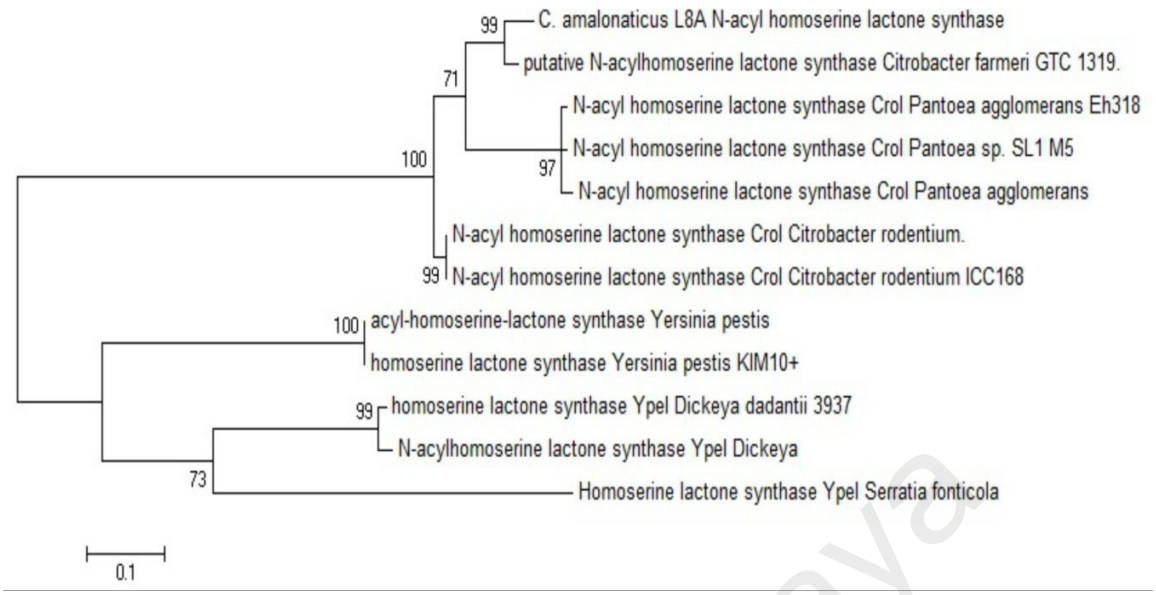

Figure 4.37: Phylogenetic tree based on QS synthase gene in *C. amalonaticus* strain L8A. *In silico* study of QS synthase gene of genome *C. amalonaticus* strain L8A was determined and represented in maximum likelihood phylogenetic tree.

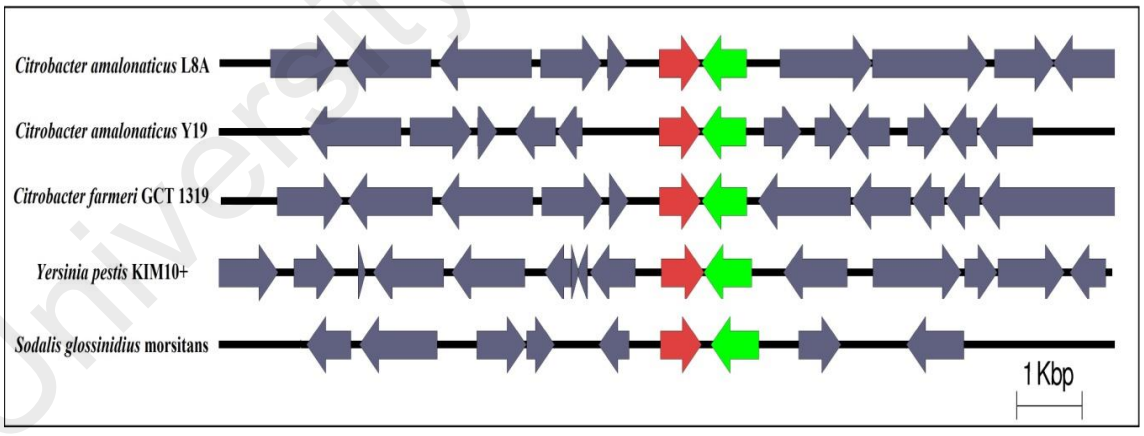

Figure 4.38: QS synthase gene and regulatory gene in the genome sequence of *C. amalonaticus* strain L8A. QS genes of *C. amalonaticus* strain L8A were synteny analysed using Easyfig (red arrow indicated the QS synthase gene and green arrow indicated the QS transcriptional regulator gene).

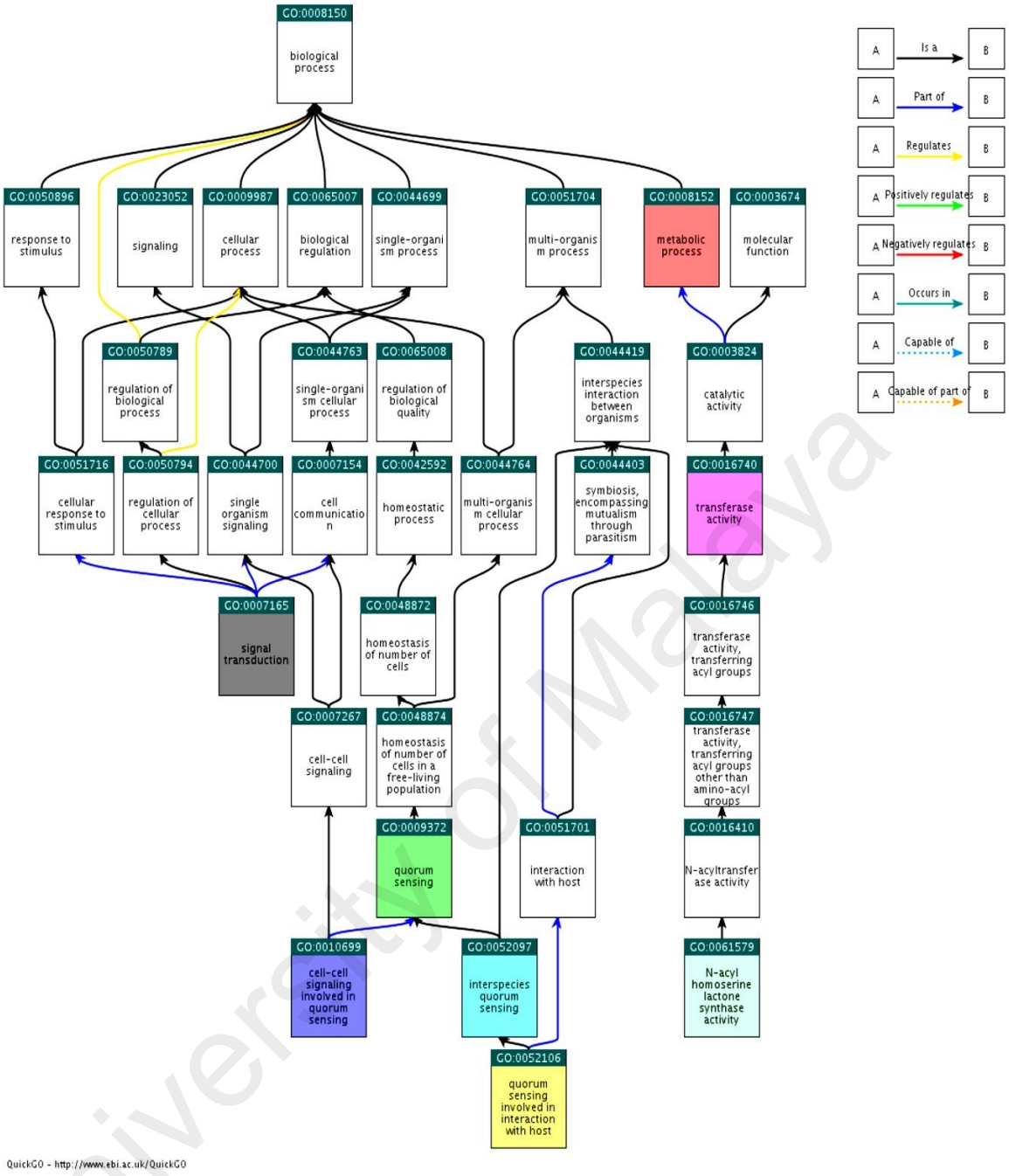

Figure 4.39: Comparison chart of eight QS-associated GO terms (showing in different highlighted colour codes) in the genome of *C. amalonaticus* strain L8A using QuickGO. Different relationships between the terms are indicated by the unique colour of arrows as depicted in the right panel.

4.4.5 *In silico* Annotation of Antibiotic Resistance Genes

The whole genome sequences of *Elizabethkingia* sp. strain B2D and *P. gergoviae* strain C7B were selected for the further study of the aspect of the drug resistant gene. The 3609 CDSs predicted by RAST in the whole genome sequences of *Elizabethkingia* sp. strain B2D were divided into subsystems in Figure 4.40 and most of the genes were assigned for nutrient metabolism. The predicted 5497 DNA coding sequences in the whole genome sequence of *P. gergoviae* strain C7B were distributed and shown in Figure 4.41.

From the automated RAST annotation system, 72 gene counts were assigned to subsystem resistance to antibiotics and toxic compounds (Figure 4.42) in *Elizabethkingia* sp. strain B2D. Among these counts, drug-resistance genes including vancomycin (n=1), resistance to fluoroquinolones (n=4), beta-lactamases (n=15) and multidrug resistance efflux pumps (n=12) have been predicted. In *P. gergoviae* strain C7B, a total of 115 genes were assigned to this subsystem (Figure 4.43).

In addition to the whole genome sequence of *Elizabethkingia* sp. strain B2D, four clusters of genes conferring to putative multidrug resistant efflux pump systems had been predicted by RAST. All the genes were further studied by PSI-BLAST against NCBI database. The results showed that all the gene clusters were components of the Resistance-Nodulation-Division (RND) efflux pumps.

Using SRST2 approach, antimicrobial resistance genes in genome *Elizabethkingia* sp. strain B2D were identified, including the three *bla* genes encoding for the extended-spectrum serine-β-lactamase CME (class D) and two metallo-β-lactamases, BlaB (subclass B1) and GOB (subclass B3). To further confirm the annotation, genomic assembled sequences of *Elizabethkingia* sp. strain B2D were local searched against ARG-ANNOT and NCBI database. These three *bla* genes were found in three different contigs as represented in Figure 4.44.

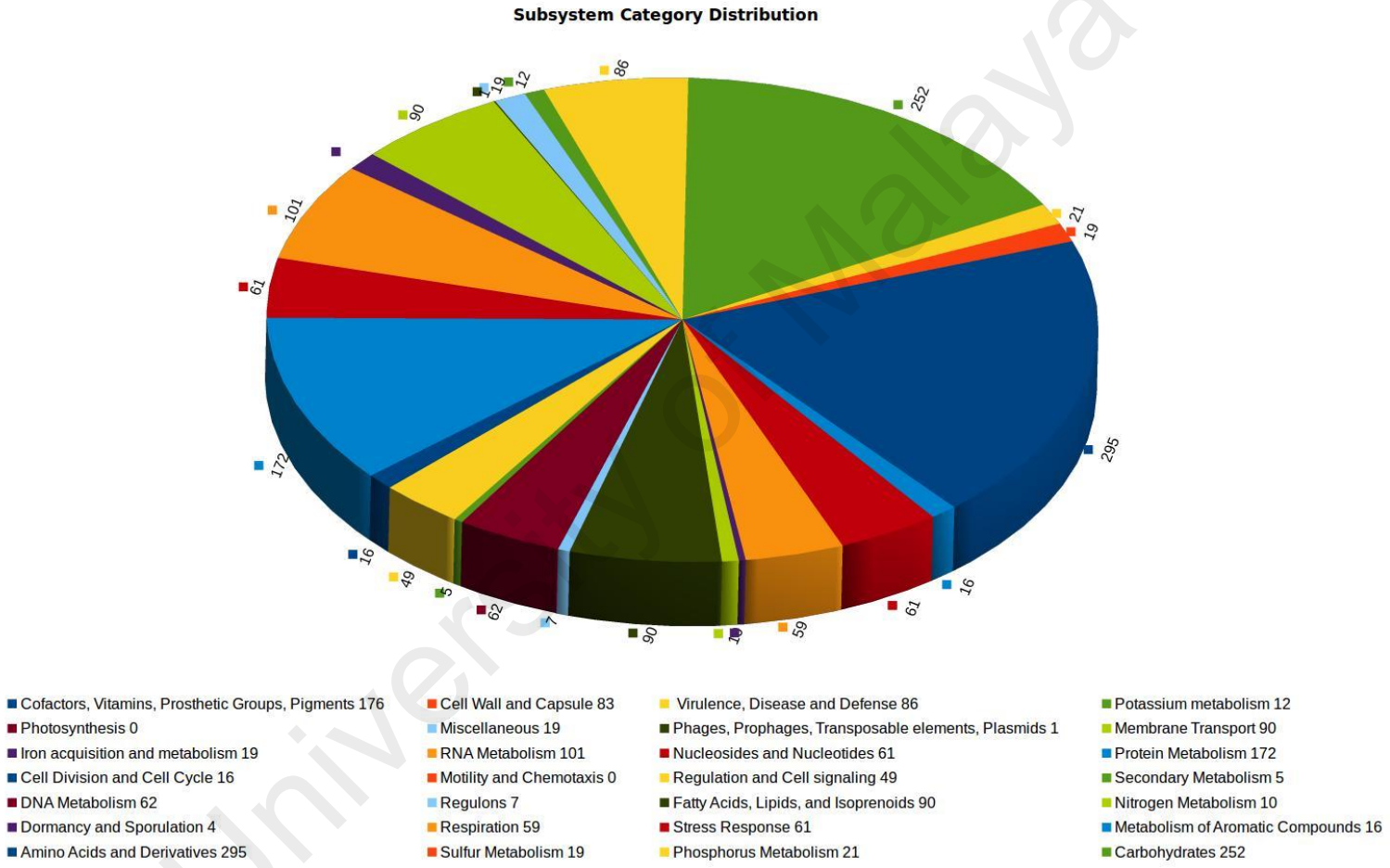

Figure 4.40: Subsystem category distribution of *Elizabethkingia* sp. strain B2D genome. The whole genome sequence was annotated and the subsystem distribution was generated from RAST.

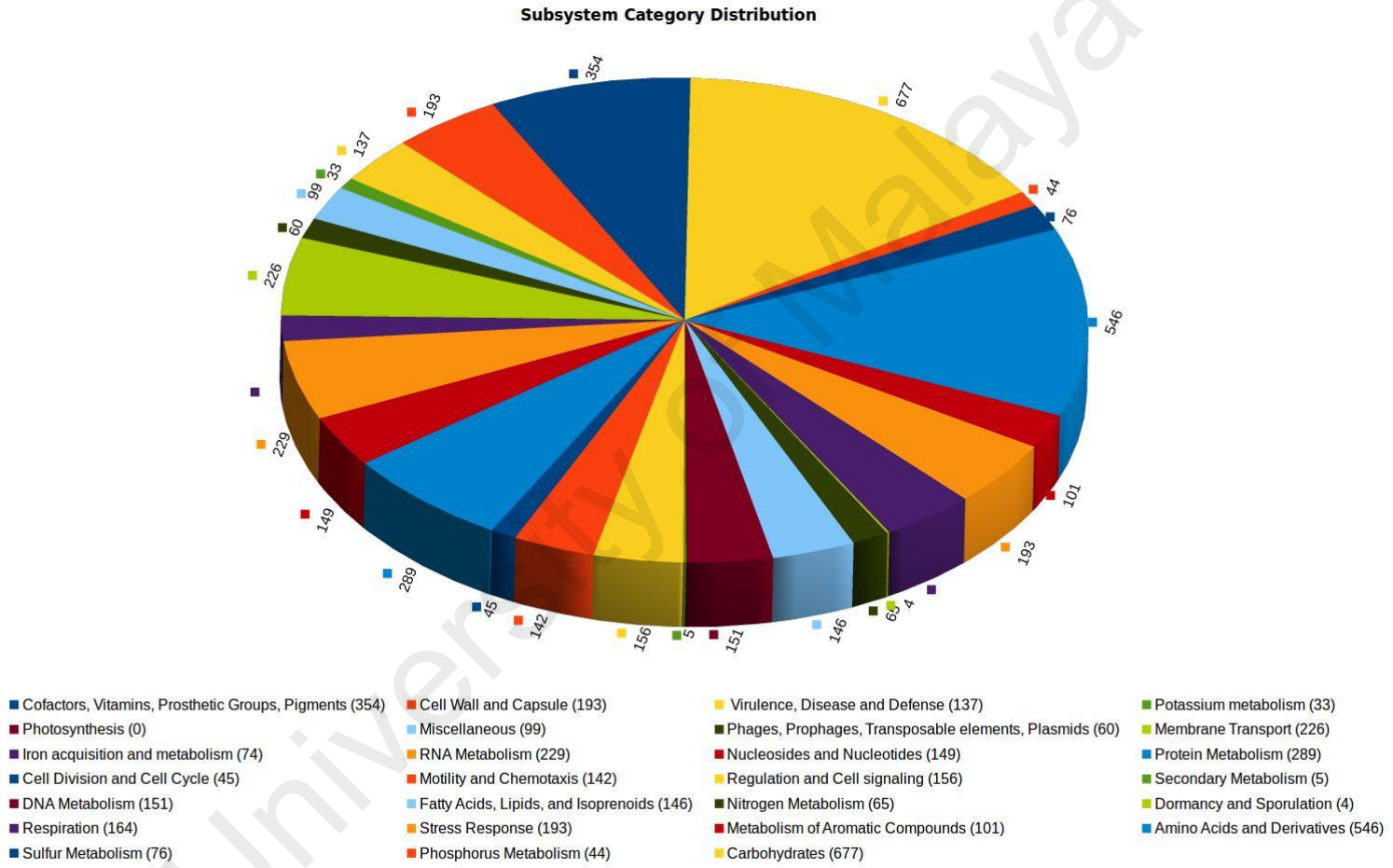

Figure 4.41: Subsystem category distribution of *P. gergoviae* strain C7B genome. The whole genome sequence of *P. gergoviae* strain C7B was annotated and the subsystem distribution was generated from RAST.

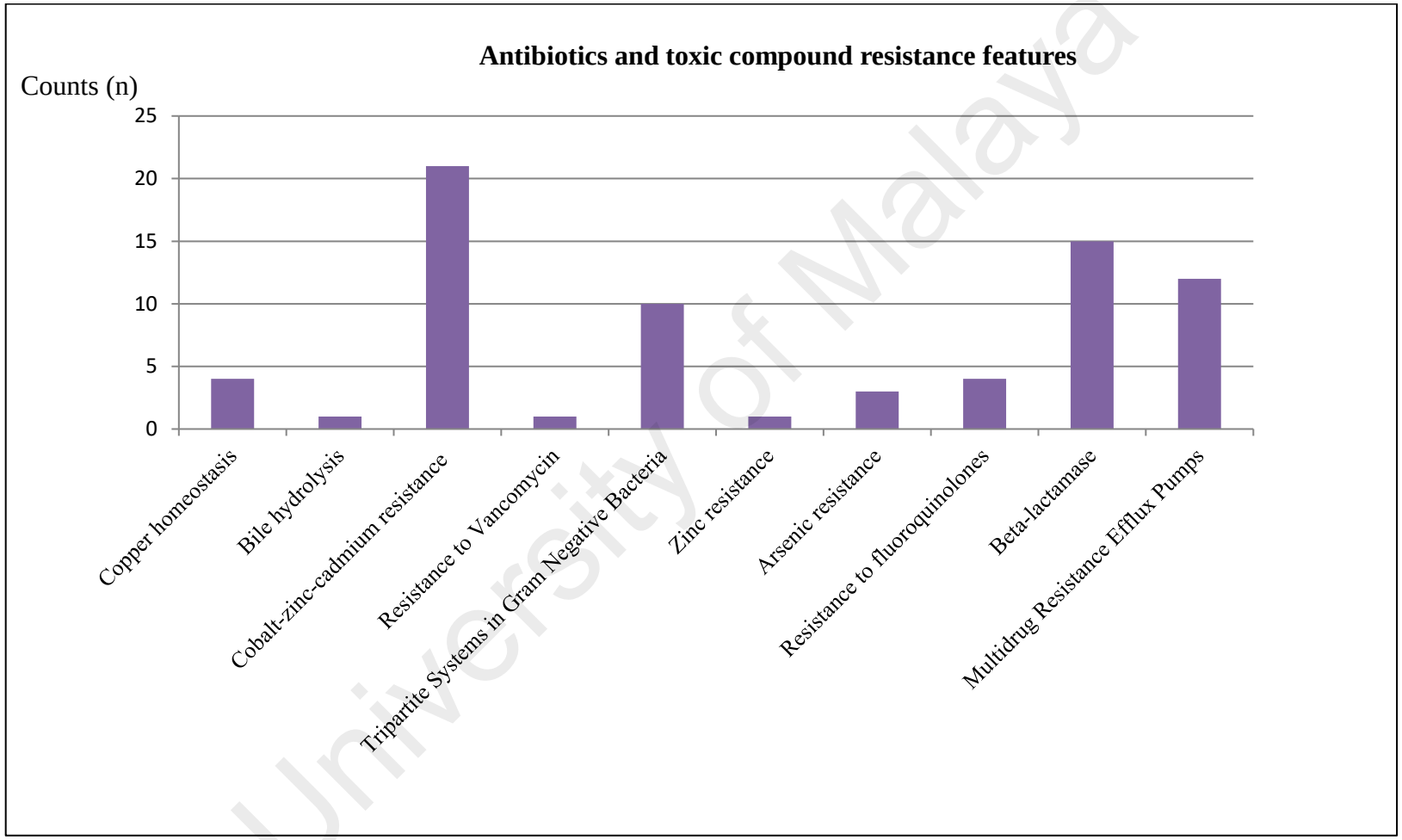

Figure 4.42: Features of antibiotics and toxic compound resistance in *Elizabethkingia* sp. strain B2D. A total of 72 gene counts were predicted by RAST and assigned to subsystem resistance to antibiotics and toxic compounds.

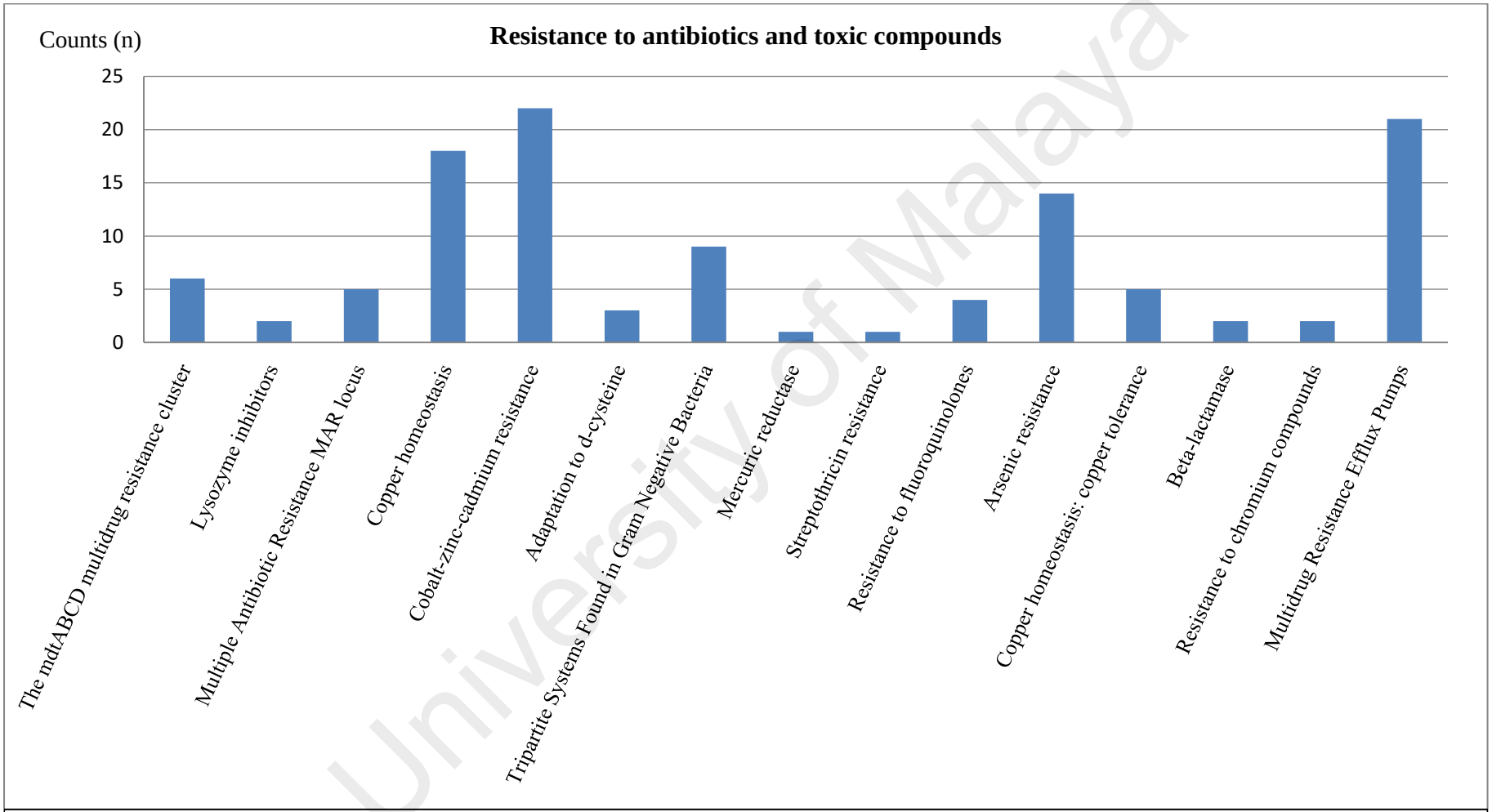

Figure 4.43: Features of antibiotics and toxic compound resistance in *P. gergoviae* strain C7B. A total of 115 genes were predicted by RAST and assigned to subsystem resistance to antibiotics and toxic compounds.

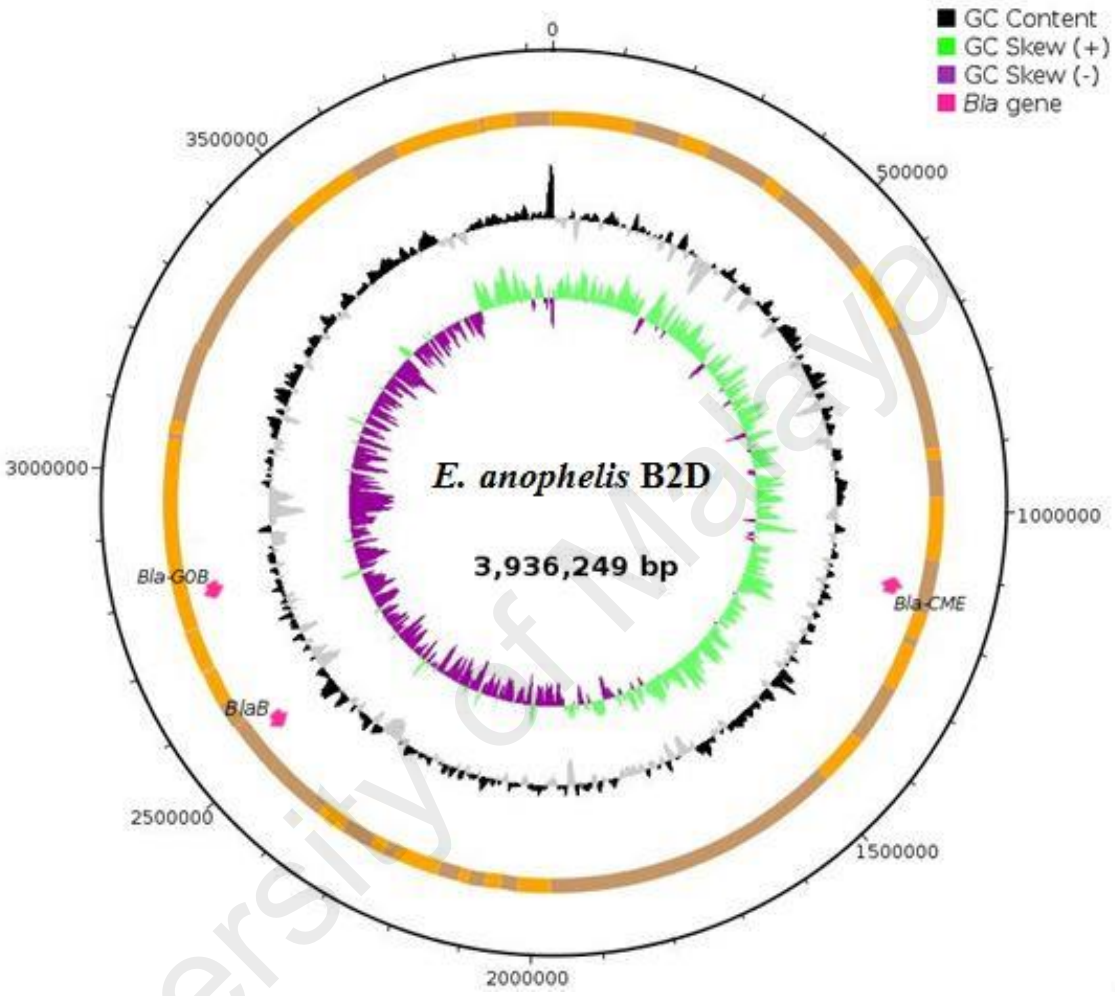

Figure 4.43: Overview of whole genome sequences *Elizabethkingia* sp. strain B2D with three identified *bla* genes. Innermost rings show GC skew (purple/green) and GC content (black/grey). The outermost ring shows *Elizabethkingia* sp. strain B2D genome (orange). *Bla* genes were in the antisense direction.

4.4.6 Comparative Genome Studies of *C. amalonaticus* strain L8A

The comparative genome analysis was performed on *C. amalonaticus* strain L8A. To date, there are only seven whole genome sequences publicly available from NCBI database include whole genome sequence of *C. amalonaticus* strain L8A. Genome comparison chart of these seven *C. amalonaticus* genomes with clinical isolates (n = 3), environmental (n = 2), food (n = 1) and industrial (n = 1) was constructed using BRIG (Figure 4.45) in circular visualization. BRIG uses BLAST to compare the pairwise genome sequence and converts the results coupling with customized feature information or analysis information into publication quality image. Among the seven genome sequences, six were draft genomes and only one was complete genome which was used as reference genome in this study. The output image shows differences and similarity between the query genome and reference genome, where BLAST matches are indicated in the form of coloured sliding scale at the side. The average genome size was 5.139 Mb with GC content of 53.35%.

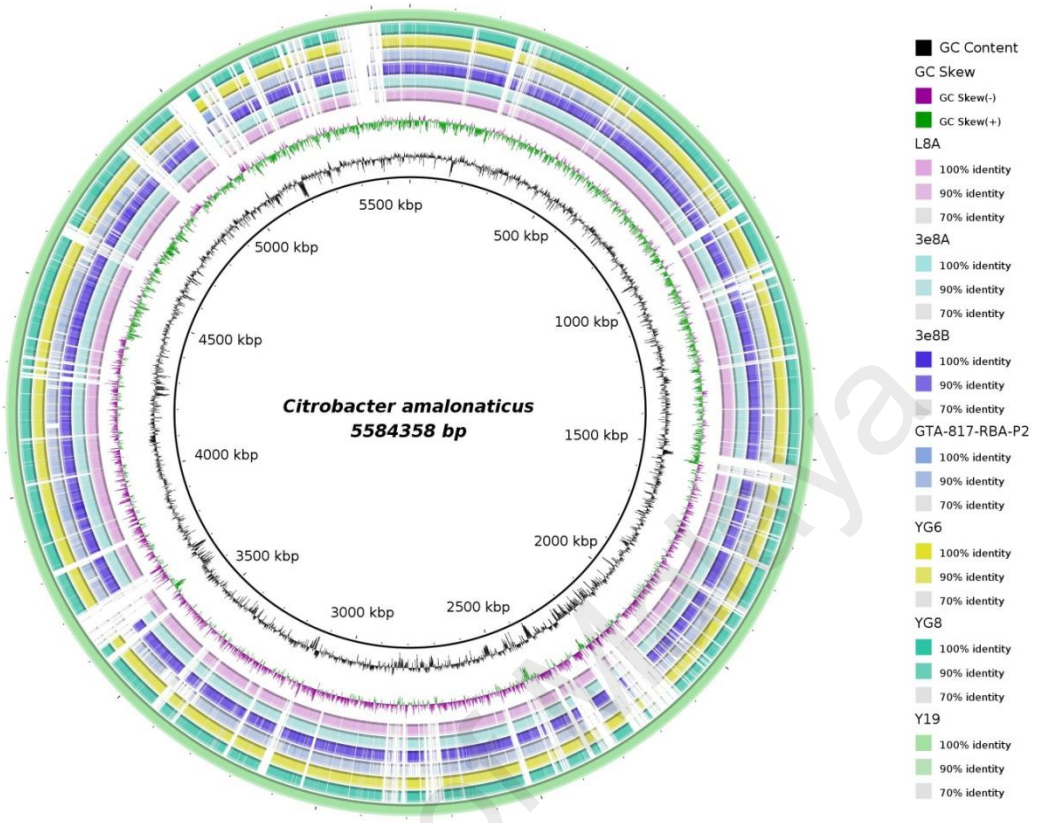

Figure 4.44: BRIG visualisation of multiple *Citrobacter amalonaticus* genomes available from NCBI database. The innermost rings show GC skew (purple/green) and GC content (black). The third innermost ring shows *C. amalonaticus* strain L8A genome and followed by *C. amalonaticus* strains 3e8A (CDQV01) and 3e8B (CDQX01) isolated from undernourished Malawian children's gut. The remaining rings represent the ground beef isolate *C. amalonaticus* strain GTA-817-RBA-P2 (LAMY01) and environmental *C. amalonaticus* strains YG6 (LIGA01) and YG8 (LIGB01) prior the outermost ring with complete genome of *C. amalonaticus* strain Y19.

CHAPTER 5: DISCUSSION

5.1 Oral Isolates and Identification

Dentine caries has a polymicrobial etiology and is a significant public health problem to human beings in order to ameliorate health caries, maintain oral health and expectancy life (Du et al., 2009; Preza et al., 2008). As its name implied, dentine caries took place in dentine layer and was caused by supragingival dental plaque (Chen et al., 2015). However, thorough understandings of the effect of microbiological factors remain elusive due to the limitation knowledge in their structure, functioning and ecosystem complexity of microbial communities.

The primary gateway to our body is through oral cavity where it is the desired habitat for microbial proliferation (Dewhirst et al., 2010). The microorganisms that inhabit in the oral cavity are capable of passing through the circulatory system and spread to different body sites. In addition, oral microbes could be transmitted into the external environment via saliva droplets from speaking, coughing, sneezing and breathing (Leão-Vasconcelos et al., 2015). Hence, the oral cavity is a vital site for research in order to understand the carriers in public health settings.

Oral bacterial genomes study using culture-dependent methods coupling with next generation sequencing and advanced computational approaches has made it possible to comprehensively elucidate the putative pathogens and probiotics in the oral cavity. Cultivation and isolation of bacterial strains on selective culture media in this study showed the variance bacteria colonized in dentine caries and dental plaque. Bacteria were isolated based on their diverse morphologies such as the colour of the bacterial colony, shape, size, surface appearance, margin, opacity and so on.

MALDI-TOF MS serves as a reliable method that provides high throughput for the classification and identification of microorganisms in a shorter turnover time (Dworzanski & Snyder, 2005; Fountoulakis, 2001). MALDI-TOF MS can support rapid and it is a promising means to accurately identify microorganisms. A number of researchers reported the feasibility of MALDI-TOF MS analysis was able to classify and differentiate clinical isolates to species level (Cherkaoui et al., 2010; Nagy et al., 2009). MALDI-TOF MS was used to identify the bacterial strains rapidly and resulted a total of 21 different species were identified from 13 genera namely, *Acinetobacter*, *Burkholderia*, *Candida*, *Citrobacter*, *Elizabethkingia*, *Enterobacter*, *Enterococcus*, *Klebsiella*, *Lactobacillus*, *Proteus*, *Pseudomonas*, *Stenotrophomonas* and *Streptococcus*. More variable community membership was observed in dentine caries than that in dental plaque. This result indicated that the bacteria composition in dental plaque is less diverse than in dentine caries.

Based on the rapid identification results from MALDI-TOF MS, strains of interest like oral isolate with QS characteristic or which is uncommonness to the oral cavity, *C. amalonaticus* strain L8A, *Enterobacter* spp. strain R8E and R2A, *Elizabethkingia* sp. strain B2D, *P. mirabilis* strain T1C, *L. paracasei* strain L9D, *P. gergoviae* strain C7B, *Klebsiella* sp. strain R8A, *C. koseri* strain B1B, *S. maltophilia* strain R5G were selected for next generation sequencing. A better understanding of genome profiling in oral isolates was gained from their draft genome sequences.

Through the downstream analysis of genome sequences, the 16S rRNA gene sequences of strain B2D exhibited 99 % nucleotide identities to *E. anophelis* type strain R26^{T} (GenBank accession no. EF426425) and 95 % –96 % nucleotide identities to those of *E. meningoseptica* strains deposited in GenBank. Furthermore, based on ANI analysis performed by NCBI, some of the strains were misidentified at species level by using

MALDI-TOF MS identification tool which included strain B2D to *Elizabethkingia anophelis*, strain R8A to *Klebsiella michiganensis*, strain R8E and strain R2A to *Enterobacter cloacae* complex 'Hoffmann cluster IV'.

Compared to Human Oral Microbiome Database (HOMD), numerous strains were isolated and identified as uncommon to the human oral cavity which included *C. amalonaticus* strain L8A, *C. koseri* strain B1B, *Elizabethkingia* sp. strain B2D, *P. gergoviae* strain C7B, *Klebsiella* sp. strain R8A, *Enterobacter* sp. strains R8E and R2A. Among these bacterial species, *C. amalonaticus*, *C. koseri*, *P. gergoviae*, *K. oxytoca*, *E. cloacae*, *E. sakasaki*, *E. aerogenes* were reported in previous studies (Leão-Vasconcelos et al., 2015; Prado-Palos et al., 2011). Hence, for the first time, the isolation of *Elizabethkingia* sp. in oral cavity was disclosed here.

Elizabethkingia is phylogenetically close to *Chryseobacterium*, *Riemerella* and *Flavobacterium* genera. *E. meningoseptica* was previously named as *Flavobacterium meningosepticum* and then *Chryseobacterium meningosepticum* prior to transfer into a new genus *Elizabethkingia* together with *C. miricola* when revealed genetically heterogeneous by 16S rRNA gene analysis from members of *Chryseobacterium*. The new genus was named in the honour of Elizabeth O. King who first described the bacteria in a case of infant meningitis (Holmes, 1987; Kim et al., 2005). After a systematic reclassification in 2005, *Elizabethkingia* is now represented by four species, included *Elizabethkingia miricola* (Li et al., 2003), *Elizabethkingia meningoseptica* (Brody et al., 1958; King, 1959), *Elizabethkingia anophelis* (Kämpfer et al., 2011), and *Elizabethkingia endophytica* (Kämpfer et al., 2015).

Genus *Elizabethkingia* is an aerobic Gram-negative microorganism that commonly found in the environment with its rod-shaped, non-motile and non-spore-forming properties. In 2011, *E. anophelis* was firstly discovered by Kämpfer and colleagues from the midgut of the mosquito *Anopheles gambiae* as its name implied (Kämpfer et al., 2011). This bacterium rarely causes illness in human and uncommon to be isolated in the respiratory tract. *E. anophelis* has not been described as a commensal microorganism or opportunistic pathogen. It is an emerging microbe related to neonatal meningitis, bloodstream and respiratory infections in immunocompromised patients and nosocomial outbreaks. The first documentation reported the bacterial meningitis was in 2013 which occurred in the Central African Republic (Frank et al., 2013) and a nosocomial outbreak in an intensive care unit in Singapore (Teo et al., 2013). Perinatal vertical transmission of *E. anopheles* from a mother to her neonate was studied in Hong Kong since 2012 (Lau et al., 2015). However, the possibility of mosquitoes or other sources plays a vital role in transmission and maintenance of *E. anophelis* remain an uncertainty, as well as the pivotal role of *Elizabethkingia* sp. playing in the oral cavity, is yet to be investigated.

Burkholderia cepacia is an opportunistic pathogen that primarily infects immunocompromised patients with cystic fibrosis (CF), chronic granulomatous disease, burns or patients with indwelling devices and cancer (Lewenza & Sokol, 2001). Members of *Burkholderia* are ubiquitous and can be found in many different environmental habitats, ranging from water, soil, water, plants and animals to humans. It has been described that *B. cepacia* can be isolated from oral cavities in newborns (Uehara et al., 2001) and sputa from CF patients (Bittar et al., 2008) but none reports that *B. cepacia* isolates from dentine caries in a healthy adult.

Similar to *B. cepacia*, *Pseudomonas aeruginosa* also an opportunistic pathogen that often infects patients with immunocompromised conditions with cystic fibrosis, cancers or burns (Worlitzsch et al., 2002). *P. aeruginosa* is environmental ubiquitous and epidermal infections often result from this pathogen infiltrating through a human host's first line of defences. Transmission of *P. aeruginosa* could be broad such as via host of fomites, vectors and hospital personnel that could be potential carriers (da Silva Filho et al., 2007). Colonization of *P. aeruginosa* in the oral cavity is the common occurrence such as the isolation of *P. aeruginosa* strain L10A from dentine caries in this study.

Over a century, *Lactobacillus acidophilus* and *Streptococcus mutans* have been implicated in dental caries. The members of *Streptococcus* and *Lactobacillus* are acidogenic organisms that can produce acid rapidly and able to survive at acidic pH environment (Van Houte et al., 1996). They produce intra and extracellular polysaccharides and polymers to facilitate them for adherence. These acidogenic bacteria regulate the overall capabilities of the microflora environment and create a possibility for a mixed bacterial system residing in the low pH environment (Kleinberg, 2002). This explains the isolation of *S. pneumonia, S. salivarius, L. paracasei* and *L. pentosus* in the human oral cavity.

The oral cavity can serve as a potential reservoir for *Enterobacteriaceae*, which can spread to susceptible individuals and surrounding environment through saliva. This fact becomes a matter of grave concern when considering the hospital environment due to *Enterobacteriaceae* infections may take place. *Klebsiella* is a genus of non-motile, rod-shaped, Gram-negative bacteria that belongs to the family *Enterobacteriaceae*. This genus consists of seven species: *K. pneumoniae, K. azaenae, K. rhinoscleromatis, K. oxytoca, K. planticola, K. terrigena,* and *K. ornithinolytica*, of which *K. pneumonia* and *K. oxytoca* are clinically more pronounced than the others (Fraser et al., 2016).

K. oxytoca is an opportunistic pathogen that can survive in the environment as well as skin surfaces of humans and animals (Fraser et al., 2016). In fact, many of *K. oxytoca* outbreak sources have been found to be the environment, including catheters, medications, and equipment in medical facilities (Jeong et al., 2001; Lowe, 2012; Zárate et al., 2008). *K. oxytoca* has also been reported as one of the most common pathogens that infect patients in neonatal intensive care units, causing neonatal bacteremia (Fraser et al., 2016). It has also been found to be associated with hemorrhagic colitis (Högenauer et al., 2006). Nevertheless, a new member of *Klebsiella*, *K. michiganensis* was described relatively recently which was isolated from a toothbrush holder and is closely related by sequence comparison to *Klebsiella oxytoca* (Saha et al., 2013). The related studies about these species in the oral cavity are deficient. For the first time, my data suggest that the existence of *K. michiganensis* in dental plaque, and thus, warrant more attention.

Citrobacter sp. is an opportunistic pathogen that infrequent infects neonates and in immunocompromised adults or older children (Holt et al., 1994) who constantly follow the prophylactic β-lactam therapy (Underwood & Avison, 2004). Members of *Citrobacter* genus are currently divided into 11 species (Doran, 1999). They are genotypically and biochemically diverse. The finding of *C. amalonaticus* and *C. koseri* in oral cavity agrees with Leão-Vasconcelos and colleagues who found similar results with additional *C. freundii* oral isolate (Leão-Vasconcelos et al., 2015).

Enterobacter spp. are a Gram-negative, facultatively anaerobic, rod-shaped, non-spore-forming bacteria of the family Enterobacteriaceae. They cause various chronic bacterial infections in the healthcare environment due to their high rates of antimicrobial resistance such as cefoxitin, colistin and aminoglycosides (Bouza & Cercenado, 2002; Paterson, 2006; Saurina et al., 2000). The genus of *Enterobacter* was created in 1960 in order to reclassify the strains which previously were inappropriately identified such as

Aerobacter aerogenes and *Aerobacter cloacae*, also known as "Cloaca B" and "Cloaca A" respectively (Hormaeche & Edwards, 1960). The taxonomy of *Enterobacter* has a long and confusing history, with several transfers of species from genus to genus over the past 20 years. Phenotypic identification of the *Enterobacter* spp. are usually problematic and not reliable. Thus, rendering the identification of *Enterobacter* spp. through MALDI-TOF MS solely is a challenging task, which explains the vague identification of *Enterobacter* sp. strains R8E and R2A in the present work.

Enterobacter gergoviae was reclassified as *Pluralibacter gergoviae* after taxonomic evaluation of the genus *Enterobacter* based on multilocus sequence analysis (MLSA), proposed by Brady and colleagues (Brady et al., 2013). This Gram-negative straight rod shape bacterium can be isolated from sewage, human and animal faeces samples, soil, water, water and cosmetic products (Chan et al., 2014; Périamé & Davin-Regli, 2014). It is recognized as a factor of common nosocomial infections of the blood, respiratory tract and urinary tract of a human, which arising from the contamination of medical devices and personnel (Hammami et al., 2012). *Enterobacter* spp. also, have been associated with antibiotic-resistant outbreaks in hospitals due to the presence of inducible β-lactamases (Sanders & Sanders, 1988). The outbreak of this type of bacterium infection in Malaysia is rare. The first documentation was reported in 2003, which happened in a neonatal intensive care unit of a general hospital and 11 babies were infected (Ganeswire et al., 2003). Hence, the presence of *P. gergoviae* strain C7B in the oral cavity constitutes a risk factor for infections such as bacteremia.

Lastly, the present finding demonstrated that the occurrence of *P. mirabilis* was determined in dentine caries of patients studied. In agreement with previous investigations, the detection of *P. mirabilis* on the colonization of dental plaque independent elderly (Sumi et al., 2007) and the saliva of health workers in a school

hospital (Prado-Palos et al., 2011) were revealed. *P. mirabilis* has fascinated microbiologists for over a century since 1885 (Hauser, 1885). This Gram-negative rod-shaped pathogen is commonly encountered in clinical specimens especially in the intestinal tracts of humans and cause urinary tract infection (Coker et al., 2000). It produces urease which hydrolyzes urea to ammonia and carbon dioxide, led to the urine more alkaline. The prolonged increased alkalinity of urine might initiate the developing of kidney calculus formation and result in renal failure (Jones & Mobley, 1989). In the aspect of treatment, this bacterium is susceptible to a wide range of antibiotics but intrinsically resistant to tetracycline and nitrofurantoin (Endimiani et al., 2005; O'Hara et al., 2000).

5.2 Quorum Sensing Activity of Oral Bacteria

Proteobacteria communicate via expression of exogenous small diffusible molecules known as *N*-acyl homoserine lactone (AHL) to mediate gene expression in a cell density-dependent manner. Frias and colleagues reported that QS in *Porphyromonas gingivalis* plays a role in controlling the production of its virulence factor in the oral cavity (Frias et al., 2001). Some *Burkholderia* species were reported to use QS to regulate their virulence factors to express pathogenicity. One of them was *B. pseudomallei* that cause human and animal melioidosis (Ulrich et al., 2004). *B. mallei* which is a causative agent of the equine disease glanders employed QS as a regulatory system to express its pathogenicity (Majerczyk et al., 2013).

By using the bacterial AHL biosensors for preliminary screening of AHL production, 5 oral isolates among the 21 strains were tested with positive results namely, *B. cepacia* strain C10B, *C. amalonaticus* strain L8A, *P. aeruginosa* strain L10A, *Enterobacter* sp. strains R8E and R2A. *C. violaceum* CV026 and *E. coli* [pSB401] AHL biosensors which were used in this study are sensitive to short chain AHLs production.

Previous reports showed that bacteria can synthesise more than one different type of AHL molecules (Hong et al., 2012; Ortori et al., 2010; Ulrich et al., 2004). Therefore, LCMS/MS was employed to profile the chemical AHLs signalling molecules of the extracts spent supernatant from the QS positive strains. The application of LCMS/MS is superior with simple sample preparation, better detection sensitivity and selectivity (Wei, 2007).

In this study, the LCMS/MS analysis result of *B. cepacia* strain C10B supernatant indicates the presence of 4 different types of AHLs. McKenney and colleagues reported that cell culture fluids from *B. cepacia* 10661 contained at least three types of signaling molecules namely C4-HSL, C6-HSL and 3-oxo-C6-HSL (McKenney et al., 1995); Gotschlich and colleagues reported *B. cepacia* H111 produces two AHL molecules, C8-HSL and C6-HSL (Gotschlich et al., 2001) whereas Lewenza and colleagues detected a single autoinducer molecule, C8-HSL in *B. cepacia* strain K56-2 (Lewenza et al., 1999). The C10-HSL and C12-HSL are the 2 unusual long chain AHLs detected in *B. cepacia* strain C10B. Hitherto, no clear pattern is observed in the production of autoinducer molecules by *B. cepacia*. There may be strain variation in the production of AHLs and thus it would be interesting to examine the types of AHLs produced by different genomovars.

Besides, a hypothesis was proposed that AHLs act as the universal language used by Gram-negative bacteria for communication (Swift et al., 1994). The first example of interspecies communication between *P. aeruginosa* PAO1 and *B. cepacia* 10661 in a type- and amount-dependent manner of autoinducer activity was performed by McKenney and colleagues (McKenney et al., 1995). *P. aeruginosa* strain L10A in this study produced 2 types of AHLs that similar to *B. cepacia* strain C10B namely C6-HSL and C8-HSL. The types of AHLs known to be produced by *P. aeruginosa* are C4-HSL, C6-HSL, 3-oxo-C6-HSL, C8-HSL and 3-oxo-C12-HSL (Geisenberger et al., 2000; Pearson et al., 1994; Winson et al., 1995). Interestingly, the production of 3-oxo-C8-HSL is uncommon to *P. aeruginosa* strain.

The result of LC chromatogram analysis on *C. amalonaticus* strain L8A, to my best knowledge, is the first documentation elucidates the detection of 4 types of AHLs in *C. amalonaticus*. Another member of *Citrobacter*, *C. rodentium* was reported to produce 2 types of AHLs namely *N*-butanoyl-L-homoserine lactone (C4-HSL) and *N*-hexanoyl-L-homoserine lactone (C6-HSL). The AHL gene in this *C. rodentium* strain has an unexpected role in affecting the mouse's virulence factors in the manner of attaching and effacing lesions through QS (Coulthurst et al., 2007). Thus, the detection of AHLs profile on *C. amalonaticus* represents the key step to understanding the potential role of QS-dependent *C. amalonaticus* plays in the oral cavity. C8-HSL and C16-HSL are the additional AHLs synthesized by *C. amalonaticus* strain L8A.

The profile of AHLs production in both *Enterobacter* sp. strains R8E and R2A are different. It has been reported that *Enterobacter* sp. strain T1-1 can produce C12-HSL (Yin et al., 2012); *Enterobacter* sp. strain M004 produce 3-oxo-C6-HSL and 3-oxo-C8-HSL (Tan et al., 2014); *E. asburiae* strain L1 produced C4-HSL and C6-HSL (Lau et al., 2013); *E. cancerogenus* strain M004 produced 3-oxo-C6-HSL and 3-oxo-C8-HSL (Chan

& Tan, 2015); and *E. sakazakii* produces 3-oxo-C6-HSL and 3-oxo-C8-HSL (Lehner et al., 2005). This finding indicates the presence of C8-HSL and 3-oxo-C12-HSL in *Enterobacter* sp. strain R8E is uncommon.

Typically, both Lux-I like autoinducer synthase and Lux-R like receptor are clustered in a pair. There are documents reported that functional *luxI*/*luxR* pairs are not shared the same bacterial chromosome or plasmids (Brameyer et al., 2015; Subramoni & Venturi, 2009). Some even reported with the absence of *luxI* autoinducer synthase in bacterial QS circuit (Patankar & González, 2009). In order to understand the mechanism of QS, whole genome sequence of *C. amalonaticus* strain L8A was selected for further analysis on its complete QS signal synthase and cognate receptor genes sequences with 636 bp and 693 bp respectively. *In silico* synteny analysis of its genome sequence, I found that functional pair of *luxI*/*luxR* in *C. amalonaticus* strain L8A is genetically clustered adjacently on the same chromosome with opposite orientation.

The phylogenetic analysis of QS synthase gene in *C. amalonaticus* strain L8A revealed that the AHL synthase extracted from whole genome sequence of *C. amalonaticus* strain L8A was clustered with putative synthase gene of *Citrobacter farmeri* GTC 1319. This finding disclosed that for the first time, the unusual various types of AHLs produced by *C. amalonaticus* strain L8A isolated from dental plaque.

Additionally, the analyses of Gene Ontology (GO) terms had conveyed a simplified GO biological pathway insight for large datasets of protein and genetic interactions. Multiple GO terms could be used for a single gene annotation manually or electronically with references. The QS pathway showing that interspecies QS and QS interplay with host occurred in *C. amalonaticus* strain L8A genome. Interspecies signalling between bacterial species via small diffusible AHL molecules has been suggested by Eberl and Tummler (2004); it happened in patients with cystic fibrosis

between *P. aeruginosa* and *B. cepacia*. QS involves in interaction with the host, in particular, a process applied by a community of single-cell *C. amalonaticus* strain L8A microorganism living in intimate contact with the host and controlling the population density with the concentration of signalling molecules. The host here referred to a larger organism which responded to symbiotic interaction (Ashburner et al., 2000; Gene Ontology Consortium, 2015). In this case, *C. amalonaticus* strain L8A is able to use their available proteins to adhere to each other and form layer of biofilm. However, the information about the possible role of AHL-regulated QS of *C. amalonaticus* is still a mystery. Therefore, it is vital to link the possible proteins to signalling pathway for later experiment design and evaluation and future work in finding out an anti-QS-based solution to control the oral cavity diseases.

5.3 Antibiotic Resistome Analysis

At present, the large majority of antibiotics used for treating infections were associated with the antibiotic resistance genes acquired by human pathogens. Transformation in natural ecosystems such as the release of vast numbers of antimicrobials might shift the development of microorganisms' population, including a selection of resistance, with the consequences of unpredictable human health. Thus, finding the appropriate antimicrobial agents and profiling the antibiotic resistance genes in microbial become the current issue for human medicine (WHO, 2012).

The recent outbreaks of *E. anophelis* infections were reported in the Midwest United States and conducted the investigation and response by Centers for Disease Control and Prevention (CDC) (2016). Previous studies reported *Elizabethkingia* spp. resistant to most β-lactam antibiotics (González & Vila, 2012; Tak et al., 2013) such as carbapenems which is the last resort of medicine treatment (Papp-Wallace et al., 2011;

Van Boeckel et al., 2014). In general, a single chromosomally encoded β-lactamase gene can be induced by β-lactams and allows the bacterial pathogen to resist the action of related antimicrobial agents (Bush, 2013; Kunz & Brook, 2010; Matyi et al., 2015). It has raised our concern and hence we aimed to study this strain by characterising its genome profile in order to gain more insights on its antimicrobial resistance followed by confirming its antimicrobial susceptibilities. The early prognostication of antimicrobial susceptibility pattern and resistance genes analysis in *Elizabethkingia* strain could help to prevent the possible emerging pathogen and infections.

Besides, the antimicrobial susceptibility patterns are different in each individual microbe and thus, there is no particular or conserved therapy for a species (Hayek et al., 2013). To select an appropriate antibiotic, a physician considered few conditions, including infection site, Gram stain and culture's information, host responses to drugs (pharmacokinetics) and interaction of drug towards infecting pathogen (pharmacodynamics) with common MIC measurement (McKinnon & Davis, 2004; Scheetz et al., 2006). The previous literature described *E. anophelis* resistant to a number of antibiotics; namely ampicillin, kanamycin, streptomycin, chloramphenicol and tetracycline (Kämpfer et al., 2011). My analyses demonstrated that *E. anophelis* strain B2D also resistant to most of the antibiotics and only susceptible to ciprofloxacin.

The VITEK 2 results in Table 1 suggested the multidrug-resistant phenotype of *E. anophelis* strain B2D and its capability of being a life-threatening pathogen. Previous work has reported that VITEK 2 system is capable of providing rapid, reliable, and highly reproducible results on microbial identification as well as antimicrobial susceptibility test (Ling et al., 2001). It has been reported that this fully automated VITEK 2 system allows the standardisation of all the critical parameters for antimicrobial susceptibility testing starting from colony and inoculum preparation to the MIC endpoint determination. As

compared to antibiotic disc diffusion assay which is very subjective and inaccurate (Chan et al., 2014), hence in this study, I turned to the automated antimicrobial susceptibility using VITEK 2 which allows clinical laboratories to perform such antimicrobial susceptibility testing simultaneously not only in an unmanned manner but also fully standardised (Pfaller et al., 2007).

Upon confirmation of the antimicrobial phenotype, the genome evidence that gave us the genotypic evidence on the molecular basis of these resistance properties was further investigated. As a result, the genomic data of *E. anophelis* strain B2D showed putative genes conferring to antibiotic resistance were present throughout its genome. The only vancomycin-resistant gene predicted by RAST encodes for Vancomycin B-type resistance protein *vanW* (705bp, 235 amino acids). A further PSI-BLAST showed its identity as vancomycin B-type resistance protein *Elizabethkingia anophelis* (98% identity). However, it is believed that the putative protein does not confer resistance to vancomycin. Vancomycin B-type resistance in *Enterococcus faecalis* V583 is determined by a 7160 bp DNA fragment with seven open reading frames. Although *vanW* is one of the proteins encoded, its function is unknown (Evers & Courvalin, 1996).

Other than vancomycin, fluoroquinolones are antibiotics targeting bacterial enzymes DNA gyrase and topoisomerase IV, interfering DNA replication. The DNA gyrase is composed of two subunits encoded by *gryA* and *gryB* while topoisomerase IV is composed of two subunits encoded by *parC* and *parE* genes (Moudgal & Kaatz, 2009). Although all the mentioned genes in *E. anophelis* strain B2D were identified as a putative resistant variant, the strain was revealed susceptible to fluoroquinolones like ciprofloxacin but not to levofloxacin, as tested in VITEK 2 system.

In this analysis, a total of 15 putative genes conferring to beta-lactamases had been annotated by RAST. These genes were further searched using PSI-BLAST against NCBI database. Of these, 13 putative beta-lactamase proteins were identified but two were recognised as hypothetical proteins in *E. anophelis* strain B2D genome sequences. Those putative beta-lactamases were revealed with high amino acid identical to *E. anophelis* as well as *Chryseobacterium gleum* and *Aquimarina* sp. that belonged to the same Family Flavobacteriaceae. Besides, a putative CME-2 beta-lactamase and a putative BlaB-14 Metallo-beta-lactamase had been detected by RAST.

According to previous reports, the uncommon antibiotics sensitivity of *E. meningoseptica* is due to the simultaneous presence of two different broad substrate spectra metalloenzymes coupled with extended-spectrum serine-β-lactamase CME (González & Vila, 2012; Vessillier et al., 2002). CME-2 beta-lactamase has been reported responsible for extended-spectrum cephalosporin resistance in *E. meningoseptica* whereas metallo-β-lactamase GOB-1 (Bellais et al., 2000) and BlaB-14 Metallo-beta-lactamase mediated carbapenem-resistance in the species with GOB's characteristic property was hindered by higher cellular levels of BlaB (González & Vila, 2012). The existence of these three *bla* genes was detected in the whole genome sequence of *E. anophelis* strain B2D while screening against ARG-ANNOT database. These findings complement the antibiotic resistance of *E. anophelis* strain B2D towards most members of beta-lactam antibiotics proven through VITEK 2. It is believed that this strain may act as potential reservoirs of novel beta-lactamase genes in the human oral cavity.

It is also observed clusters of putative genes that might confer multidrug resistance efflux pump in the genome of *E. anophelis* strain B2D. In Gram-negative bacteria, these RND efflux pumps are one of the most important determinants of multidrug resistance. They consist of an outer membrane protein, a membrane fusion protein and an inner

membrane component that function as a tripartite protein complex. The complex forms a channel across the cell membrane to allow pumping out of molecules (such as an antibiotic) across periplasm and outer membrane. The process happens effectively with energy derived from proton-gradient. The findings were remarkable as it showed the molecular evidence of multidrug resistance in the genome of *E. anophelis* strain B2D (Femando & Kumar, 2013).

Other than *E. anophelis* strain B2D, the genome sequences of *P. gergoviae* strain C7B were also annotated with six putative genes of beta-lactamase associated function in our analyses. This putative gene included Metallo-beta-lactamase superfamily protein, beta-lactamase protein precursor and beta-lactamase class C and other penicillin binding proteins, encoded in few numbers of contigs. Some of them have been classified as not being functional in the genome by automated RAST server annotation.

5.4 Comparative Genome Studies

In this study, the whole genome sequence of *C. amalonaticus* strain L8A and its closest relative genomes sequences were studied using a comparative genomic analysis approach. The overview of sequence feature information is able to be visualized in the context of sequence analysis results generated through the comparative genomics tool. The sole complete genome *C. amalonaticus* strain Y19 (CP011132) was assigned as reference genome in this comparative study. This strain was reported with the capability to produce hydrogen from oxidizing toxic carbon monoxide (Ainala et al., 2015). Besides, *C. amalonaticus* strain Y19 also possess the genes for glycerol metabolism and production of 1,3-propanediol (1,3-PDO) (Ainala et al., 2013). *C. amalonaticus* strain GTA-817-RBA-P2 (LAMY01) was isolated from ground beef enriched in modified tryptic soy broth (mTSB) containing vancomycin (10 μg/ml) and cefsulodin (3 μg/ml).

Whereas, *C. amalonaticus* strains 3e8A (CDQV01) and 3e8B (CDQX01) with functional characterization of immunoglobulin A (IgA) were isolated from the fecal microbiota of undernourished Malawian children who exhibited a diet-dependent enteropathy (Kau et al., 2015).

Regardless of the limitation of online available genome sequences, it is possible to compare three diverse sources of the genome sequences. From the comparison image, the co-conservation profiles across these six draft genomes illustrated that they are closely related associated in some form. There are more breakpoints were observed in the genome profile of *C. amalonaticus* strain GTA-817-RBA-P2 which due to genome complexity, different sources of isolation and other factors. By observing the existence of a gene in a genome across multiple genomes, one can expect that closely related functional gene would tend to appear and disappear in a correlated manner (Wei et al., 2002). Thus, expanding investigate and predict the possible missing gene, gap, phages, antimicrobial resistance gene and other genomic features are strongly recommended.

5.5 Other Genomic Features

Research on human health relatedness microbes to improve health and to prevent or cure illness always the concern topic of global dimensions. Besides the QS and antibiotic resistant aspect, other genomic features from whole genome sequences of oral isolates were analysed in this research.

The study of probiotic bacteria (e.g. *Lactobacillus* and *Bifidobacterium*) is vital to human as they play a key role in satisfying public demand such as dairy product fermentation in order to support dental health, alleviate chronic fatigue syndrome (Sullivan et al., 2009), treat ulcerative colitis (Zocco et al., 2006), and alter fat storage

(Aronsson et al., 2010). However, in Miller hypothesis, he proposed that oral microorganisms which produce acid might be one of the etiologies of dental caries (Miller, 1891). Whole genome sequences of *Lactobacillus paracasei* strain L9D was annotated with operon encoding osmotically activated L-carnitine/choline ATP-Binding Cassette transporter in contig 68. This operon has a high level of sequence similarity to the operons encoding protein OpuB and OpuC in *Bacillus subtilis* which involved in the oxidation of choline to glycine betaine pathway (Kappes et al., 1999). The operon in *L. paracasei* strain L9D was predicted consists of 4 genes which are encoded for ATP binding protein (OpuCA), extracellular substrate binding protein (OpuCC) precursor and two membrane-associated permease protein (OpuCB and OpuCD). The *opuC* operon plays a vital role in response to osmotic stress which is capable of transporting carnitine in this bacterium. Carnitine is an important amino acid involved in lipid metabolism and generates energy. Carnitine deficiency and causes it cannot be transported into tissue may lead to chronic muscle weakness (Myopathy, 1973), hypoglycemia and other symptoms. Possible beneficial effects of exogenous carnitine and probiotics have been the focus of intensive research (Hegazy & El-Bedewy, 2010; Moeinian et al., 2014). This finding suggests that existing of an alternative transport system for carnitine accumulation in *L. paracasei* should encourage further studies with interventions with probiotics treatment.

On the other hand, in agreement with the previous report on *Proteus mirabilis*, a total of seven putative urease subunits were identified and profiled in the subsystem of *P. mirabilis* strain T1C. Urease activity prone to increase the circumstance pH and they are nickel-containing metalloenzymes that have a profound impact on medical and agricultural (Krajewska et al., 2012). This genome also predicted the features of resistance to fluoroquinolones and extended-spectrum cephalosporins due to the production of beta-lactamases (Stürenburg & Mack, 2003).

5.6 Future Work

These data describing the whole genome sequences of oral isolates from dentine caries and dental plaque, and AHLs producing QS positive strains in this thesis provide a useful basis for the elucidation of the potential reservoir for QS pathogens in the human oral cavity. Hence, in future, the QS relatedness genes in these QS strains will be studied through gene knockout, expression analysis and RNAseq to investigate the regulatory role of the AHLs particularly on their virulence factors which are used by the bacteria to express their pathogenicity. The comparison of phenotypes changes between the mutants and wildtype will be performed by using Omnilog system.

In addition, the presence of the multidrug resistant phenotype of *E. anophelis* strain B2D on dental plaque leads to the speculation that strain B2D could be an opportunistic pathogen and might contribute to other associated diseases in the human body. As such, further analysis of this strain should provide the model to understand this potential pathogen in the oral cavity and perhaps to other human body parts. This could be done by a combination of recent advances in third generation sequencing to generate a complete genome with full genomics information of the strain.

CHAPTER 6: CONCLUSION

A total of twenty-one different species were isolated from dentine caries and dental plaque of human oral cavity and identified by MALDI-TOF MS. Among the oral isolates, five isolates showed QS activity namely, *Burkholderia cepacia* strain C10B, *Citrobacter amalonaticus* strain L8A, *Pseudomonas aeruginosa* strain L10A, *Enterobacter* sp. strains R8E and strain R2A. The high-resolution triple quadrupole LCMS mass spectrometry analysis of extracts spent supernatant of each QS positive strain confirmed *B. cepacia* strain C10B produced four types of AHLs (C6-HSL, C8-HSL, C10-HSL and C12-HSL), *C. amalonaticus* strain L8A produced four types of AHLs (C4-HSL, C6-HSL, C8-HSL and C16-HSL), *P. aeruginosa* strain L10A produced five type of AHLs (C4-HSL, C6-HSL, C8-HSL, 3-oxo-C8-HSL and 3-oxo-C12-HSL), *Enterobacter* sp. strain R8E produced four types of AHLs (C4-HSL, C6-HSL, C8-HSL and 3-oxo-C12-HSL) and *Enterobacter* sp. strain R2A produced two types of AHLs (C4-HSL and C12-HSL). For the first time, this finding unveils the existence of *Elizabethkingia anophelis*, *Klebsiella michiganensis* and *Enterobacter cloacae complex* 'Hoffmann cluster IV' in the human oral cavity.

Coupling with next generation sequencing technology and systematic bioinformatics analysis, this study will provide insights into oral bacteria genome features and invaluable information about these bacterial strains notably on the QS system and antibiotics resistance genes. Besides this, the genome sequences of oral microbial could be exploited as a reference genome for future comparative, functional and clinical genomics study. The genome sequences may be useful for research on any related genes through its nucleotide sequences analysis, orientation, upstream and downstream regulatory elements and gene products, as well as its miscellaneous genomics features. By elucidating the QS genes in the genome sequence, it will pave the way to understand

the QS regulation in these isolates. In addition to this, this work will also illustrate that oral cavity could be a potential reservoir for multidrug resistance genes and hence more intense research should be carried out.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

A LIST OF PUBLICATIONS

Goh, S. Y., Tan, W. S., Khan, S. A., Chew, H. P., Kasim, N. H. A., Yin, W. F., & Chan, K. G. (2014). Unusual multiple production of *N*-acylhomoserine lactones a by *Burkholderia* sp. strain C10B isolated from dentine caries. *Sensors*, *14*(5), 8940-8949.

Goh, S. Y., Khan, S. A., Tee, K. K., Kasim, N. H. A., Yin, W. F., & Chan, K. G. (2016). Quorum sensing activity of *Citrobacter amalonaticus* L8A, a bacterium isolated from dental plaque. *Scientific Reports*, *6*.

B LIST OF PRESENTATIONS

Goh, Share Yuan, Yin, W. F., & Chan, K. G. (2013). *Human Oral Microbiome in Dental Caries and Plaque.* Poster Presentation at Bioinfosummer 2013. School of Molecular and Biomedical Science, University of Adelaide. 5th December 2013, Andreas W. Schreiber, Bioinformatics group leader, ACRF Cancer Genomics Facility.

Goh, Share Yuan, Khan, S. A., Chew, H. P., Kasim, N. H. A., Yin, W. F., & Chan, K. G. (2014). *Metagenomics Analysis of Oral Microbiome*. Poster Presentation at Monash Science Symposium 2014. School of Science Monash University Malaysia.