STUDY OF BACTERIAL DIVERSITY IN THE ORAL CAVITY OF MALAYSIAN SUBJECTS

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Abstract

About 700 species of bacteria can inhabit the human oral cavity. This project initially investigated the bacterial diversity within the oral cavity of Malaysian subjects by using culture-dependent techniques and 16S rDNA sequence analysis. The second part of the research profiled the antibiotic resistance of the identified oral bacteria by the Etest method. Three sampling sites of the oral cavity from nine healthy subjects were analyzed: the tooth surface, gingival crevice, and dorsum of the tongue. The tooth surface was shown to harbor 41 species, gingival crevice harbored 51 species and tongue dorsum 41 species. On average, each subject carried 27 species from all the three sites. *Streptococcus mutans*, a putative cariogenic pathogen and *Prevotella intermedia*, a putative periodontal pathogen were detected. In this study, no novel species of oral bacteria were identified. The bacteria that inhabit in the oral cavity are a diverse and dynamic community. Some bacterial species showed a pattern of site-specificity and subject-specificity while some are common in all sites of the oral cavity and in all subjects. All 411 isolates were susceptible to amoxicillin-clavulanic acid except *Pseudomonas aeruginosa* which showed high resistance (MIC >256μg/ml). 24 of the 411 isolates (5.8%) were resistant to clindamycin. The resistant strains included *Capnocytophaga gingivalis*, *Eikenella corrodens*, *Haemophilus parainfluenzae*, *Lautropia* sp., *Neisseria* sp., *N. meningitidis*, *N. flavescens*, *N. subflava*, *Streptococcus sanguinis*, *S. mitis* and *P. aeruginosa*.

Abstrak

Sekitar 700 spesies bakteria boleh mendiami rongga mulut manusia. Projek ini awalnya menyiasat kepelbagaian bakteria dalam rongga mulut daripada subjek Malaysia dengan menggunakan teknik bergantung pada kultur dan analisis urutan 16S rDNA. Bahagian kedua projek ini adalah kajian memprofilkan antibiotik resistensi daripada bakteria mulut yang dikenalpasti dengan kaedah Etest. Tiga lokasi sampling daripada rongga mulut sembilan subjek sihat dianalisiskan. Lokasi termasuk permukaan gigi, celah gusi, dan lidah dorsum. Permukaan gigi didapati mengandungi 41 spesies, celah gusi mengandungi 51 spesies dan 41 spesies daripada lidah dorsum. Secara purata, setiap subjek mempunyai 27 spesies daripada semua tiga lokasi sampling. *Streptococcus mutans*, yang dipercayai patogen cariogenic dan *Prevotella intermedia*, yang dipercayai patogen periodontal dikesan. Dalam kajian ini, tidak ada novel spesies mulut bakteria yang dapat dikenalpasti. Bakteria yang mendiami di dalam rongga mulut adalah terdiri daripada pelbagai dan dinamik komuniti. Beberapa spesies bakteria menunjukkan tempat-kekhususan dan subjek-kekhususan sementara beberapa yang umum di semua lokasi sampling dari rongga mulut dan dalam semua subjek. Semua 411 isolat tidak resisten terhadap amoxicillin-clavulanic asid kecuali *Pseudomonas aeruginosa* yang menunjukkan resistansi kuality (MIC> 256μg/ml) bagi amoxicillin-clavulanic asid. 24 daripada 411 isolat (5.8%) resisten terhadap clindamycin. Strain resisten termasuk *Capnocytophaga gingivalis*, *Eikenella corrodens*, *Haemophilus parainfluenzae*, *Lautropia* sp., *Neisseria* sp., *N. meningitidis*, *N. flavescens*, *N. subflava*, *Streptococcus sanguinis*, *S. mitis* dan *P. aeruginosa*.

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List of abbreviations

ANOVA	Analysis of variance
CFU	Colony forming unit
EDTA	Ethylenediaminetetraacetic acid
HOMD	Human Oral Microbiome Database
K2HPO4	Dipotassium hydrogenphosphate/ Dipotassium phosphate
KH2PO4	Potassium dihydrogen phosphate/ Potassium phosphate monobasic
MIC	Minimum inhibition concentration
MR-VP	Methyl red and Voges-Proskauer
Na2CO3	Sodium carbonate
NaHCO3	Sodium bicarbonate/ Sodium hydrogen carbonate
NCBI	National Center for Biotechnology Information
(NH4)H2PO4	Ammonium dihydrogenphosphate/ Ammonium phosphate monobasic
OF	Oxidative/Fermentation
RTF	Reduced Transport Fluid
SEM	Scanning Electron Microscopy
SIM	Sulfide production, indole formation and motility
TEM	Transmission Electron Microscopy

Chapter 1

Introduction

Oral health is vital to general health and well-being. A healthy oral cavity is effectively maintained by good oral hygiene. Daily oral hygiene measures are important to ensure the balanced interaction between the normal oral microflora and the host as oral health is strongly linked to the microbial community that inhabits the oral cavity. This fact has been known for a very long time. Many studies had been carried out to profile the human oral microflora. Even in the public domain, a database is created to provide comprehensive information on the bacterial species that are present in the human oral cavity. The database is called Human Oral Microbiome Database (HOMD). It is created and maintained by scientists at the Forsyth Institute in Boston and King's College London in England (http://www.HOMD.org.)

Based on both culture-dependent and culture-independent methods done by many studies around the world, it has been estimated that about 700 species of bacteria are capable of inhabiting the human oral cavity. It appears that with the advent of molecular techniques the diversity of microflora in the human oral cavity has reached a point of saturation. Fewer novel bacterial species are now being discovered from the oral cavity. Many studies have involved the profiling of the oral microbiota of different ethnic backgrounds such as Colombians, Brazilians, Sudanese, Americans, Swedish, Mexicans, Chinese and so on (Botero *et al*., 2007; Colombo *et al*., 2002; Darout *et al*., 2003; Haffajee *et al*., 2005; Mayorga-Fayad *et al*., 2007). The results of these studies showed the distribution and composition of oral microflora differed between the various geographical locations and within and between racial or ethnic groups. This is may be related to dietary and genetic differences. The majority of oral microflora studies have been done in Western Caucasian populations and only limited study has been done on other ethnic groups. Studies have also been done on antibiotic resistance of oral bacteria. The results showed that oral bacteria resistant to antibiotics are

especially prevalent in several countries (Arvand *et al*., 2000; Cha *et al*., 2001; Eisenblatter *et al*., 2008; Mosca *et al*., 2007). The antibiotic susceptibility of oral bacteria differs among the different countries. This may relate to differences in the genetics of the bacteria and the usage of the particular antibiotic in the country. For example *Fusobacterium nucleatum* in Spanish populations show higher minimum inhibitory concentration (MIC) values for penicillin and ciprofloxacin compared with Dutch strains (van Winkelhoff *et al*., 2005). *Prevotella intermedia* in Spanish populations show higher MIC values for penicillin, amoxicillin and tetracycline compared with Dutch strains. *Porphyromonas gingivalis* in Spanish populations show higher MIC values for tetracycline and ciprofloxacin compared with Dutch strains. From the study, it showed that *Fusobacterium nucleatum* and *Prevotella intermedia* are more frequently resistant in Spain than in the Netherlands (van Winkelhoff *et al*., 2005). Based on one study, it showed that the antibiotic resistance of oral bacteria to tetracycline was significantly higher in Japanese and South Asian children compared with white children (Ready *et al*., 2006).

The first part of this project investigated the diversity existing within the oral bacteria in three ethnic groups in Malaysia. This was anticipated to generate some information on the distribution pattern of the oral microflora among Malaysians. The second objective was to profile the antibiotic resistance of the identified oral bacteria. This was to provide some information to local dentists in their prescriptions of antibiotics if the subjects do not respond well to mechanical therapy of plaque-related oral diseases. This project was carried out mainly due to the currently scarce information available on the oral microbial diversity and antibiotic susceptibility of oral bacteria in Malaysia. In this project, a culture dependent method was used to isolate the oral bacteria from Malaysian subjects. The pure isolates were identified using 16S

rDNA sequence analysis and their antibiotic resistance profiles were determined. This project also included the characterization of *Streptococcus salivarius* using electron microscope. This was done to view the morphology of the *S. salivarius* isolated from Malaysian subjects.

Chapter 2

Literature review

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2.1 Normal oral microflora

Normal oral microbial flora consists of a great diversity of microorganisms which includes bacteria and fungi. However, bacteria are the predominant group in the oral cavity. In gingival crevice, it is estimated to have 1.8×10^{11} anaerobes per gram (Moore *et al*., 1984). The anaerobes outnumber aerobic bacteria by a ratio of 10:1 to 100:1. Anaerobes dominate our indigenous oral microflora and are therefore mostly associated with oral diseases (Moore *et al*., 1984). The microorganisms isolated from the odontogenic infections usually reflect the host's normal oral flora (Finegold, 1977). Under certain circumstances, their virulent features can lead to diseases. Therefore, it is worthwhile to know all the oral microorganisms for the purpose of diagnosis and treatment.

In the oral cavity there is culturable and unculturable microflora. Based on both culture-dependent methods and culture-independent methods, it has been estimated that about 700 species of bacteria are capable of inhabiting the human oral cavity (Jenkinson and Lamont, 2005; Paster *et al*., 2001; Smoot *et al*., 2005). About 400 bacterial species have been isolated from subgingival pockets and about 300 species have been identified from tongue, oral mucous membranes, caries, and endodontic infections (Paster *et al*., 2006). A single individual oral cavity can harbor 150-200 bacterial species (Giannobile *et al*., 2009). It is estimated that cultured oral bacterial species account for about 50% of the microorganisms identified by culture-independent methods, indicating that the oral cavity is an environment where most species can be studied by routine culture methods (Kolenbrander *et al*., 2002; Paster *et al*., 2006). Dental plaque is one of the best-described mixed-species of bacterial communities. Since it is easily accessible, it is convenient to study this complex model system.

Majority of these oral microorganisms are commensals while some of them are opportunistic pathogens that can cause systemic diseases. Oral bacteria have been implicated in bacterial endocarditis, aspiration pneumonia, osteomyelitis in children, preterm low birth weight, coronary heart disease and cerebral infarction (Paster *et al*., 2001). The incidence of bacteremia following dental procedures has been well documented (Li *et al*., 2000). The oral microbiota plays critical roles in oral health and is directly linked to major human diseases such as dental caries and periodontal disease (Li *et al*., 2000; Paster *et al*., 2001; Pihlstrom *et al*., 2005; Selwitz *et al*., 2007; Smoot *et al*., 2005).

Predominant cultivable bacterial phyla include Firmicutes (e.g. *Streptococcus*, *Gemella*, *Selenomonas*), Fusobacteria, Actinobacteria (e.g. *Actinomyces*, *Rothia*), Spirochaetes (e.g. *Treponema*), Proteobacteria (e.g. *A. actinomycetemcomitans*, *Neisseria*, *Eikenella*, *Haemophilus*, *Campylobacter*), and Bacteroidetes (e.g. *Porphyromonas*, *Prevotella*, *Tannerella*). Uncultivable bacterial phyla include Obsidian Pool OP11 (rare), TM7 and Synergistes (Paster *et al*., 2001 and 2006).

Spirochaetes include species of the genus *Treponema*. About 60 species have been identified (Paster *et al*., 2001). *Treponema* are Gram-negative helical cells and are strict anaerobes. The predominantly cultivable and known *Treponema* species included *T. denticola*, *T. maltophilum* and *T. socranskii*. *T. denticola*, *T. macrodentium*, *T. skoliodontium*, *T. socranskii*, *T. maltophilum*, *T. amylovarum*, and *T. vincentii* are found in the gingival crevice (Aas *et al*., 2005; Kazor *et al*., 2003; Paster *et al*., 2001 and 2006).

Fusobacteria include the genera *Fusobacterium* and *Leptotrichia*. *Fusobacterium* is Gram-negative rod and a strict anaerobe. Main species isolated from gingival crevice are *F. nucleatum*, *F. alocis*, *F. sulci*, and *F. periodonticum*. *Leptotrichia* is Gram-negative filament, strict anaerobe. Main species is *L. buccalis* (Aas *et al*., 2005; Kazor *et al*., 2003; Paster *et al*., 2001 and 2006).

Actinobacteria include the genera *Actinomyces*, *Atopobium*, *Bifidobacterium*, *Corynebacterium*, *Propionibacterium* and *Rothia*. *Actinomyces* is Gram-positive pleomorphic rod, facultative anaerobe. Main species are *A. israelii*, *A. gerencseriae*, *A. odontolyticus*, *A. naeslundii*, *A. myeri*, and *A. georgiae*. They are found in dental plaque. *Corynebacterium matruchotii*, *Atopobium parvulum* and *A. rimae* are detected in oral cavity. *Rothia* is Gram-positive branching filament, facultative anaerobe. *R. dentocariosa* is commonly found in dental plaque and *R. mucilaginosa* is isolated from tongue and gingival crevice. *Propionibacterium* is Gram-positive bacillus, strict anaerobe. Main species are *P. acnes*, and *P. propionicus* (formerly *Arachnia propionica*). *Bifidobacterium dentium* is Gram-positive strict anaerobe found on teeth (Aas *et al*., 2005; Kazor *et al*., 2003; Paster *et al*., 2001 and 2006).

The Firmicutes include the genera *Streptococcus*, *Micromonas* (formerly *Peptostreptococcus*), *Gemella*, *Eubacterium*, *Lactobacillus*, *Abiotrophia*, *Granulicatella*, *Selenomonas*, *Pseudoramibacter* and *Veillonella*. Streptococci are Gram-positive cocci in chains, non-motile, usually have surface fibrils, occasionally capsulated and facultative anaerobes. The major tooth surface cultivable streptococci include *S. mutans*, *S. sobrinus*, *S. cricetus*, *S. rattus*, *S. ferus*, *S. macacae*, *S. downei*, *S. mitis*, *S. sanguis*, *S. gordonii*, *S. oralis*, and *S. crista*. On dorsum of the tongue, the main cultivable streptococci are *S. salivarius*, *S. vestibularis*, *S. mitis*, *S. sanguis*, *S.*

gordonii, *S. oralis*, and *S. crista*. The main streptococci isolated from the gingival crevice include *S. constellatus*, *S. intermedius*, and *S. anginosus*. *Micromonas* main species is *M. micros*. *Gemella haemolysans* and *G. morbillorum* are commonly detected in the oral cavity. *Eubacterium* is a pleomorphic, Gram-variable rod or filament and is strict anaerobe. Main species are *E. brachy*, *E. nodatum* and *E. saphenum*. Lactobacilli are Gram-positive bacillus-shaped, microaerophilic bacteria. The main species found in dental plaque are *L. casei*, *L. fermentum*, *L. acidophilus*, *L. salivarius*, and *L. rhamnosus*. *Abiotrophia* main species is *A. defective*. *Granulicatella* main isolate is *G. adiacens* (formerly *Abiotrophia adiacens*). *Selenomonas* is Gram-negative curved cell, strict anaerobe. *S. sputigena*, *S. noxia*, *S. flueggei*, *S. inflexi*, and *S. diane* are the main species from gingival crevice. *Veillonella* is Gram-negative coccus strict anaerobe, most isolated species from tongue and teeth are *V. parvula*, *V. dispar*, and *V. atypica* (Aas *et al*., 2005; Kazor *et al*., 2003; Paster *et al*., 2001 and 2006). *Pseudoramibacter* main species is *P. alactolyticus* (Willems and Collins, 1996).

The Proteobacteria include the genera *Haemophilus*, *Campylobacter* (formerly *Wolinella*), *Neisseria*, *Actinobacillus*, *Lautropia*, *Eikenella* and *Kingella*. *Haemophilus* is a genus of Gram-negative coccobacilli, facultative anaerobe. Main isolated species are *H. parainfluenzae*, *H. segnis*, *H. aphrophilus*, *H. haemolyticus*, and *H. parahaemolyticus*. *Campylobacter* is Gram-negative curved bacillus, strict anaerobe. *C. rectus*, *C. gracilis* and *C. concisus* are detected in oral cavity. *Neisseria* are Gram-negative diplococci, facultative anaerobes. Main species isolated from tongue are *N. subflava*, *N. mucosa*, and *N. sicca*. *Actinobacillus* are Gram-negative coccobacilli, microaerophilic microbes. The main species is *A. actinomycetemcomitans*. *Lautropia* is Gram-negative coccus, pleomorphic, facultative anaerobe. Main species is *L. mirabilis* (Rossmann *et al*., 1998). *Eikenella* is Gram-negative coccobacilli, microaerophilic

bacteria. Main species is *E. corrodens*. In the genus of *Kingella*, the main isolated species are *K. denitrificans*, and *K. oralis* (Aas *et al*., 2005; Kazor *et al*., 2003; Paster *et al*., 2001 and 2006).

Bacteroidetes include the genera *Porphyromonas*, *Prevotella*, *Tannerella*, and *Capnocytophaga*. *Porphyromonas* is Gram-negative pleomorphic rod, black-pigmented strict anaerobe. Main isolates from gingival crevice are *P. gingivalis*, *P. endodontalis*, and *P. catoniae*. *Prevotella* is Gram-negative pleomorphic rod black-pigmented or non-black-pigmented strict anaerobe. Majority gingival crevice isolates are *P. intermedia*, *P. nigrescens*, *P. loeschii*, *P. corporis*, and *P. melaninogenica*. Non-black-pigmented species are *P. buccae*, *P. oralis*, *P. oris*, *P. oulora*, *P. veroralis*, and *P. dentalis*. *Capnocytophaga* is Gram-negative fusiform rod, capnophilic (CO_2 dependent) bacteria. Main species are *C. gingivalis*, *C. sputigena*, *C. ochracea*, *C. granulosa*, and *C. haemolytica*. *Tannerella* main species is *T. forsythia* (Aas *et al*., 2005; Kazor *et al*., 2003; Paster *et al*., 2001 and 2006).

Generally, the genera of *Gemella*, *Granulicatella* (e.g. *G. adiacens*), *Streptococcus* (e.g. *S. mitis*), and *Veillonella* are commonly detected at all sites of the oral cavity. *Rothia dentocariosa*, *Actinomyces* sp., *Streptococcus sanguinis*, *S. gordonii* and *Abiotrophia defectiva* are frequently detected on teeth surface (supragingival plaque). *Streptococcus salivarius* is typically detected on the dorsum of the tongue. *Neisseria* species are mostly isolated from all sites except subgingival plaque. In most of the subjects, *Streptococcus intermedius* is detected only in subgingival plaque (Aas *et al*., 2005; Paster *et al*., 2006).

Table 2.1 Overview of cultivable oral microbiota

Phylum	**Genus**	**Species**
Spirochaetes	*Treponema*	*T. denticola, T. maltophilum, T. socranskii, T. macrodentium, T. skoliodontium, T. amylovarum, T. vincentii*
Fusobacteria	*Fusobacterium*	*F. nucleatum, F. alocis, F. sulci, F. periodonticum*
	Leptotrichia	*L. buccalis*
Actinobacteria	*Actinomyces*	*A. israelii, A. gerencseriae, A. odontolyticus, A. naeslundii, A. myeri, A. georgiae*
	Atopobium	*A. parvulum, A. rimae*
	Bifidobacterium	*B. dentium*
	Corynebacterium	*C. matruchotii,*
	Propionibacterium	*P. acnes, P. propionicus*
	Rothia	*R. mucilaginosa, R. dentocariosa*
Firmicutes	*Abiotrophia*	*A. defective*
	Eubacterium	*E. brachy, E. nodatum, E. saphenum*
	Gemella	*G. haemolysans, G. morbillorum*
	Granulicatella	*G. adiacens*
	Lactobacillus	*L. casei, L. fermentum, L. acidophilus, L. salivarius, L. rhamnosus*
	Micromonas	*M. micros*
	Pseudoramibacter	*P. alactolyticus*
	Selenomonas	*S. sputigena, S. noxia, S. flueggei, S. inflexi, S. diane*
	Streptococcus	*S. mutans, S. sobrinus, S. cricetus, S. rattus, S. macacae, S. downei, S. mitis, S. sanguis, S. crista, S. gordonii, S. oralis, S. salivarius, S. vestibularis, S. constellatus, S. intermedius, S. anginosus, S. ferus*
	Veillonella	*V. parvula, V. dispar, V. atypica*
Proteobacteria	*Actinobacillus*	*A. actinomycetemcomitans*
	Campylobacter	*C. rectus, C. gracilis, C. concisus*
	Eikenella	*E. corrodens*
	Haemophilus	*H. parainfluenzae, H. segnis, H. aphrophilus, H. haemolyticus, H. parahaemolyticus*
	Kingella	*K. denitrificans, K. oralis*
	Lautropia	*L. mirabilis*
	Neisseria	*N. subflava, N. mucosa, N. sicca*
Bacteroidetes	*Capnocytophaga*	*C. gingivalis, C. sputigena, C. ochracea, C. granulosa, C. haemolytica*
	Porphyromonas	*P. gingivalis, P. endodontalis, P. catoniae*
	Prevotella	*P. intermedia, P. nigrescens, P. loeschii, P. corporis, P. melaninogenica, P. buccae, P. oralis, P. oris, P. oulora, P. veroralis, P. dentalis*
	Tannerella	*T. forsythia*

2.2 Factors determining the composition of the oral microflora

2.2.1 Nutritional support in microbial communities

Nutrition is vital to support the mixed microbial communities. The microorganisms possess metabolic pathways that utilize nutrients available within the oral cavity. The intrinsic sources of nutrient for the oral microbes are the carbohydrates (hyaluronic acid and chondroitin sulfate) of the dentin, degradation of epithelial cells, saliva and gingival crevicular fluid (Marsh, 1995). Cariogenic streptococci can breakdown the carbohydrate of the dentin. Saliva has been found to contain a mixture of inorganic ions (sodium, potassium, calcium, chloride, bicarbonate and phosphate), eighteen amino acids (aspartic acid, glutamic acids, threonine, serine, glycine, alanine, phenylalanine, leucine, isoleucine, proline, cystine, valine, methionine, tyrosine, tryptophan, histidine, lysine and arginine), proteins and glycoproteins (mucin) (Nolte, 1982; Samaranayake, 2006).

The composition of crevicular fluid is similar to serum. Proteolytic and saccharolytic bacteria can obtain peptides, amino acids, carbohydrates and cofactors (haemin by degradation of haemoglobin) from the crevicular fluid. The extrinsic source of nutrients will be the food which we are taking. Humans consume foods of animal and plant origin which are complex and can favor the development and support of mixed microbial communities in the oral cavity. However, intrinsic sources of nutrients play the major role in supporting the complex microbial communities.

2.2.2 Interactions between the oral bacterial species

In mixed microbial communities, the interrelationships between the microorganisms are complex and can be divided into competition for nutrients, symbiosis, commensalism, antagonism and synergism (Grenier, 1996; Marsh, 1989). In

competition for nutrients, oral bacteria which can metabolize the available nutrients more efficiently will have competitive advantage or better viability. For example, *Streptococcus mutans* can metabolize sucrose more efficiently than other oral bacteria (Hamada and Slade, 1980). Symbiosis means mutual beneficial interaction. As an example, the growth of *Porphyromonas gingivalis* can be stimulated by succinate produced by *Treponema denticola*. The growth of *T. denticola* can also be stimulated by isobutyric acid provided by *P. gingivalis* (Grenier, 1992).

Commensalism is a relationship in which one species benefits while the other species is unaffected. For example, aerobes utilize atmospheric oxygen and create a low oxidation-reduction potential which is favorable to anaerobes. This association also can be shown between *Bacteroides melaninogenicus* (most of the *Bacteroides* species are now designated in the genera *Prevotella*, *Porphyromonas* and *Campylobacter*) and *Staphylococcus aureus*. *Prevotella melaninogenica* (formerly *Bacteroides melaninogenicus*) require vitamin K-like substance which can be provided by *Staphylococcus*. Another example is *Veillonella* species that can metabolise lactic acids produced by saccharolytic *Streptococcus* and *Actinomyces* species (Mikx and van der Hoeven, 1975). *Veillonella* species can also induce *Streptococcus* to produce amylase (Egland *et al*., 2004). In addition, highly proteolytic *Porphyromonas gingivalis* can benefit other oral bacteria which are inefficient in proteolytic activity (Potempa *et al*., 1995). A study showed that *Fusobacterium nucleatum* and *Prevotella intermedia* are able to metabolize amino acids to ammonia. This will prevent the lowering of pH and protect pH sensitive bacteria like *P. gingivalis* (Takahashi, 2003).

Antagonism is a relationship where one organism inhibits or kills other organisms. For example, *Streptococcus mutans* can inhibit *S. sanguinis* by producing

lactic acid and mutacins while *S. sanguinis* can inhibit *S. mutans* with H_2O_2. *S. sanguinis* is generally correlated with good oral health because many periodontal pathogens are susceptible to H_2O_2 (Hillman *et al*., 1985). Noncariogenic *S. oligofermentas* can convert lactic acids to H_2O_2 and inhibit *S. mutans* (Tong *et al*., 2007). Besides that, accumulation of acids that result from carbohydrate breakdown by lactobacilli also can inhibit the proteolytic microbes. *Streptococcus salivarius* can produce bacteriocins that are active against *S. pyogenes* and some *Enterococcus* strains but not against unrelated bacteria. *S. pyogenes* also produce structurally similar bacteriocins against *S. salivarius* (Upton *et al*., 2001). Bacteriocins usually have narrow killing spetrum compared to antibiotics (Riley and Wertz, 2002). It was found that nigrescin, a 41-kDa bacteriocin produced by *Prevotella nigrescens,* is able to inhibit *P. gingivalis, P. intermedia, Tannerella forsythia* and *Actinomyces* sp. (Kaewsrichan *et al*., 2004).

Synergism is a relationship in which several species need to be together to enhance survival or growth. Synergism involves various factors like providing nutrients, growth factors and enzymes, creating favorable pH and oxidation-reduction potential, and defense against host resistance.

2.2.3 Tissue specificity of the oral bacterial species

Members of the normal bacterial flora prefer to colonize certain sites or tissues. This can be due to the availability of the required oxygen level, pH, temperature, nutrient source or growth factors for the growth of the microorganism are present at certain sites. Besides that, the microbes are able to colonize specific sites because they can adhere to that site specifically. The bacterial surface has ligands or adhesins which can interact with the host cell receptor. The bacterial adhesin can be the component of

the capsule, fimbriae or cell wall. As for host receptor, it is usually the glycoprotein on the surface of the host cell surface that plays a role in adherence. The bacterial adhesins bind specifically to the receptors on the given oral cavity surface (Gibbons *et al*., 1976). Some bacteria tend to cluster at specific locations within the oral cavity such as the tongue because of coaggregation. The late colonizers are able to adhere to early colonizers of the specific location and form the clustering of bacterial species. It was shown that *Porphyromonas gingivalis* usually coaggregate with several *Streptococcus* sp. to colonize certain locations (Kolenbrander, 1988). *Tannerella forsythia* and *F. nucleatum* were often found together in the same dental plaque (Sharma *et al*., 2005).

2.2.4 Changes in normal oral microflora with age

The oral cavity's ecological properties change with age and this affects the composition of the normal bacterial flora. The oral microflora can be influenced by tooth eruption, loss of teeth, oral hygiene, hormonal and immunity changes of the host. At birth, the oral cavity is composed mainly of the soft tissues of the lips, cheeks, tongue and palate. At birth the oral cavity is sterile but rapidly acquires microorganisms from the environment especially from mother or caregiver (Kobayashi *et al*., 2008; Lee *et al*., 2006). *Streptococcus salivarius* are the dominant bacteria and make up 98% of the total oral normal flora until the appearance of the milk teeth (Todar, 2008). The eruption of the teeth leads to the colonization of *Streptococcus gordonii*, *S. oralis*, *S. mitis* and *S. sanguinis*. These species are the early colonizers of teeth surfaces. *S. gordonii* attach to the tooth surface by using sspA/B (adhesin) which binds to salivary agglutinin (glycoprotein) on the tooth surface (Kolenbrander *et al*., 2002). Other species of bacteria will start to adhere to the early colonizer through adhesin and receptor interaction. This initiates the establishment of early dental plaque. The creation of gingival crevice increases the habitat for various anaerobic species of bacteria. The

complexity of the normal oral flora will continue to increase with time. The Bacteriodetes and Spirochaetes will begin to colonize the anaerobic gingival crevice. Studies showed that black-pigmented bacteria, Spirochaetes and *Actinomyces viscosus* were isolated more frequently in young adults than in children (Percival *et al*., 1991). It is also shown that elderly oral microflora were more diverse than young and middle-aged adults. It was suggested that the immunity changes in the elderly may result in higher bacterial diversity (Preza *et al*., 2009).

2.3 Development of the dental plaque

Dental plaque is the material which adheres to the solid substratum; it consists of bacterial cells (60-70% of the volume of the plaque), bacterial extracellular products and salivary polymers. Dental plaque is generally divided into supragingival plaque (on tooth surfaces) and subgingival plaque (within periodontal pockets) (Nishihara and Koseki, 2004). Supragingival plaque is dominated by Gram-positive bacteria while subgingival plaque is dominated by Gram-negative anaerobic bacteria (Aas *et al*., 2005). Plaque which is mineralized with calcium and phosphate is called calculus.

Plaque formation on teeth is initiated by a weak adhesion of the streptococci (cariogenic bacteria) to the salivary agglutinin (glycoproteins) on the teeth surface. Then stronger adhesion is formed by the secretion of extracellular glucans from the bacteria. The production of the extracellular glucans is upregulated when the bacteria adhere to any solid surfaces (Donlan and Costerton, 2002). For example, *algC* gene involved in extracellular polysaccharide synthesis was upregulated after adhesion to surfaces (Davies and Geesey, 1995). This will be followed by the coaggregation of additional bacterial species. Mixtures of bacterial species will embed in the matrix of salivary and bacterial extracellular polymers. The process will continue and leads to

formation of mature biofilm-dental plaque (Shen *et al*., 2005). The accumulation of the bacterial plaque can result in dental diseases such as dental caries and periodontal disease.

2.4 Disruption of microbial homeostasis and its role in oral diseases

In a homeostatic state, the normal microflora is important in protecting the host from exogenous pathogens. The normal oral flora makes it difficult for other microbes to establish on the oral cavity surface. They produce inhibitory substances (organic acids, hydrogen peroxide and bacteriocins) against the abnormal flora and induce the immune system to produce antibodies which cross-react with certain related pathogens. However, when homeostasis is disrupted, it will lead to disease. Interspecies interactions play a role in determining whether the virulence factors of the indigenous oral bacteria will cause damage in the healthy host (Kuramitsu *et al*., 2007). For example, the number of acidogenic and acid-tolerating species is higher in caries-associated dental plaque compared to normal dental plaque (Marsh, 2006). The acidogenic and acid-tolerating species like *Streptococcus mutans* and *Lactobacillus* have optimum growth at low pH. This is usually caused by high fermentable sugar content diet which disturbs the local habitat and selection that occurs on them. Healthy dental plaque will shift to disease (tooth decay) causing dental plaque.

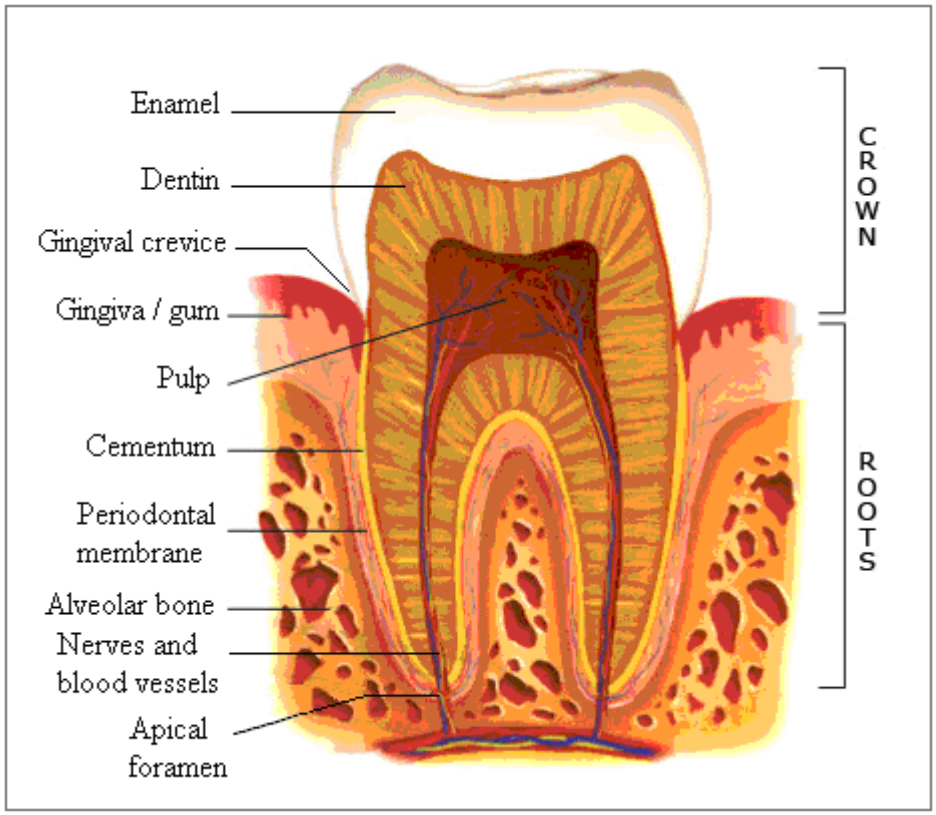

Figure 2.1 Tooth anatomy.

Dental caries is the destruction of the enamel, dentin or cementum of the teeth. Caries are caused by demineralization of the enamel due to lactic acid and other organic acids which accumulates in the dental plaque. Lactic acid bacteria like *Streptococcus mutans* ferment carbohydrates into lactic acid in the dental plaque. *S. mutans* adhere to tooth surface using SpaP adhesin (Kelly *et al*., 1999). *S. mutans* play an important role in initiation of dental caries because they can colonize the teeth surface and lead to plaque formation and then demineralization of enamel (Selwitz *et al*., 2007). Besides, *S. mutans* are able to convert sucrose to lactic acid more rapidly than other oral bacteria (Hamada and Slade, 1980). Various oral bacteria like *Lactobacillus* species and *Actinomyces* species can enter the exposed dentin and subsequently into dental pulp and contribute to the progression of lesions. The major factor that contributes to caries is a high fermentable sugar diet.

Periodontal diseases are bacterial infections that affect the supporting structure of the teeth which are the gingiva, cementum, periodontal membrane and alveolar bone. Gingivitis is a common periodontal disease. This leads to inflamed gingiva caused by the bacterial plaque inside the gingival margin or gingival crevice. The symptoms are bleeding, redness and swelling of the gingiva. Gingivitis is a reversible condition (Kobayashi *et al*., 2008). The serious form of periodontal disease affects periodontal membrane and alveolar bone that can result in tooth loss. It is also called periodontitis and is irreversible. The shift of gingivitis to periodontitis is caused by imbalanced interaction between the microbial load within the periodontal pocket and the host defense mechanisms which favor tissue degradation (Sbordone and Bortolaia, 2003). This is because of the overgrowth of the putative pathogens that overwhelm the host defense mechanism (Teughels *et al*., 2009). In most cases, periodontitis is chronic, with only small percentage of patients having aggressive or acute periodontitis. The pathogenesis of periodontal disease is associated with the bacterial hydrolytic enzymes (callogenase and hyaluronidase), endotoxins and metabolites. Gram-positive (*Actinomyces*, *Streptococcus*) and Gram-negative (Spirochaetes, Bacteroidetes) bacteria are associated in periodontal diseases. *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans*, *Tannerella forsythia* (formerly *Bacteroides forsythus*), *Prevotella intermedia, Dialister pneumosintes, Eubacterium nodatum* and *Treponema denticola* are strongly associated with periodontitis (Shaddox and Walker, 2009; Slots, 2004). *A. actinomycetemcomitans* possess an important virulence factor, RTX leukotoxin. This 114kDa leukotoxin can help in evading the host immune response and lyses erythrocytes (Balashova *et al*., 2006). In addition, the severity of the periodontal disease is associated with the presence of *T. denticola* and *T. forsythia* (Miyamoto *et al*., 2009).

Halitosis or oral malodor (bad breath) is a common problem following incidences of dental caries and periodontal diseases (Rayman and Almas, 2008; Scully and Greenman, 2008). Odor in breath can happen in all age groups. Bad breath in the morning is common. Persistent halitosis can affect individual social communication and it can happen to periodontally healthy subjects (Riggio *et al*., 2008). Halitosis is caused by the microbial conversion of cysteine, methionine, tryptophan, arginine and lysine into hydrogen sulfide, methylmercaptan, indole, putrescine and cadaverine respectively (Greenman, 1999; van den Broek *et al*., 2007). Hydrogen sulfide and methylmercaptan are the main volatile sulfur compounds (VSCs) which contribute to halitosis. A study showed that oral malodor compounds can be one of the factors that lead to periodontal disease and cancer (Yaegaki, 2008). VSCs cause the enhancement of oxidative stress and activation of mitogen activated protein kinase signaling pathway which could lead to cancer (Yaegaki, 2008). Microorganisms associated with halitosis are *Centipeda periodontii*, *Eikenella corrodens*, *Fusobacterium nucleatum*, *F. periodoticum*, *Porphyromonas endodontalis*, *P. gingivalis*, *Prevotella* (formerly *Bacteroides*) *melaninogenica*, *P. intermedia*, *P. loescheii*, *Solobacterium moorei*, *Tannerella forsythia* (formerly *Tannerella forsythensis*) and *Treponema denticola* (Scully and Greenman, 2008). These anaerobes and an anaerobic environment are important in generating volatile sulfur compounds in halitosis.

Oral diseases such as dental caries, periodontal diseases and halitosis are polymicrobial diseases and mostly putative pathogens are indigenous to human oral cavity unlike many medical pathogens that are foreign virulent microorganisms to our body. There is no specific single species which causes the particular disease mentioned above (Marsh, 2006; Slots, 2004; Teughels *et al*., 2009). *Streptococcus mutans* are strongly associated with dental caries. However, caries can occur in the absence of the

species while this species can persist without the detectable demineralization of the enamel (Marsh, 2006). However, the proportion of the putative pathogen in the dental plaque contributes to the diseases. Higher number of *S. mutans* and *Lactobacillus* in the plaque will enhance the demineralization of the enamel. But it is not necessary that only these species can cause caries. Other species with similar cariogenic characteristic can also lead to caries. In normal or healthy plaque, cariogenic putative pathogens are also detected but in low numbers (Marsh, 2006). *A. actinomycetemcomitans* has strong association with periodontitis, but healthy individuals harbor this bacteria in lower numbers compared to the dental plaque of patients with periodontitis (Shaddox and Walker, 2009; Socransky and Haffajee, 2005). Halitosis also is linked to the microbial load on the tongue rather than the presence of specific bacteria (Riggio *et al*., 2008; Scully and Greenman, 2008).

2.5 Antibiotics in the treatment of plaque-related oral diseases

Antibiotics are naturally occurring or synthetic organic substances that can inhibit (bacteriostatic) or kill (bacteriocidal) microorganisms. For example, tetracyclines are bacteriostatic and β-lactam antibiotics are bacteriocidal (Eliopoulos, 1989; Shaddox and Walker, 2009). Mechanically removing plaque by scaling and root planing are still the most effective way in the treatment and prevention of plaque-related diseases (Sbordone and Bortolaia, 2003). Antibiotics will be put into use when the conventional mechanical removal of bacteria gives unsatisfactory results. Usually antibiotics are used for acute periodontitis or recurring dental diseases (Shaddox and Walker, 2009; Slots, 2004).

After disrupting the biofilm, antibiotics can be used to achieve greater effectiveness. This is because intact biofilms are more resistant to antibiotics. The

highly hydrated anionic exopolymer matrix can prevent or delay the antibiotics from reaching the targeted bacteria within the biofilm. Study by Suci *et al*. (1994) showed delayed penetration of ciprofloxacin into *Pseudomonas aeruginosa* biofilms. This is because most of the antibiotics are positively charged and the polysaccharides of the matrix are neutral or negatively charged. Besides, some species within the biofilm may be able to produce enzymes that inactivate the antibiotic and this will provide protection for other species within the same biofilm. The antibiotic will also be less effective in killing the bacteria which have much slower growth rate in biofilm state comparing with their planktonic state (Donlan and Costerton, 2002; Sbordone and Bortolaia, 2003). The behavior of the bacteria growing in pure culture is very different from the same bacteria growing in mixed cultures on solid surface. This is due to the communication between the mixed species (Donlan and Costerton, 2002). *Streptococcus sanguis* surface expression of fibronectin and collagen adhesins was upregulated in biofilm state compared to planktonic state (Black *et al*., 2004).

Antibiotics commonly used in oral diseases are tetracycline, amoxicillin, spiramycin-metronidazone, erythromycin, azithromycin, clarithromycin, ciprofloxacin, amoxicillin-clavulanic acid and clindamycin. Amoxicillin-clavulanic acid is the most frequently prescribed treatment, followed by spiramycin-metronidazone and lastly clindamycin (Poveda-Roda *et al*., 2007; Slots, 2004). It is found that amoxicillin-clavulanic acid, clindamycin and moxifloxacin are the most effective treatment for odontogenic infections (Poveda-Roda *et al*., 2007).

Different countries have different management methods for the use of antibiotics. So the resistant rates may differ between countries. Horizontal gene transfer can be one potential mechanism which leads to unwanted resistance. Genetic

exchange of antibiotic resistance genes via conjugation or transformation could happen. This is because of the close proximity of the bacteria residing in the biofilm (Kuramitsu and Trapa, 1984). It has been shown that DNA fragments from lysed *S. mutans* in the *in vitro* biofilm can transform nearby bacteria (Li *et al*., 2001).

Antibiotic resistance of oral bacteria is gradually increasing, especially in *Porphyromonas* and *Prevotella* (Poveda-Roda *et al*., 2007). *Prevotella* species have been found to be resistant to ampicillin and amoxicillin (Handal *et al*., 2003). Many studies have found that oral bacteria show increasing penicillin resistance. Studies in United Kingdom found penicillin resistance rose from 3% in 1986 to 23% of the isolates in 1995 (Lewis *et al*., 1989 and 1995). Similar cases were reported in United States, Sweden, and Japan (Brook *et al*., 2005). A study has found that 15% of the isolates are resistant to penicillin V and 55% of the isolates are resistant to metronidazole (Baumgartner and Xia, 2003). The frequency of β-lactamase producing bacteria is increased in odontogenic infection patients who have previously been given β-lactam antibiotics (Kuriyama *et al*., 2000). In Kuwait, it was found that 25% of the viridans group streptococci (VS) were resistant to penicillin and 35% of VS were resistant to erythromycin (Mokaddas *et al*., 2007). *Eikenella corrodens* (Gram-negative facultative anaerobic bacillus) is resistant to clindamycin (Walker and Gordon, 1990). *A. actinomycetemcomitans* is found to be resistant to tetracycline (Shaddox and Walker, 2009). In one Taiwan study, 11% of *Fusobacterium nucleatum* isolates were resistant to ampicillin and amoxicillin. 8.4% of *E. corrodens* isolates and 11.4% of *F. nucleatum* isolates were resistant to amoxicillin-clavulanate. 30% of *E. corrodens* isolates and 17% of *F. nucleatum* isolates were resistant to clindamycin. Tetracycline and erythromycin are not effective against oral pathogenic bacteria (Chan and Chan, 2003). In Japan, 88.9% of *Neiserria subflava* isolates have intermediate resistance to penicillin

and 8.8% are resistant to it. 60% of *N. subflava* have intermediate resistance to tetracycline and 28.9% are resistant (Furuya *et al*., 2007). It was found that penicillin G resistant *F. nucleatum* strains increased with age and usage of the antibiotic in children (Nyfors *et al*., 2003).

Antibiotic resistance among human pathogens is emerging. Therefore, the use of antibiotics should be restrictive and conservative. However, if mechanical therapy is not working then the use of antibiotics is reasonable. The use of antibiotics can increase the number of untargeted or harmless normal oral flora and prevent colonization by putative pathogens. Susceptibility or resistance profiles of the oral bacterial species can be a valuable guide to determine the effectiveness of certain antibiotics against certain bacterial profiles.

2.6 Antibiotics to prevent infections

The use of naturally occurring or synthetic organic substances to prevent microbial infection is known as chemoprophylaxis. In dentistry, it is used to prevent infection at the surgical site and different sites of the body including infective endocarditis and joint infections.

Infective endocarditis is a microbial infection of the endocardial surface (usually damaged heart valves). In most cases of infective endocarditis and joint infections, *Staphylococcus aureus* and oral streptococci (α-hemolytic viridans streptococci) are the most frequent isolates. It is found that about 50% of bacterial endocarditis cases are caused by oral streptococci while 5% of joint infections cases are also caused by oral streptococci (Moreillon and Que, 2004; Seymour and Hogg, 2008). Many dental procedures (tooth extraction and surgery) and even normal tooth brushing

and chewing can result in bacteremia. It is very rare that invasive dental procedures and care measures cause bacterial endocarditis and joint infections (Poveda-Roda *et al*., 2007; Seymour and Hogg, 2008). But it is not less important as the risk is still there especially with patients using prosthetic heart valves or prosthetic joints. Poor oral health is one of the risk factors that lead to infective endocarditis and joint infections (Seymour and Hogg, 2008).

Chapter 3

Materials and methods

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3.1 Preparation of Columbia blood agar

The appropriate weight of Columbia blood agar base (Oxoid, Basingstoke, England) was taken at the rate of 39g per liter. The medium was dissolved in the right amount of distilled water. Medium solution was loaded into Schott bottle (autoclave tape was stuck on the bottle) and sent for autoclaving (15psi, 121^{o}C for 15 minutes). After autoclaving, the medium was cooled down to 45^{o}C in a water bath. 5% (final v/v) of rabbit blood was loaded into the medium. Sterile filtered (using 0.2μm pore size cellulose acetate sterile syringe filter) 0.0005 % (final w/v) hemin solution and 0.00005% (final w/v) vitamin K_3 solution was added into the medium. The medium was shaken well and poured into Petri dishes under aseptic condition. All procedures were done in a laminar flow cabinet. The medium was left to solidify. The Petri dishes were inverted and sealed with parafilm (Parafilm, USA). The media were incubated at 37^{o}C for 48 hours to detect contamination. The media were stored at 4^{o}C.

3.1.1 Preparation of rabbit blood

The study was approved by University of Malaya ethics committee (reference no: ISB/05/08/2009/TWY (R)). The New Zealand White rabbit was placed into a restrainer. The fur covering the ear central artery was removed. The central artery area was wiped with 70% ethanol (Merck, Darmstadt, Germany). The central artery was dilated by tapping it. A 26G (1/2 inch length) needle (Terumo, Tokyo) attached with a 5ml syringe was inserted into the artery as distally as possible. If another insertion was required, the needle was moved about 1 inch away from the previous insertion site (that is, tip to base of the ear). The blood should flow into the syringe (Nipro, Japan) if inserted correctly. The syringe was held firmly to the ear to avoid any movement of the needle thereby injuring the ear. The blood was drawn gently. In the possible event that the blood flow is too slow, massaging or pushing the blood towards the syringe may

help. Once the syringe was full (this step must be done rapidly to avoid any blood clotting), the needle was taken out and the bleeding stopped by pressing on the insertion site with sterile tissue paper. The insertion site was wiped with 70% ethanol after the bleeding was stopped. The cap of the universal bottle containing glass beads was loosened and the mouth of the bottle was flamed using a Bunsen burner. The syringe was disconnected from the needle and put near to the Bunsen flame. The blood was ejected into the universal bottle. The cap of the universal bottle was tightened. The bottle with blood was shaken for three minutes. 20ml of blood will be collected from each rabbit (~3kg)*. The same procedures were repeated for the other ear. After collecting 20ml of blood, the rabbit was returned to the cage.

* Note: rabbit blood volume is generally 56ml/kg body weight; blood sampling limit at 15% of the total blood volume (McGuill and Rowan, 1989)

3.1.2 Preparation of hemin stock solution 0.05% (w/v)

0.05g of bovine hemin (Sigma, Steinheim, Germany) was dissolved in 5ml of 1M NaOH (Merck, Darmstadt, Germany) (0.2g NaOH pellet in 5ml distilled water). The hemin solution was dissolved in 100ml of distilled water. The final solution was kept in an amber bottle at 4^{o}C. This solution should not be kept for over two months.

3.1.3 Preparation of vitamin K_3 (menadione) stock solution 0.005% (w/v)

0.01g of menadione (Fluka, Steinheim, Germany) was dissolved in 2ml of 95% ethanol (Merck, Darmstadt, Germany). 198ml of distilled water was used to dissolve the vitamin K_3 solution. The final solution was kept in an amber bottle at 4^{o}C to protect from light and heat.

3.2 Sampling and storage of oral bacterial cultures

3.2.1 Inclusion criteria for study subjects

An information sheet and consent form was given to each volunteer. All volunteers in the study consented to take part in the study. The volunteers consisted of three Malay, three Chinese and three Indian subjects. Their ages ranged from 21-26 years (mean = 22). All participants were males and had no observable symptoms like tooth decay, gingival inflammation, deep periodontal pocket and severe oral malodor based on their dental records. The subjects did not receive any antibiotics for three months preceding the sampling and they were non-smokers. All subjects had no general health problems.

3.2.2 Sampling of oral bacteria

The sampling was done in the morning between 9 and 10am. The subjects refrained from oral hygiene measures and consumption of food on the sampling day until sampling was completed. Oral bacteria were collected from teeth surfaces and gingival crevices of the volunteers using sterile Gracey curette (Nordent, USA). Six teeth (two premolars and four molars) were randomly selected for sampling and the samples were pooled. This method was also adopted for the sampling of the gingival crevices. Sterile cotton swabs were used to collect tongue cultures by swabbing the tongue six times and then pooling them. Collected samples were suspended in a universal bottle which contained 3ml of Reduced Transport Fluid (RTF) and glass beads. After collection, the bottle was shaken for two minutes. The sample suspension was diluted to 10^{-1}, 10^{-2} and 10^{-3}. 100µl of each diluted samples were spread on Columbia blood agar plates. Each dilution was done in duplicate. The plates were incubated aerobically and anaerobically at 37^{o}C for four days. The anaerobic condition was achieved by using AnaeroJar™ (Oxoid, Basingstoke, England) and AnaeroGen™

(Oxoid, Basingstoke, England). The colony forming unit (CFU) was taken for plates that had 30-300 colonies. Distinct colonies (1-2 colonies of apparently same morphology) were randomly selected and streaked separately on Columbia blood agar plates. Pure cultures were obtained by several streak plates.

3.2.3 Preparation of Reduced Transport Fluid (RTF)

Solution A was prepared by dissolving 0.6g of K_2HPO_4 (Sigma, Steinheim, Germany) in 100ml of distilled water. Solution B was prepared by dissolving 1.2g of NaCl (BDH, Poole, England), 1.2g of $(NH_4)_2SO_4$ (Sigma, Steinheim, Germany), 0.6g of KH_2PO_4 (BDH, Poole, England), and 0.5g of $MgSO_4.7H_2O$ (Merck, Darmstadt, Germany) in 100ml of distilled water. RTF was prepared freshly by dissolving 1.5ml of solution A, 1.5ml of solution B, 0.0058g of EDTA (BDH, Poole, England), 0.008g of Na_2CO_3 (BDH, Poole, England), and 0.004g of DL-Dithiothreitol (Fluka, Steinheim, Germany) in 17ml of distilled water to make up to a total volume of 20ml. The RTF was filtered using 0.2µm cellulose acetate syringe filter (Sartorius Stedium, Goettingen, Germany) aseptically.

3.2.4 Preparation of 15% (v/v) glycerol stock for aerobic bacteria

Pure colonies were taken using sterile inoculation loops. Then it was suspended in 769µl of Brain Heart Infusion (Oxoid, Basingstoke, England). 231µl of sterile 65% (v/v) glycerol was added and mixed well. The 1.5ml microcentrifuge tube containing the bacteria stock culture in the glycerol solution was kept at -80^{o}C.

3.2.5 Preparation of 20% (w/v) skimmed milk for anaerobic bacteria stock

20g of Skim milk powder (Oxoid, Basingstoke, England) was weighed and dissolved in 100ml of distilled water. The skim milk was autoclaved at 121^{o}C for 5

minutes and cooled down before using. Pure colonies of anaerobes were picked and suspended in 1ml of 20% skim milk and kept at -80^{o}C.

3.3 Bacterial identification by conventional methods

3.3.1 Gram Staining

One drop of sterile distilled water was placed on the glass slides. Single colonies were picked and smeared on the distilled water. The slides were air dried. The samples were fixed on the slides by briefly flaming. 1% (w/v) methyl violet solution was dropped on to the samples. Two to three drop of 5% (w/v) $NaHCO_3$ solution was added to the methyl violet solution on the samples. After 45 seconds, the slides were rinsed with water. Iodine solution was dropped on the samples. After 45 seconds, the slides were rinsed with water. The slides were again rinsed with acetone (BDH, Poole, England) for 10 seconds to avoid excessive de-staining. Then water was used to wash away the excess acetone on the slides. 2% (w/v) safranin solution was dropped on to the samples. After 45 seconds, the slides were rinsed with water. After that the slides were air dried. The samples were observed under a light microscope. 1% (w/v) methyl violet was prepared by dissolving 1g of methyl violet (Merck, Darmstadt, Germany) in 100ml of distilled water. 5% (w/v) $NaHCO_3$ was prepared by dissolving 1g of $NaHCO_3$ (Sigma, Steinheim, Germany) in 20ml of distilled water. Iodine solution was prepared by dissolving 2g of potassium iodide (Merck, Darmstadt, Germany) in 100ml of distilled water. 2% (w/v) safranin was prepared by dissolving 2g of safranine (Merck, Darmstadt, Germany) in 100ml of distilled water.

3.3.2 Catalase test

Pure colonies were picked and smeared on the glass slide. 3% (v/v) Hydrogen peroxide (BDH, Poole, England) was dropped on the sample. Positive result was indicated by bubbling.

3.3.3 Oxidase test

Freshy prepared 1% (w/v) tetramethyl-p-phenylenediamine dihydrochloride (Fluka, Steinheim, Germany) solution was dropped on filter paper. Pure colonies were picked and smeared on the spot of filter paper with reagent using sterile tooth picks. Positive result was indicated by purple color formation.

3.3.4 Indole test

Sulfide production, indole formation and motility (SIM) medium was prepared by dissolving 2g of peptone (Difco, France), 0.61g of beef extract (Oxoid, Basingstoke, England), 0.02g of ferrous ammonium sulfate (Fluka, Steinheim, Germany), 0.02g of sodium thiosulfate (Sigma, Steinheim, Germany) and 0.35g of agar (Difco, France) in 100ml of distilled water. The solution was boiled. 3ml of SIM medium was added into each test tube. After the tubes were capped, the tubes were sent for autoclaving. After autoclaving, the SIM tubes were incubated at 37^{o}C to detect for any contamination. Pure culture was inoculated in SIM tubes by using inoculation needle and incubated aerobically at 37^{o}C for one day. Kovac's reagent was prepared by dissolving 0.5g of 4-dimehylamino benzaldehyde (BDH, Poole, England) in 7.5ml of Isoamyl alcohol (Merck, Darmstadt, Germany) and then added with 2.5ml of HCl (BDH, Poole, England). 2ml of Kovac's reagent was added onto the SIM medium. Positive result was indicated by red color formation from yellow color.

3.3.5 H_2S production test

Each culture was inoculated in duplicate SIM tubes and incubated aerobically at 37^{o}C for one day. Positive result was indicated by black color formation in the SIM tubes.

3.3.6 Methyl red and Voges-Proskauer test (MR-VP test)

MR-VP broth was prepared by dissolving 0.7g of peptone, 0.5g of K_2HPO_4 and 0.5g of glucose (BDH, Poole, England) in 100ml of distilled water. 3ml of MR-VP broth was loaded into duplicate test tubes and capped. The broth was autoclaved and incubated at 37^{o}C to detect for any contamination. MR-VP broth was inoculated with culture and incubated aerobically at 37^{o}C for one day. 1ml of the broth was transferred into a new sterile test tube. One tube was used for MR test and the other tube was used for VP test. For MR test, MR reagent was prepared by dissolving 0.001g of methyl red (Sigma, Steinheim, Germany) in 3 ml of 95% ethanol and topped up to 5ml with sterile distilled water. Five to six drops of MR reagent was introduced into the broth using a dropper. Positive result was indicated by red color. For VP test, reagent A was prepared by dissolving 0.5g of α-napthol (BDH, Poole, England) in 10ml of 95% ethanol. Reagent B was prepared by dissolving 4g of KOH pellet (BDH, Poole, England) in 10ml of sterile distilled water. Five to six drops of reagent A and reagent B were added into the broth and mixed. The mixture was incubated at room temperature for 20 minutes. Positive result was indicated by the formation of a red layer on the broth surface.

3.3.7 Nitrate test

Nitrate broth was prepared by dissolving 0.3g of beef extract, 0.5g of peptone and 0.2g of potassium nitrate (BDH, Poole, England) in 100ml of distilled water. 3ml

of broth was loaded into test tube and Durham tube (Samco, USA) was also included into the test tube. The tubes were capped and autoclaved. The broth was incubated at 37^{o}C to detect for any contamination. The broth was inoculated with pure culture and incubated aerobically at 37^{o}C for one day. Nitrate reagent A was prepared by dissolving 0.08g sulfanilic acid (Sigma, Steinheim, Germany) in 10ml of 5N acetic acid (5ml of acetic acid added with 12.5ml of distilled water). Nitrate reagent B was prepared by dissolving 0.05g of N-(1-naphthyl)-ethylenediamine (Sigma, Steinheim, Germany) in 10ml of 5N acetic acid (Merck, Darmstadt, Germany). Five to six drops of nitrate reagent A and nitrate reagent B was added into the broth. Positive result was indicated by red color. Production of gas was detected by the presence of bubbles in the Durham tubes. Negative result was colorless. If negative, zinc dust (Merck, Darmstadt, Germany) was added into the broth. After adding zinc dust, red color indicated that negative result is confirmed.

3.3.8 Esculin hydrolysis

Esculin agar slant was prepared by dissolving 0.1g of esculin (Sigma, Steinheim, Germany), 0.3g of beef extract, 0.5g of peptone, 0.05g of ferric citrate (BDH, Poole, England) and 1.5g of agar in 100ml of distilled water. The solution was boiled. 3ml of the solution was loaded into test tube. The tubes were capped and autoclaved. After autoclaving, the tubes were placed slanted and left to solidify. The slants were incubated at 37^{o}C to detect for any contamination. The slant was inoculated with culture and incubated aerobically at 37^{o}C for one day. Positive result was indicated by black color formation.

3.3.9 Starch hydrolysis

Starch agar plate was prepared by dissolving 0.3g of beef extract, 1g of soluble starch (Merck, Darmstadt, Germany) and 1.2g of agar in 100ml of distilled water. The starch medium was autoclaved and poured into Petri dish. The Petri dish was left to solidify. The Petri dish was sealed with parafilm and incubated at 37^{o}C to detect for any contamination. The culture was streaked in a single line on the starch medium and incubated aerobically at 37^{o}C for one day. Iodine solution was dropped on the culture. Positive result was indicated by clearing zones around the culture. The iodine stains starch agar with dark blue color.

3.3.10 Gelatin hydrolysis

Gelatin tube was prepared by dissolving 0.15g of beef extract, 0.25g of peptone and 6g of gelatin (Merck, Darmstadt, Germany) in 50ml of distilled water. The solution was boiled. 3ml of solution was loaded into the test tube. The tubes were capped and autoclaved. The gelatin tube was incubated at 37^{o}C to detect for any contamination. The gelatin tube was inoculated with culture and incubated aerobically at 37^{o}C for one day. The gelatin tube was put into a refrigerator (4^{o}C) for 20 minutes. After that, positive result was indicated by liquefaction and negative result was indicated by solidification.

3.3.11 Phenol red fermentation

Phenol red broth was prepared by dissolving 1g of peptone, 0.5g of NaCl, 0.0018g of phenol red (Sigma, Steinheim, Germany) and 0.5g of sugar (glucose/lactose/sucrose/mannitol/trehalose/sorbitol) (Sigma, Steinheim, Germany) in 100ml of distilled water. 3ml of broth was loaded into test tube and then a Durham tube was introduced. The tubes were capped and autoclaved. The broth was incubated at

37^{o}C to detect for any contamination. Culture was inoculated into the phenol red broth and incubated aerobically at 37^{o}C for one day. Positive result was indicated by a change from red to yellow color. Production of gas was detected by the presence of a bubble in each Durham tube.

3.3.12 Simmon citrate utilization

Simmon citrate agar slant was prepared by dissolving 1.5g of agar, 0.5g of NaCl, 0.2g of sodium citrate (Sigma, Steinheim, Germany), 0.1g of K_2HPO_4, 0.1g of $(NH_4)H_2PO_4$ (Sigma, Steinheim, Germany), 0.02g of $MgSO_4.7H_2O$ and 0.008g of Bromothymol blue (BDH, Poole, England) in 100ml of distilled water. The mixture was boiled. 3ml of the mixture was loaded into test tube. The tube was capped and autoclaved. After autoclaving, the tubes were placed slanted and left to solidify. The media were incubated at 37^{o}C to detect for any contamination. Culture was streaked on the agar slant and incubated aerobically at 37^{o}C for one day. Positive result was indicated by the formation of blue color from green color medium.

3.3.13 Amino acid decarboxylase or dihydrolase test

Amino acid decarboxyalse/dihyrolase broth was prepared by dissolving 0.5g of peptone, 0.5g of beef extract, 0.05g of glucose, 0.01g of bromocresol purple (BDH, Poole, England), 0.0005g of cresol red (BDH, Poole, England), 0.0005g of pyridoxal (Sigma, Steinheim, Germany) and 1g of amino acid (lysine/ornithine/arginine) (Sigma, Steinheim, Germany) in 100ml of distilled water. 3ml of the mixture was loaded into test tubes. The tubes were capped and autoclaved. The broth was incubated at 37^{o}C to detect for any contamination. Each culture was then inoculated into the broth. The broth was overlaid with sterile mineral oil (BDH, Poole, England) and incubated aerobically at 37^{o}C for one day. Positive result was indicated by purple color.

3.3.14 Oxidative/Fermentation (OF) medium test

OF medium was prepared by dissolving 0.2g of peptone, 1g of glucose, 0.5g of NaCl, 0.03g of K_2HPO_4, 0.008g of bromothymol blue and 0.2g of agar in 100ml of distilled water. The mixture was boiled. 3ml of OF medium was added into the test tube. The tubes were capped and autoclaved. The media were incubated at 37^{o}C to detect for any contamination. Culture was inoculated into two tubes. One of the tubes was overlaid with sterile mineral oil and the other without mineral oil. The media were incubated aerobically at 37^{o}C for one day. Oxidative result was indicated by yellow color in tube without mineral oil and green color in tube with mineral oil. Fermentation result was indicated by yellow color in tube with mineral oil and green color in tube without mineral oil.

3.4 Bacterial identification by 16S rDNA sequence analysis

3.4.1 Genomic DNA extraction from Gram-negative bacteria

Genomic DNA extraction of the Gram-negative bacteria was done by using i-genomic CTB DNA extraction mini kit (iNtRON Biotechnology, Seongnam). Culture was grown on agar plates. Pure colonies were picked and suspended in 3ml sterile distilled water. The optical density was adjusted to 0.8-1.2 at 600nm wavelength. 2ml of culture suspension was transferred into 2ml micro-centrifuge tubes. Then centrifugation was done at 11337 x g for five minutes. The supernatant was discarded and the pellet was re-suspended by tapping the tube. 200µl of buffer CG, 10µl of proteinase K and 3µl of RNase A were added into the tube. The mixture was placed on a vortex for 10 seconds. The mixture was incubated at 65^{o}C for 30 minutes (the tube was shaken for every five minutes). 250µl of buffer CB was added into the mixture. The mixture was further mixed by inverting a few times. The mixture was left for 2 minutes and then 250µl of 80% ethanol was added into it. The mixture was mixed by

inverting and centrifuging for 10 seconds. 750µl of the mixture was transferred to the spin column provided in the kit. The spin column was then centrifuged at 11337 x g for one minute. The flow-through was discarded. The spin column was placed into new 2ml collection tubes provided in the kit. 700µl of buffer CW (added 40ml of 95% ethanol) was loaded into the spin column. The spin column was centrifuged at 11337 x g for one minute. The flow-through was discarded. The spin column was again centrifuged at 11337 x g for one minute to dry the column. The flow-through along with the 2ml collection tube was discarded. The spin column was placed into new 1.5ml microcentrifuge tubes. 100µl of buffer CE was loaded onto the columns and incubated for five minutes at room temperature. The spin column was centrifuged at 11337 x g for one minute. The spin column was discarded. The flow-through in the 1.5ml microcentrifuge tube was stored at -20^{o}C.

3.4.2 Genomic DNA extraction from Gram-positive bacteria

Genomic DNA extraction of the Gram-positive bacteria was done by using i-genomic BYF DNA extraction mini kit (iNtRON Biotechnology, Seongnam). Culture was grown on agar plates. Pure colonies were picked and suspended in 3ml sterile distilled water. The optical density was adjusted to 0.8-1 at 600nm wavelength. 2ml of culture suspension was transferred into 2ml microcentrifuge tubes. Then centrifugation was done at 11337 x g for five minutes. The supernatant was discarded and the pellet was re-suspended by tapping the tube. 100µl of buffer MP and 5µl of lysozyme were added into the tube. The mixture was mixed by vortex for 10 seconds. The mixture was incubated at 37^{o}C for one hour (the tube was shaken every 10 minutes). The tube was centrifuged at 11337 x g for three minutes. The supernatant was discarded and pellet was re-suspended by tapping. 200µl of buffer MG, 10µl of proteinase K and 5µl of RNase A was added into the tube. The mixture was placed on a vortex for 10 seconds.

The mixture was incubated at 65^{o}C for 30 minutes (the tube was shaken every five minutes). 250µl of buffer MB was added into the mixture. The mixture was mixed by inverting. The mixture was left for two minutes and then 250µl of 80% ethanol was added into it. The mixture was mixed further by inverting and centrifuging for 10 seconds. 750µl of the mixture was transferred to the spin column provided in the kit. The spin column was centrifuged at 11337 x g for one minute. The flow-through was discarded. The spin column was placed into new 2ml collection tubes provided in the kit. 700µl of buffer MW (added with 40ml of 95% ethanol) was loaded into the spin column. The spin column was centrifuged at 11337 x g for one minute. The flow-through was discarded. The spin column was again centrifuged at 11337 x g for one minute to dry the column. The flow-through along with the 2ml collection tube was discarded. The spin column was placed into new 1.5ml microcentrifuge tube. 50µl of buffer ME was loaded onto the column and incubated for five minutes at room temperature. The spin column was centrifuged at 11337 x g for one minute. The spin column was discarded. The flow-through in the 1.5ml microcentrifuge tube was stored at -20^{o}C.

3.4.3 Amplification of 16S rDNA by PCR

Universal primers for 16S rDNA (27f: 5'-AGA GTT TGA TCA TGG CTC AG-3' and 1492r: 5'-TAC GGC TAC CTT GTT ACG ACT T-3') were used for PCR amplification. PCR reaction mixtures (total volume of 20µl) contained 2µl of 10x PCR buffer, 2µl of dNTP mix (2.5mM each), 1µl of each primer (10pmoles), 1ng of DNA template, and 0.5µl of i-Taq^{TM} DNA polymerase (5U/µl) (iNtRON Biotechnology, Seongnam). PCR conditions were: initial denaturation at 94^{o}C for five minutes, 32 cycles of denaturation at 94^{o}C for 30 seconds, annealing at 50^{o}C for 40 seconds, extension at 72^{o}C for one minute and final extension at 72^{o}C for five minutes. The

amplification was done by Eppendorf Mastercycler personal (Eppendorf, Hamburg, Germany).

3.4.4 Amplification of partial *rnpB* gene from *Streptococcus* species

strF (5'-CGT GCA ATT TTT GGA TAA T-3') and strR (5'-TTC TAT AAG CCA TGT TTT GT-3') were used to amplify *rnpB* gene from *Streptococcus* species with ambiguous identity. PCR reaction mixtures (total volume of 20µl) contained 2µl of 10x PCR buffer, 2µl of dNTP mix (2.5mM each), 1µl of each primer (10pmoles), 1ng of DNA template, and 0.5µl of i-Taq^{TM} DNA polymerase (5U/µl). PCR conditions were: initial denaturation at 95^{o}C for five minutes, 32 cycles of denaturation at 94^{o}C for 40 seconds, annealing at 45^{o}C for 40 seconds, extension at 72^{o}C for 40 seconds and final extension at 72^{o}C for five minutes. The amplification was done by Eppendorf Mastercycler personal.

3.4.5 Agarose gel electrophoresis

0.5X TBE buffer was prepared by dissolving 2.7g of Tris-base (Sigma, Steinheim, Germany), 1.38g of boric acid (BDH, Poole, England), and 0.15g of EDTA in 500ml of distilled water. The pH of the buffer was adjusted to pH 8.3 with 1M HCl or 1M NaOH. The buffer was loaded into Schott bottle and autoclaved. 1.5% agarose gel was prepared by adding 0.6g of agarose (Sigma, Steinheim, Germany) into 40ml of 0.5X TBE buffer and boiled in microwave for two minutes. The gel solution in the 100ml flask was cooled down under running tap water. 2µl of ethidium bromide (Sigma, Steinheim, Germany) was added into the gel solution and mixed. The gel solution was loaded into an electrophoresis tray bearing combs each with 17 wells. The gel was left for 30 minutes to solidify. The comb was removed and the tray along with the gel was placed into the RunOne ™ System DNA electrophoresis equipment

(KomaBiotech, Korea). 0.5X TBE buffer was loaded into the tank to immerse the gel. 1µl of 6x loading dye (Sigma, Steinheim, Germany) was dropped on parafilm. 2µl of the PCR product was mixed with the loading dye on the parafilm. The mixture was loaded into the well of the gel. 1µl of 1Kb ladder marker (Bioneer, Daejeon, Korea) was loaded into separate wells of the gel for 16S rDNA PCR product. As for *rnpB* gene PCR product, 100bp DNA ladder GeneRuler^{TM} (Fermentas, Ontario, Canada) was used. Electrophoresis was run at 100V for 30 minutes. The gel was observed under UV. The PCR product (16S rDNA) should be in 1.4kbp size. The PCR product for *rnpB* gene should be about 400bp in size.

3.4.6 PCR product purification

The PCR product was purified by MEGAquick-spin PCR & Agarose Gel DNA Extraction System (iNtRON Biotechnology, Seongnam). 100µl of BNL buffer was loaded into PCR product solution (20µl). The mixture was mixed by pipetting and then left for two minutes. The spin column was placed into 2ml collection tube provided by the kit. The PCR product mixture was transferred into the spin column and centrifuged at 11337 x g for one minute. The flow-through was discarded. The spin column was placed into the same 2ml collection tube. 700µl of washing buffer was loaded into the spin column. The spin column was centrifuged at 11337 x g for one minute. The flow-through was discarded. The spin column was again centrifuged at 11337 x g for one minute to dry the column. The spin column was placed into new 1.5ml microcentrifuge tubes. 40µl of elution buffer was loaded to the column and incubated at room temperature for five minutes. The spin column was centrifuged at 11337 x g for one minute. The spin column was discarded. The flow-through in the 1.5ml microcentrifuge tube was stored at -20^{o}C.

3.4.7 Sequencing and bacterial identification

The purified PCR products and primers (forward and reverse) were sent to Macrogen Inc., Seoul to run the DNA sequencing by using ABI 3730xl DNA analyzer. The obtained sequences were analysed by using QualTrace program (www.nucleics.com/qualtrace-dna-sequencing-demo/index.html). Only sequences which passed the analysis were used to generate the full 1.4k bases 16S rDNA sequence for bacteria identification. The obtained reverse sequences were converted to antisense reverse sequence by FastPCR© program (www.biocenter.helsinki.fi/bi/Programs/fastpcr.htm). The forward sequence and antisense reverse sequence were aligned using bl2seq program from NCBI web site. After knowing the matching sequence, full length of 1.4k bases sequence was manually generated using Microsoft word program. The full length sequence was blasted at National Center for Biotechnology Information (NCBI) nucleotide collection (nr/nt) databases using megablast tool. The bacterial species were identified on the basis of at least 98% similarity to 16S rDNA sequences database. Cross-reference was also made to the Human Oral Microbiome Database (HOMD) (www.homd.org). As for *rnpB* gene sequences, forward and antisense reverse sequences were blasted in NCBI nucleotide collection (nr/nt) databases using megablast tool for identification of *Streptococcus* species.

3.5 Statistical analysis

Mean CFU/ml of the sampling sites were analysed for significant difference. SPSS 13 was used in the analysis. One-Way ANOVA Tukey test was used to analyse the significance. *P* values of <0.05 were considered statistically significant.

3.6 Phylogram construction

16S rDNA sequences of this study which matched with different NCBI database 16S rDNA sequences were selected for generating the phylogenetic tree. Clustalx (www.clustal.org) was used to carry out the multiple sequence alignment of the selected 16S rDNA sequences. The unrooted bootstrap neighbor-joining tree was generated by clustalx. The bootstrap trials were set at 1000. The tree was viewed by TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html)

3.7 Etest for antibiotics susceptibility

Pure cultures were picked and suspended in the broth*. The suspension turbidity was adjusted to one Mcfarland standard (Ab_{600} ~ 0.25). Sterile cotton swab was dipped into the suspension and then pressed against the wall of the container to remove the excess fluid. Then the cultures were streaked on the entire agar** surface, the plate was rotated while streaking to spread the culture evenly. The agar surface was dried before applying the strip. The Etest strips of amoxicillin-clavulanic acid (AB bioMerieux, Solna, Sweden) and clindamycin (AB bioMerieux, Solna, Sweden) were placed on the agar surface by forceps. The whole strip should be completely in contact with the agar surface. Air pockets under the strip were removed by pressing the strip. The plates were incubated at 37^{o}C for 48 hours under anaerobic or aerobic condition according to the bacteria. The anaerobic condition was achieved by using AnaeroJar™ and AnaeroGen ™. After incubation, an ellipse shaped zone of inhibition was seen around the strip. The minimum inhibition concentration (MIC) was read from the scale where the edge of inhibition ellipse intersects with the strip.

* Mueller Hinton broth (Difco, France) was used for *Streptococcus* sp., *Haemophilus* sp., *Campylobacter* sp., *Actinomyces* sp., *Staphylococcus* sp., *Pseudomonas* sp.

* Brucella broth (Difco, France) was used for anaerobes

** Mueller Hinton agar (MHA) (Difco, France) +5 % rabbit blood was used for *Streptococcus* sp., *Haemophilus* sp., *Campylobacter* sp., *Actinomyces* sp.

** MHA +2 % NaCl was used for *Staphylococcus* sp.

** MHA was used for *Pseudomonas* sp.

** Brucella agar (Difco, France) + 5% rabbit blood + vitamin K (1µg/ml) + hemin (5µg/ml) was used for anaerobes

3.8 Transmission Electron Microscopy (TEM)

3.8.1 Preparation of *Streptococcus salivarius* for Fixation

Pure colonies were suspended in 0.1M sodium cacodylate buffer (pH 7.4) inside 1.5mL microcentrifuge tubes. The suspension was centrifuged at 11337 x g for three minutes. The supernatant was discarded. Cacodylate buffer was loaded into the same tube to re-suspend the bacteria. These steps were repeated three times to wash the bacteria. About 3mm pellet was immersed in 4% (v/v) glutaraldehyde and then left at 4^{o}C for more than one day. 0.1M sodium cacodylate buffer was prepared by dissolving 21.4g of sodium cacodylate (Agar Scientific limited, Stansted, England) in 1L double distilled water. The pH was adjusted to 7.4 with HCl and stored at 4^{o}C. 4% (v/v) glutaraldehyde (Agar Scientific limited, Stansted, England) was prepared by mixing 80ml of filtered 25% glutaraldehyde and 420ml of 0.1M sodium cacodylate buffer. The solution was stored in amber bottle at 4^{o}C.

3.8.2 Washing

Cacodylate buffer was added into the microcentrifuge tube containing the pellet and left for 10 minutes. The content of the microcentrifuge tube was transferred into new mould. The supernatant was discarded and cacodylate buffer was again added and left for 10 minutes. These steps were repeated for three times. The pellet was immersed

with 1% (v/v) osmium tetroxide for two hours. The supernatant was discarded. The pellet was immersed with cacodylate buffer and left overnight. 1% (v/v) osmium tetroxide was prepared by mixing 1ml of 0.1M sodium cacodylate buffer and 1ml of 2% (w/v) osmium tetroxide. 2% (w/v) osmium tetroxide was prepared by dissolving 1g of osmium tetroxide in 50ml of double distilled water at room temperature for about 30 hours. The 2% (w/v) osmium tetroxide solution was stored at 4^{o}C in amber bottle.

3.8.3 Dehydration

The supernatant was discarded and added with double distilled water and left for five minutes. The distilled water was discarded and added with double distilled water again. The washing steps were repeated three times. 8% (w/v) uranyl acetate was added into the sample and left for 10 minutes. The sample was again washed by using double distilled water for three times. 35% ethanol was added into the sample and left for 10 minutes. After 10 minutes, 35% ethanol was replaced by 50% ethanol then left for 10 minutes. These steps were repeated for 70% ethanol, 95% ethanol and finally three times with 100% ethanol. 95% and 100% ethanol dehydration was done for 15 minutes each time. After removing from the third 100% ethanol dehydration, propylene oxide (Agar Scientific limited, Stansted, England) was added into the sample and left for 15 minutes. This step was repeated once more. After removing propylene oxide, propylene oxide: Epon (1:1 ratio) was added into the sample and left for 1 hour. After removing propylene oxide: Epon (1:1 ratio), propylene oxide: Epon (1:3 ratio) was added into the sample and left for 2 hours. After removing propylene oxide: Epon (1:3 ratio), Epon was added into the sample and left overnight. The next day, sample was embedded in Epon at 37^{o}C for five hours. Then it was overnight at 60^{o}C. 8% (w/v) uranyl acetate was prepared by dissolving 4g of uranyl acetate (Agar Scientific limited, Stansted, England) in 50ml of double distilled water. The solution was filtered with

0.22µm nitrocellulose filter (Agar Scientific limited, Stansted, England) and stored in an amber bottle at 4^{o}C.

3.8.4 Trimming

The block was clamped into a block holder and placed under a stereomicroscope. The excess embedding medium was trimmed away by razor blade. The block was trimmed to a trapezium shape.

3.8.5 Semithin sectioning and staining

The trimmed block was put into a microtome (Leica, Wetzlar, Germany) chuck. The glass knife was put into the knife holder. The microtome was set for 1µm semithin sections. The knife angle and block was adjusted to an appropriate position. The block surface was trimmed using 'E' region of the knife edge. The debris on the knife edge was removed by using a blower. Sections were picked up with fine forceps and transferred onto a drop of double distilled water on the clean glass slide. The glass slide was put on a hot plate to fix the section onto the glass slide. Toluidine Blue stain was dropped onto the dried semithin sections on the glass slide and left for one minute. The stain was washed off with distilled water. The sections were washed with 95% ethanol. The glass slide with the sections was washed with distilled water. The glass slide was air dried and observed under light microscope. Toluidine blue stain was prepared by mixing 4ml of solution A (1g of toluidine blue dissolved in 100ml of 1% borax solution) and 1ml of solution B (1g of pyronin G dissolved in 100ml of 1% borax solution). The toluidine blue stain was stored at room temperature in amber bottle. The stain was filtered through 0.22µm nitrocellulose before use.

3.8.6 Ultrathin sectioning, staining and viewing

The trimmed block was put into ultramicrotome (Leica, Wetzlar, Germany) chuck and clamped securely. Diamond knife (Drukker, The Netherlands) was put into the knife holder. Viewing through the stereomicroscope, the knife was adjusted very near to the specimen. Cutting speed was set at 1.2mm/s and cutting thickness was set at 70nm. The diamond knife was filled with double distilled water. Ultramicrotome was switched to the automatic cutting mode. After sufficient ultrathin sections have been cut, the automatic cutting mode was turned off. Forceps was used to grip copper grid (Agar Scientific limited, Stansted, England). The ultrathin sections were picked up by pushing clean copper grids down upon the sections which were floating on the water. The grids were air dried on filter paper. 8% uranyl acetate was dropped onto a piece of dental wax (Cavex, Haarle, The Netherlands) which was placed in a Petri dish. Grids were immersed in uranyl acetate and left for five minutes. The grids were washed with double distilled water three times and dried with filter paper. NaOH pellets were put around the dental wax. Lead citrate solution was dropped onto the dental wax. Grids were immersed in the lead citrate. The Petri dish was covered and left for 10 minutes. The grids were washed with double distilled water for three times and dried with filter paper. The grids were ready for viewing in Leo Libra 120 TEM (Germany) at 120kV with SI viewer software. Lead citrate solution was prepared by mixing 0.25g of lead citrate, 10ml of 1N NaOH and 100ml of double distilled water. The mixture was filtered and kept in amber bottle at 4^{o}C.

3.9 Scanning Electron Microscopy (SEM)

3.9.1 Preparation of *Streptococcus salivarius* for Fixation

Fixation steps for SEM were similar to the fixation steps used in TEM preparation.

3.9.2 Membrane and micro-carrier fixation and washing

Some of the pellets were transferred into a micro-carrier (Agar Scientific limited, Stansted, England). The carrier was capped properly and immersed in 4% (v/v) glutaraldehyde for one hour. The remaining pellet was re-suspended then dropped on nucleopore Track-Etch membrane (Whatman, Kent, UK). The membrane was immersed in 4% (v/v) glutaraldehyde for one hour. After one hour, 4% (v/v) glutaraldehyde was removed and added with cacodylate buffer. The washing steps were repeated three times. After washing, samples were immersed in 1% (v/v) osmium tetroxide for one hour. After one hour, the samples were washed with cacodylate buffer for three times. The samples were immersed in cacodylate buffer overnight at 4^{o}C.

3.9.3 Dehydration

The samples were restored to room temperature. The samples were washed with double distilled water two times. Each time the samples were immersed in double distilled water for 15 minutes. The samples were dehydrated in increasing concentrations of ethanol at 30%, 40%, 50%, 60%, 70%, 80% 90% and 95%. Each time the samples were immersed for 15 minutes. After that, samples were immersed in 100% ethanol for 15 minutes twice. Dehydration was continued with ethanol: acetone mixture in the ratios of 3:1, 1:1 and 1:3. Each time the samples were immersed for 15 minutes. After that, samples were immersed in pure acetone for 20 minutes thrice.

3.9.4 Critical Point Drying

The pressure chamber of the critical point dryer (Bal-Tec CPD 030, Leica, Germany) was cooled to 10^{o}C. The pressure chamber was filled with acetone. Membrane was transferred into a metal basket and placed into the pressure chamber along with the micro-carrier. The pressure chamber was filled with CO_2 up to the upper edge of the front sight glass. The liquid was drained till the specimens were just covered by the liquid. The filling and draining steps were repeated till acetone is completely replaced by liquid CO_2 (indicated by dry ice formation). The specimens were left in liquid CO_2 for 30 minutes. After that, filling and draining steps were repeated till dry ice was formed. The pressure chamber was heated till 40^{o}C (indicated by the liquid transforming to gas). The gas was released till zero pressure as indicated by the pressure gauge. Samples were then taken out.

3.9.5 Gold coating and viewing

The membrane was mounted on a stub with double-sided tape. Pellets from the micro-carrier were stuck on the double-sided tape and mounted on another stub. After mounting, the samples were coated with gold using Sputter coater (Leica EM SCD005, Wetzlar, Germany) for 30 seconds. The samples were viewed in the scanning electron microscope model Quanta 200 FFSEM (FEI, Germany).

Chapter 4

Results

Chapter 4: Results

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4.1 The distribution of anaerobic bacteria isolated from the oral cavity

The distribution and proportion of the isolated anaerobic bacterial species is tabulated below. The bacterial species were grouped according to the phylum. The phyla included Actinobacteria, Bacteroidetes, Fusobacteria, Firmicutes, and Proteobacteria. The number of the isolated bacterial species from three of the subjects of a given race was pooled.

Table 4.1 Number of anaerobic bacteria isolated from gingival crevices, teeth surfaces and tongue dorsa

Bacterial species	Sampling sites											
	Gingival crevices				Teeth surfaces				Tongue dorsa			
	M	C	I	Total (%)	M	C	I	Total (%)	M	C	I	Total (%)
Actinobacteria												
Actinomyces viscosus	1	1		2 (1.4)	1			1 (0.9)				0
Actinomyces naeslundii	1	7	5	13 (8.9)	2	4	5	11 (9.8)	1			1 (0.8)
Actinomyces sp.	1		1	2 (1.4)	2		1	3 (2.7)	1			1 (0.8)
Actinomyces odontolyticus		1	3	4 (2.7)	2	1	2	5 (4.5)	5	4	4	13 (11)
Actinomyces georgiae	1	2		3 (2.1)	1	2		3 (2.7)				0
Actinomyces cardiffensis			1	1 (0.7)				0				0
Actinomyces meyeri				0		1		1 (0.9)				0
Actinomyces oris		1	1	2 (1.4)	1		1	2 (1.8)				0
Corynebacterium durum				0		1		1 (0.9)				0
Rothia dentocariosa			2	2 (1.4)	2	1	4	7 (6.3)	1			1 (0.8)
Rothia aeria				0	4	2	1	7 (6.3)			1	1 (0.8)
Rothia mucilaginosa				0				0	1	2	1	4 (3.4)
Total* (%)				29 (19.9)				41 (36.6)				21 (17.8)
Bacteroidetes												
Capnocytophaga ochracea	3		1	4 (2.7)	1			1 (0.9)				0
Capnocytophaga granulosa	1	1	1	3 (2.1)	1	2	1	4 (3.6)				0
Capnocytophaga gingivalis	1	3	1	5 (3.4)		2	1	3 (2.7)		1		1 (0.8)
Capnocytophaga sp.	1		1	2 (1.4)				0		1		1 (0.8)
Capnocytophaga sputigena				0		1		1 (0.9)				0
Prevotella nigrescens	1	1		2 (1.4)		1		1 (0.9)				0
Prevotella intermedia	2	1	2	5 (3.4)			1	1 (0.9)				0
Prevotella buccae		2	1	3 (2.1)				0				0
Prevotella sp.	5		1	6 (4.1)				0				0
Prevotella tannerae	1			1 (0.7)				0				0
Prevotella melaninogenica	2			2 (1.4)		1		1 (0.9)	1	3		4 (3.4)
Prevotella salivae	1			1 (0.7)				0				0
Prevotella loescheii	1			1 (0.7)				0				0
Prevotella maculosa	3			3 (2.1)				0				0
Prevotella denticola				0	1			1 (0.9)				0
Prevotella nanceiensis				0				0	1			1 (0.8)
Prevotella oralis				0				0	1			1 (0.8)
Prevotella pallens				0				0			1	1 (0.8)
Total* (%)				38 (26)				13 (11.6)				9 (8)
Fusobacteria												
Fusobacterium nucleatum	3			3 (2.1)	3	2		5 (4.5)		1		1 (0.8)
Fusobacterium periodonticum				0				0	1	1		2 (1.7)
Leptotrichia trevisanii		1		1 (0.7)				0				0
Leptotrichia buccalis	1	2	2	5 (3.4)				0				0
Leptotrichia wadei	1			1 (0.7)				0		1		1 (0.8)
Leptotrichia sp.			2	2 (1.4)			1	1 (0.9)	1			1 (0.8)
Leptotrichia hofstadii	1	1	1	3 (2.1)	1	1		2 (1.8)				0
Leptotrichia goodfellowii			1	1 (0.7)				0				0
Total* (%)				16 (11)				8 (7.1)				5 (4.2)
Firmicutes												
Eubacterium sp.	1			1 (0.7)				0				0
Lachnospiraceae bacterium		1	1	2 (1.4)				0	1			1 (0.8)
Streptococcus sanguinis	1	1	1	3 (2.1)	3	5	3	11 (9.8)				0
Streptococcus mutans	1			1 (0.7)	4	1	1	6 (5.4)				0
Streptococcus anginosus	1		2	3 (2.1)			1	1 (0.9)				0
Streptococcus mitis	1	1	1	3 (2.1)			2	2 (1.8)	2	3	4	9 (7.6)
Streptococcus cristatus		2	1	3 (2.1)				0				0
Streptococcus gordonii				0	3		2	5 (4.5)				0
Streptococcus sp.				0			2	2 (1.8)	5		1	6 (5.1)
Streptococcus australis				0				0	1	1		2 (1.7)
Streptococcus infantis				0				0	1	2	1	4 (3.4)
Streptococcus parasanguis				0				0			3	3 (2.5)
Streptococcus parasanguinis				0			1	1 (0.9)	2	1	2	5 (4.2)
Streptococcus oralis	1	1	3	5 (3.4)	2		1	3 (2.7)			2	2 (1.7)
Streptococcus salivarius				0				0	2		1	3 (2.5)

Table 4.1, continued

Streptococcus constellatus			2	2 (1.4)				0				0
Streptococcus pneumoniae				0				0		1	2	3 (2.5)
Streptococcus intermedius		1		1 (0.7)				0				0
Staphylococcus pasteuri				0				0	2			2 (1.7)
Gemella morbillorum	3	3	2	8 (5.5)	1			1 (0.9)		1		1 (0.8)
Gemella haemolysans		1	2	3 (2.1)		1		1 (0.9)	1	1		2 (1.7)
Gemella sanguinis				0			1	1 (0.9)	2	4	4	10 (8.5)
Selenomonas sp.				0	1			1 (0.9)				0
Veillonella parvula				0				0	2	2	1	5 (4.2)
Veillonella dispar				0				0			1	1 (0.8)
Veillonella atyica				0				0			1	1 (0.8)
Total* (%)				35 (24)				35 (31.3)				60 (50.8)
Proteobacteria												
Campylobacter concisus	1			1 (0.7)				0				0
Campylobacter showae	2	1	1	4 (2.7)				0				0
Neisseria sp.		2		2 (1.4)		1	2	3 (2.7)			1	1 (0.8)
Neisseria meningitidis				0		1		1 (0.9)				0
Neisseria flavescens				0		1		1 (0.9)		1	2	3 (2.5)
Neisseria subflava				0				0		4	2	6 (5.1)
Neisseria flava				0		1		1 (0.9)				0
Haemophilus segnis	1	2		3 (2.1)				0		2	1	3 (2.5)
Haemophilus parainfluenzae	1	2		3 (2.1)	2	2	1	5 (4.5)	3	1	3	7 (5.9)
Haemophilus sp.	1	1		2 (1.4)				0				0
Haemophilus haemolyticus		1		1 (0.7)		1		1 (0.9)			1	1 (0.8)
Haemophilus parahaemolyticus				0		1		1 (0.9)	1			1 (0.8)
Eikenella corrodens	1	3	3	7 (4.8)		2		2 (1.8)		1		1 (0.8)
Kingella oralis	1			1 (0.7)				0				0
Aggregatibacter aphrophilus	2	2		4 (2.7)				0				0
Total* (%)				28 (19.2)				15 (13.4)				23 (19.5)
Total isolates	51	49	46	146 (100)	38	39	35	112 (100)	39	39	40	118 (100)

M = Malay; C = Chinese; I = Indian (each race consists of three subjects)

* = total isolates of the phylum

The number of the isolated bacterial species from three of the subjects of a given race was pooled.

Actinomyces georgiae, *A. oris*, *Capnocytophaga granulosa*, *Leptotrichia hofstadii*, and *Streptococcus sanguinis* were isolated only from gingival crevices and teeth surfaces in this study (Table 4.1). *Prevotella buccae*, *Leptotrichia buccalis*, *Streptococcus cristatus*, *Campylobacter showae* and *Aggregatibacter aphrophilus* were isolated only from gingival crevices. *Streptococcus gordonii* were isolated from teeth surfaces only. *Rothia mucilaginosa*, *Fusobacterium periodonticum*, *Streptococcus australis*, *S. infantis*, *S. salivarius*, *S. pneumoniae*, *Veillonella parvula* and *Neisseria subflava* were only isolated on tongue dorsa. *Haemophilus segnis* were isolated from gingival crevices and tongue dorsa only. *Actinomyces odontolyticus* and *Haemophilus parainfluenzae* were isolated from all the three sites. Of 146 isolates from gingival crevices, the predominantly isolated species were *Actinomyces naeslundii* (8.9%), *Gemella morbillorum* (5.5%) and *Eikenella corrodens* (4.8%). Of 112 isolates from teeth surfaces, the predominantly isolated species were *A. naeslundii* (9.8%), *S. sanguinis* (9.8%), *Rothia dentocariosa* (6.3%), *R. aeria* (6.3%), *Streptococcus mutans* (5.4%), *A. odontolyticus* (4.5%), *Fusobacterium nucleatum* (4.5%), *S. gordonii* (4.5%), and *H. parainfluenzae* (4.5%). Of 118 isolates from tongue dorsa, the predominantly isolated species were *A. odontolyticus* (11%), *Gemella sanguinis* (8.5%), *Streptococcus mitis* (7.6%), *H. parainfluenzae* (5.9%), and *N. subflava* (5.1%).

4.2 The distribution of the anaerobic bacterial phyla from sampling sites in the oral cavity

Bacteroidetes (26%) contributed the highest number of isolates and Fusobacteria (11%) formed the lowest number of isolates in gingival crevices of the nine Malaysian subjects (Figure 4.1). Based on figure 4.2, the teeth surfaces were colonized mainly by Actinobacteria (37%) and Firmicutes (31%). Fusobacteria (7%) constituted the lowest number of isolates on the teeth surfaces compared to other phyla. On tongue dorsa, Firmicutes were predominant which contributed 51% and Fusobacteria (4%) formed the lowest number of isolates (Figure 4.3).

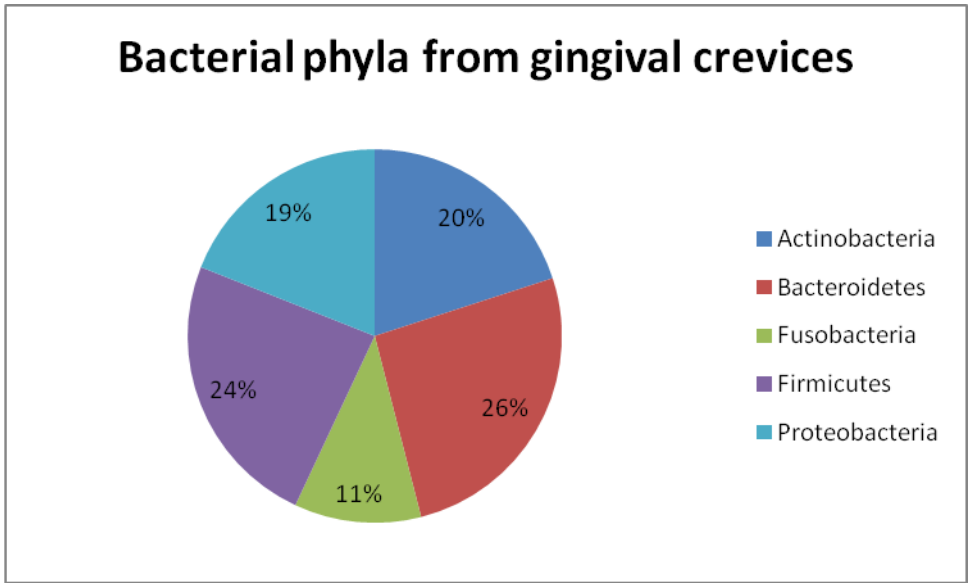


Figure 4.1 Distribution of anaerobic bacterial phyla isolated from gingival crevices. Percentages refer to the number of isolates (total number of isolates from the gingival crevices is 146) from each phylum.

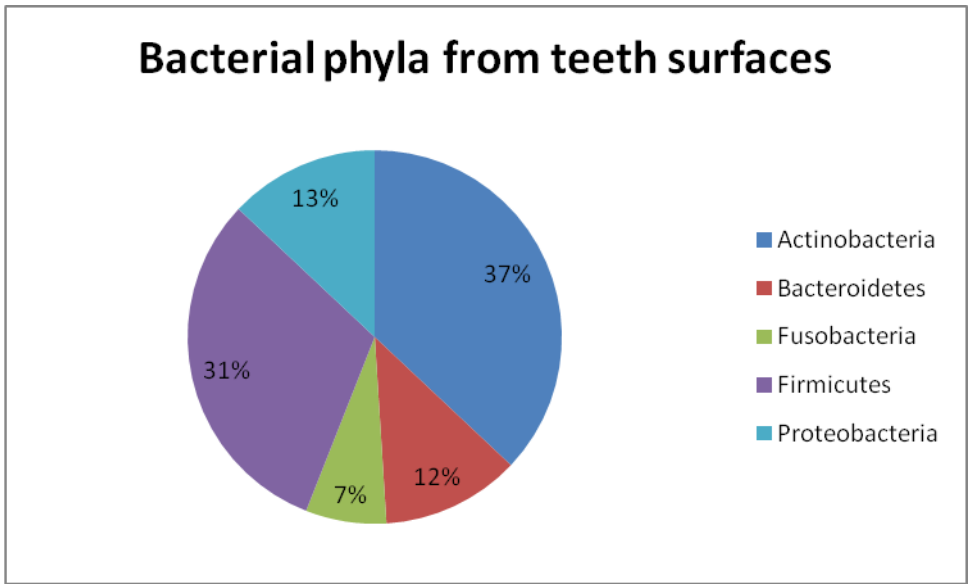


Figure 4.2 Distribution of anaerobic bacterial phyla isolated from teeth surfaces. Percentages refer to the number of isolates (total number of isolates from teeth surface is 112) from each phylum.

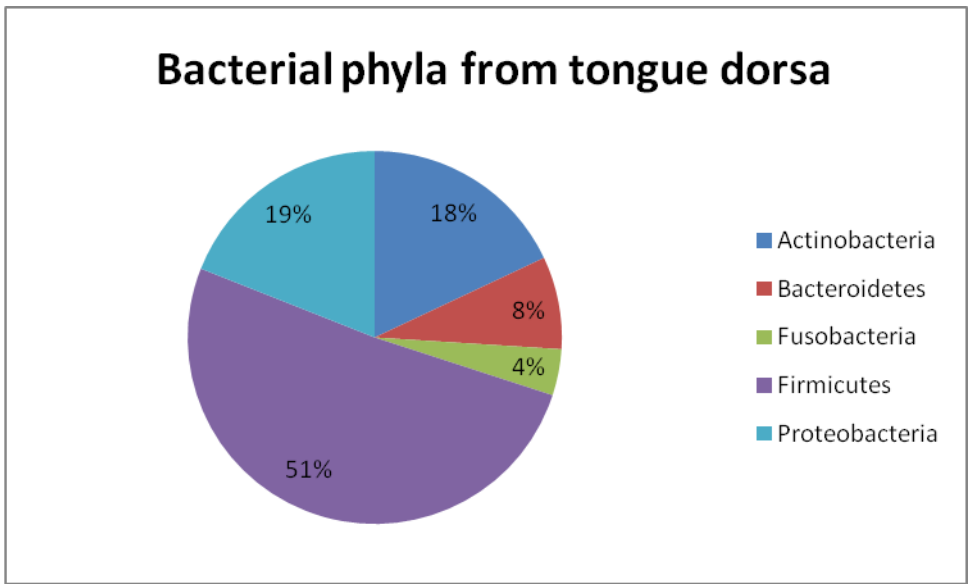


Figure 4.3 Distribution of anaerobic bacterial phyla isolated from tongue dorsa. Percentages refer to the number of isolates (total isolates from tongues is 118) from each phylum.

4.3 Distribution of anaerobic bacterial species in subject

4.3.1 Species from gingival crevice

Rothia dentocariosa and *Streptococcus constellatus* were detected in two of the Indian gingival crevices only (Table 4.2). *Prevotella melaninogenica*, *P. maculosa* and *Fusobacterium nucleatum* were detected in the gingival crevices of Malay subjects but

not in Chinese and Indian. *Aggregatibacter aphrophilus* were detected in the gingival crevices of two of the Malay and Chinese subjects only. *Leptotrichia wadei* and *Eubacterium* sp. were detected in the gingival crevices of M 1 subject only. *Prevotella salivae* and *P. loescheii* were detected in the gingival crevices of M 2 subject only. *Prevotella tannerae*, *Streptococcus mutans, Campylobacter concisus,* and *Kingella oralis* were detected in the gingival crevices of M 3 subject only. *Leptotrichia trevisanii*, *Streptococcus intermedius* and *Haemophilus haemolyticus* were detected in the gingival crevices of C 3 subject only. *Leptotrichia goodfellowii* were detected in the gingival crevices of I 2 subject only. *Actinomyces cardiffensis* were detected in gingival crevices of I 3 subject only.

Table 4.2 Anaerobic bacterial species in gingival crevices of each subject

	Subjects								
Bacterial species	**M 1**	**M 2**	**M 3**	**C 1**	**C 2**	**C 3**	**I 1**	**I 2**	**I 3**
Actinomyces viscosus		■				■			
Actinomyces naeslundii		■		■	■	■	■		■
Actinomyces sp.			■						■
Actinomyces odontolyticus					■		■		
Actinomyces georgiae	■					■			
Actinomyces cardiffensis									■
Actinomyces meyeri									
Actinomyces oris						■		■	
Corynebacterium durum									
Rothia dentocariosa							■	■	
Rothia aeria									
Rothia mucilaginosa									
Capnocytophaga ochracea	■	■							■
Capnocytophaga granulosa	■			■				■	
Capnocytophaga gingivalis	■			■		■			■
Capnocytophaga sp.			■					■	
Capnocytophaga sputigena									
Prevotella nigrescens		■				■			
Prevotella intermedia	■	■			■			■	■
Prevotella buccae					■				■
Prevotella sp.	■	■	■						■
Prevotella tannerae			■						
Prevotella melaninogenica	■	■							

Table 4.2, continued

Prevotella salivae		■							
Prevotella loescheii		■							
Prevotella maculosa		■	■						
Prevotella denticola									
Prevotella nanceiensis									
Prevotella oralis									
Prevotella pallens									
Fusobacterium nucleatum	■	■	■						
Fusobacterium periodonticum									
Leptotrichia trevisanii						■			
Leptotrichia buccalis			■	■	■		■	■	
Leptotrichia wadei	■								
Leptotrichia sp.							■		
Leptotrichia hofstadii		■				■	■		
Leptotrichia goodfellowii								■	
Eubacterium sp.	■								
Lachnospiraceae bacterium					■				■
Streptococcus sanguinis	■			■					■
Streptococcus mutans			■						
Streptococcus anginosus			■				■		
Streptococcus mitis	■				■			■	
Streptococcus cristatus						■			■
Streptococcus gordonii									
Streptococcus sp.									
Streptococcus australis									
Streptococcus infantis									
Streptococcus parasanguis									
Streptococcus parasanguinis									
Streptococcus oralis			■		■		■		
Streptococcus salivarius									
Streptococcus constellatus							■	■	
Streptococcus pneumoniae									
Streptococcus intermedius						■			
Staphylococcus pasteuri									
Gemella morbillorum			■	■	■	■	■	■	
Gemella haemolysans						■			■
Gemella sanguinis									
Selenomonas sp.									
Veillonella parvula									
Veillonella dispar									
Veillonella atypica									
Campylobacter concisus			■						
Campylobacter showae	■		■	■			■		

Table 4.2, continued

Neisseria sp.				■		■			
Neisseria meningitidis									
Neisseria flavescens									
Neisseria subflava									
Neisseria flava									
Haemophilus segnis	■					■			
Haemophilus parainfluenzae			■		■				
Haemophilus sp.		■		■					
Haemophilus haemolyticus						■			
Haemophilus parahaemolyticus									
Eikenella corrodens			■	■		■	■		■
Kingella oralis			■						
Aggregatibacter aphrophilus	■	■		■	■				

M = Malay; C = Chinese; I = Indian
The presence is indicated by the shaded box

4.3.2 Species from teeth surface

Actinomyces naeslundii and *Streptococcus sanguinis* were detected on the teeth surfaces of the three races (Table 4.3). *Fusobacterium nucleatum* were detected on teeth surfaces of two of the Malay and Chinese subjects only. *Eikenella corrodens* were detected on the teeth surfaces of two of the Chinese only. *Streptococcus mitis* were detected on teeth surfaces of two of the Indian subjects only. *Capnocytophaga ochracea, Prevotella denticola* and *Selenomonas* sp. were detected on the teeth surfaces of M 2 subject only. *Actinomyces viscosus* and *Gemella morbillorum* were detected on the teeth surfaces of M 3 subject only. *Prevotella melaninogenica* and *Neisseria flava* were detected on the teeth surfaces of C 1 subject only. *Capnocytophaga sputigena*, *Prevotella nigrescens*, and *Gemella haemolysans* were detected on the teeth surfaces of C 2 subject only. *Actinomyces meyeri, Corynebacterium durum, Neisseria meningitidis, N. flavescens, Haemophilus haemolyticus,* and *H. parahaemolyticus* were detected on the teeth surfaces of C 3 subject only. *Streptococcus anginosus, S. parasanguinis* and *Gemella sanguinis* were detected on the teeth surfaces of I 1 subject only. *Prevotella intermedia* were detected on the teeth surfaces of I 3 subject only.

Table 4.3 Anaerobic bacterial species on teeth surfaces of each subject

	Subjects								
Bacterial species	**M 1**	**M 2**	**M 3**	**C 1**	**C 2**	**C 3**	**I 1**	**I 2**	**I 3**
Actinomyces viscosus			■						
Actinomyces naeslundii	■	■			■	■	■	■	■
Actinomyces sp.	■		■						■
Actinomyces odontolyticus		■		■				■	■
Actinomyces georgiae		■		■	■				
Actinomyces cardiffensis									
Actinomyces meyeri						■			
Actinomyces oris		■							■
Corynebacterium durum						■			
Rothia dentocariosa	■	■		■			■	■	
Rothia aeria	■		■			■			■
Rothia mucilaginosa									
Capnocytophaga ochracea		■							
Capnocytophaga granulosa		■		■	■				■
Capnocytophaga gingivalis				■	■			■	
Capnocytophaga sp.									
Capnocytophaga sputigena					■				
Prevotella nigrescens					■				
Prevotella intermedia									■
Prevotella buccae									
Prevotella sp.									
Prevotella tannerae									
Prevotella melaninogenica				■					
Prevotella salivae									
Prevotella loescheii									
Prevotella maculosa									
Prevotella denticola		■							
Prevotella nanceiensis									
Prevotella oralis									
Prevotella pallens									
Fusobacterium nucleatum		■	■	■	■				
Fusobacterium periodonticum									
Leptotrichia trevisanii									
Leptotrichia buccalis									
Leptotrichia wadei									
Leptotrichia sp.							■		
Leptotrichia hofstadii	■			■					
Leptotrichia goodfellowii									
Eubacterium sp.									
Lachnospiraceae bacterium									
Streptococcus sanguinis	■	■		■	■	■	■		■

Table 4.3, continued

Streptococcus mutans		■	■		■			■	
Streptococcus anginosus							■		
Streptococcus mitis							■	■	
Streptococcus cristatus									
Streptococcus gordonii		■	■					■	
Streptococcus sp.							■		■
Streptococcus australis									
Streptococcus infantis									
Streptococcus parasanguis									
Streptococcus parasanguinis							■		
Streptococcus oralis	■		■					■	
Streptococcus salivarius									
Streptococcus constellatus									
Streptococcus pneumoniae									
Streptococcus intermedius									
Staphylococcus pasteuri									
Gemella morbillorum			■						
Gemella haemolysans					■				
Gemella sanguinis							■		
Selenomonas sp.		■							
Veillonella parvula									
Veillonella dispar									
Veillonella atypica									
Campylobacter concisus									
Campylobacter showae									
Neisseria sp.						■		■	
Neisseria meningitidis						■			
Neisseria flavescens						■			
Neisseria subflava									
Neisseria flava				■					
Haemophilus segnis									
Haemophilus parainfluenzae	■		■		■			■	
Haemophilus sp.									
Haemophilus haemolyticus						■			
Haemophilus parahaemolyticus						■			
Eikenella corrodens				■		■			
Kingella oralis									
Aggregatibacter aphrophilus									

M = Malay; C = Chinese; I = Indian

The presence is indicated by the shaded box

4.3.3 Species from tongue dorsum

Actinomyces odontolyticus, *Streptococcus mitis* and *Gemella sanguinis* were detected on the tongue dorsa of the three races (Table 4.4). *Actinomyces naeslundii*, *Prevotella nanceiensis*, and *P. oralis* were detected on the tongue dorsum of M 1 subject only. *Lachnospiraceae bacterium*, *Staphylococcus pasteuri*, and *Haemophilus parahaemolyticus* were detected on tongue dorsum of M 2 subject only. *Rothia dentocariosa* were detected on tongue dorsum of M 3 subject only. *Leptotrichia wadei* and *Gemella morbillorum* were detected on tongue dorsum of C 1 subject only. *Capnocytophaga gingivalis* were detected on tongue dorsum of C 2 subject only. *Fusobacterium nucleatum* and *Eikenella corrodens* were detected on tongue dorsum of C 3 subject only. *Prevotella pallens* and *Streptococcus parasanguis* were detected on tongue dorsum of I 1 subject only. *Veillonella dispar*, *V. atypica* and *Haemophilus haemolyticus* were detected on tongue dorsum of I 2 subject only. *Rothia aeria* and *Streptococcus oralis* were detected on tongue dorsum of I 3 subject only.

Table 4.4 Anaerobic bacterial species on tongue dorsum of each subject

	Subjects								
Bacterial species	**M 1**	**M 2**	**M 3**	**C 1**	**C 2**	**C 3**	**I 1**	**I 2**	**I 3**
Actinomyces viscosus									
Actinomyces naeslundii	■								
Actinomyces sp.			■						
Actinomyces odontolyticus		■	■	■	■		■	■	■
Actinomyces georgiae									
Actinomyces cardiffensis									
Actinomyces meyeri									
Actinomyces oris									
Corynebacterium durum									
Rothia dentocariosa			■						
Rothia aeria									■
Rothia mucilaginosa			■		■	■		■	
Capnocytophaga ochracea									
Capnocytophaga granulosa									
Capnocytophaga gingivalis					■				
Capnocytophaga sp.				■					

Table 4.4, continued

Capnocytophaga sputigena									
Prevotella nigrescens									
Prevotella intermedia									
Prevotella buccae									
Prevotella sp.									
Prevotella tannerae									
Prevotella melaninogenica		■		■	■				
Prevotella salivae									
Prevotella loescheii									
Prevotella maculosa									
Prevotella denticola									
Prevotella nanceiensis	■								
Prevotella oralis	■								
Prevotella pallens							■		
Fusobacterium nucleatum						■			
Fusobacterium periodonticum	■			■					
Leptotrichia trevisanii									
Leptotrichia buccalis									
Leptotrichia wadei				■					
Leptotrichia sp.			■						
Leptotrichia hofstadii									
Leptotrichia goodfellowii									
Eubacterium sp.									
Lachnospiraceae bacterium		■							
Streptococcus sanguinis									
Streptococcus mutans									
Streptococcus anginosus									
Streptococcus mitis	■		■	■		■	■		■
Streptococcus cristatus									
Streptococcus gordonii									
Streptococcus sp.			■						■
Streptococcus australis			■		■				
Streptococcus infantis			■		■	■			■
Streptococcus parasanguis							■		
Streptococcus parasanguinis			■			■	■	■	
Streptococcus oralis									■
Streptococcus salivarius	■		■						■
Streptococcus constellatus									
Streptococcus pneumoniae						■	■		■
Streptococcus intermedius									
Staphylococcus pasteuri		■							
Gemella morbillorum				■					
Gemella haemolysans	■			■					

Table 4.4, continued

Gemella sanguinis		■	■		■	■	■	■	■
Selenomonas sp.									
Veillonella parvula	■			■	■		■		
Veillonella dispar								■	
Veillonella atypica								■	
Campylobacter concisus									
Campylobacter showae									
Neisseria sp.								■	
Neisseria meningitidis									
Neisseria flavescens				■			■		■
Neisseria subflava				■	■				■
Neisseria flava									
Haemophilus segnis				■	■			■	
Haemophilus parainfluenzae		■	■		■		■	■	■
Haemophilus sp.									
Haemophilus haemolyticus								■	
Haemophilus parahaemolyticus		■							
Eikenella corrodens						■			
Kingella oralis									
Aggregatibacter aphrophilus									

M = Malay; C = Chinese; I = Indian
The presence is indicated by the shaded box

4.4 Bacterial diversity of each site

Table 4.5 shows that gingival crevices had the greatest bacterial diversity compared to teeth surfaces and tongue dorsa. For gingival crevices, Malays had a mean number of 15 bacterial species, Chinese had 13 bacterial species and Indians 12 bacterial species. Teeth surfaces and tongue dorsa harbored similar bacterial diversity. On teeth surfaces, Malays had average of 10 species, Chinese had average 11 species and Indians had average 9 species. 41 bacterial species had been detected on tongue dorsa. On tongue dorsa, Malays had an average of 9 species, Chinese 10 species and Indians 11 species. On average, Malays harbored 29 bacterial species, Chinese 28 species and Indians 25 species for all the sampling sites.

Table 4.5 The number of anaerobic bacterial species per site per subject

Subject	Total species per site			Total species per subject
	Gingival crevices	Teeth surfaces	Tongue dorsa	
M 1	15	8	8	28
M 2	14	13	7	29
M 3	16	9	13	30
C 1	11	11	12	27
C 2	11	11	11	28
C 3	16	11	8	30
I 1	12	9	10	23
I 2	10	10	10	25
I 3	13	9	12	27
Total detected species	51	41	41	

M = Malay; C = Chinese; I = Indian.

4.5 Statistical analysis of mean colony forming unit (CFU) from the sampling sites

Table 4.6 shows that the mean logarithm CFU/ml were 5.68 ± 0.14, 5.02 ± 0.13 and 6.02 ± 0.08 in gingival crevices, on teeth surfaces and on tongue dorsa, respectively. Based on One-Way ANOVA using Tukey test (Table 4.8), significant difference were seen between CFU/ml in gingival crevices and CFU/ml on teeth surfaces, and between CFU/ml on tongue dorsa and CFU/ml on teeth surfaces. There is no significant difference between CFU/ml in gingival crevices and CFU/ml on tongue dorsa. As the data were not completely normally distributed, non-parametric tests were carried out. Referring to appendix 8, Kruskal-Wallis test showed there was significant difference between the three groups. Mann-Whitey U tests had shown the same result as Tukey test.

Table 4.6 Log CFU per ml (anaerobic bacteria) from each oral cavity sampling site

Subject	log CFU/ml in gingival crevices	log CFU/ml on teeth surfaces	log CFU/ml on tongue dorsa
M 1	5.87	4.30	5.92
M 2	5.92	5.23	5.99
M 3	5.18	5.32	5.83
C 1	4.93	4.96	5.95
C 2	5.99	5.71	6.51
C 3	5.91	5.0	6.28
I 1	6.08	4.91	5.83
I 2	5.26	5.08	5.72
I 3	6.0	4.63	6.15
Mean ± SE	5.68 ± 0.14	5.02 ± 0.13	6.02 ± 0.08

SE = standard error

Table 4.7 ANOVA

CFU

	Sum of squares	df	Mean square	F	Sig.
Between groups	4.702	2	2.351	17.116	0.000
Within groups	3.297	24	0.137		
Total	7.999	26			

P value of <0.05 are considered statistically significant. The ANOVA result showed there is significant difference.

Table 4.8 Post Hoc test

Dependent variable: CFU

Tukey HSD

(I) site	(J) site	Mean Difference (I-J)	Std. Error	Sig.	95% confidence interval	
					Lower bound	Upper bound
GC	TS	0.66667*	0.17472	0.002	0.2304	1.1030
	TD	-0.33778	0.17472	0.151	-0.7741	0.0985
TS	GC	-0.66667*	0.17472	0.002	-1.1030	-0.2304
	TD	-1.00444*	0.17472	0.000	-1.4408	-0.5681
TD	GC	0.33778	0.17472	0.151	-0.0985	0.7741
	TS	1.00444*	0.17472	0.000	0.5681	1.4408

GC = Gingival crevices; TS = Teeth surfaces; TD = Tongue dorsa.

* The mean difference is significant at the 0.05 level.

4.6 Prevalence of the anaerobic bacterial species from gingival crevices, teeth surfaces and tongue dorsa

Actinomyces naeslundii and *Gemella morbillorum* were detected in six out of nine subjects in the gingival crevices (Table 4.9). *Prevotella intermedia*, *Leptotrichia*

buccalis, and *Eikenella corrodens* were detected in five out of nine subjects in the gingival crevices. *A. naeslundii* and *Streptococcus sanguinis* were detected in seven out of nine subjects on their teeth surfaces. *Rothia dentocariosa* were detected in five out of nine subjects on the teeth surfaces. *Actinomyces odontolyticus* and *Gemella sanguinis* were detected in seven out of nine subjects on the tongue dorsa. *Streptococcus mitis* and *Haemophilus parainfluenzae* were detected in six out of nine subjects on the tongue dorsa. *A. naeslundii* had higher occurrence in gingival crevice and on teeth surface compared with tongue dorsum. *A. odontolyticus* had higher occurrence on tongue dorsum compared with gingival crevice and teeth surface. *Rothia aeria* had higher occurrence on teeth surface compared with tongue dorsum. *R. dentocariosa* had higher occurrence on teeth surface than gingival crevice and tongue dorsum. *Capnocytophaga gingivalis* had higher occurrence in gingival crevice and on teeth surface compared with tongue dorsum. *P. intermedia* had higher occurrence in gingival crevice than on teeth surface. *Fusobacterium nucleatum* had higher occurrence on teeth surface and in gingival crevice compared with tongue dorsum. *S. sanguinis* had higher occurrence on teeth surface than in gingival crevice. *Streptococcus mutans* had higher occurrence on teeth surface than in gingival crevice. *S. mitis* had higher occurrence on tongue dorsum compared with gingival crevice and teeth surface. *Streptococcus parasanguinis* had higher occurrence on tongue dorsum than teeth surface. *Gemella morbillorum* had higher occurrence in gingival crevice compared with teeth surface and tongue dorsum. *G. sanguinis* had higher occurrence on tongue dorsum than teeth surface. *H. parainfluenzae* had higher occurrence on tongue dorsum compared with gingival crevice and teeth surface. *E. corrodens* had higher occurrence in gingival crevice compared with teeth surface and tongue dorsum.

Actinomyces cardiffensis, *A. meyeri*, *Corynebacterium durum*, *Capnocytophaga sputigena*, *Prevotella tannerae*, *P. salivae*, *P. loescheii*, *P. denticola*, *P. nanceiensis*, *P. oralis*, *P. pallens*, *Leptotrichia trevisanii*, *L. wadei*, *L. goodfellowii*, *Streptococcus parasanguis*, *S. intermedius*, *Staphylococcus pasteuri*, *Veillonella dispar*, *V. atypica*, *Campylobacter concisus*, *Neisseria meningitidis*, *N. flava*, *Haemophilus haemolyticus*, *H. parahaemolyticus*, and *Kingella oralis* were less common isolates from the subjects.

Table 4.9 The number of subjects detected with the specific anaerobic bacterial species from the sampling sites

	Number of subjects detected with the species (total subjects: 9)		
Bacterial species	**Gingival crevices**	**Teeth surfaces**	**Tongue dorsa**
Actinobacteria			
Actinomyces viscosus	2	1	-
Actinomyces naeslundii	6	7	1
Actinomyces sp.	2	3	1
Actinomyces odontolyticus	2	4	7
Actinomyces georgiae	2	3	-
Actinomyces cardiffensis	1	-	-
Actinomyces meyeri	-	1	-
Actinomyces oris	2	2	-
Corynebacterium durum	-	1	-
Rothia dentocariosa	2	5	1
Rothia aeria	-	4	1
Rothia mucilaginosa	-	-	4
Bacteroidetes			
Capnocytophaga ochracea	3	1	-
Capnocytophaga granulosa	3	4	-
Capnocytophaga gingivalis	4	3	1
Capnocytophaga sp.	2	-	1
Capnocytophaga sputigena	-	1	-
Prevotella nigrescens	2	1	-
Prevotella intermedia	5	1	-
Prevotella buccae	2	-	-
Prevotella sp.	4	-	-
Prevotella tannerae	1	-	-
Prevotella melaninogenica	2	1	3
Prevotella salivae	1	-	-
Prevotella loescheii	1	-	-
Prevotella maculosa	2	-	-
Prevotella denticola	-	1	-

Table 4.9, continued

Prevotella nanceiensis	-	-	1
Prevotella oralis	-	-	1
Prevotella pallens	-	-	1
Fusobacteria			
Fusobacterium nucleatum	3	4	1
Fusobacterium periodonticum	-	-	2
Leptotrichia trevisanii	1	-	-
Leptotrichia buccalis	5	-	-
Leptotrichia wadei	1	-	1
Leptotrichia sp.	1	1	1
Leptotrichia hofstadii	3	2	-
Leptotrichia goodfellowii	1	-	-
Firmicutes			
Eubacterium sp.	1	-	-
Lachnospiraceae bacterium	2	-	1
Streptococcus sanguinis	3	7	-
Streptococcus mutans	1	4	-
Streptococcus anginosus	2	1	-
Streptococcus mitis	3	2	6
Streptococcus cristatus	2	-	-
Streptococcus gordonii	-	3	-
Streptococcus sp.	-	2	2
Streptococcus australis	-	-	2
Streptococcus infantis	-	-	4
Streptococcus parasanguis	-	-	1
Streptococcus parasanguinis	-	1	4
Streptococcus oralis	3	3	1
Streptococcus salivarius	-	-	3
Streptococcus constellatus	2	-	-
Streptococcus pneumoniae	-	-	3
Streptococcus intermedius	1	-	-
Staphylococcus pasteuri	-	-	1
Gemella morbillorum	6	1	1
Gemella haemolysans	2	1	2
Gemella sanguinis	-	1	7
Selenomonas sp.	-	1	-
Veillonella parvula	-	-	4
Veillonella dispar	-	-	1
Veillonella atyica	-	-	1
Proteobacteria			
Campylobacter concisus	1	-	-
Campylobacter showae	4	-	-
Neisseria sp.	2	2	1
Neisseria meningitidis	-	1	-
Neisseria flavescens	-	1	3

Table 4.9, continued

Neisseria subflava	-	-	3
Neisseria flava	-	1	-
Haemophilus segnis	2	-	3
Haemophilus parainfluenzae	2	4	6
Haemophilus sp.	2	-	-
Haemophilus haemolyticus	1	1	1
Haemophilus parahaemolyticus	-	1	1
Eikenella corrodens	5	2	1
Kingella oralis	1	-	-
Aggregatibacter aphrophilus	4	-	-

Bacterial species were grouped into five phyla. The five phyla are in bold letter. The subjects comprised three Malays, three Chinese and three Indians.

4.7 Phylogenetic relationship between bacterial species

Figure 4.4 shows that the major families were Streptococcaceae and Prevotellaceae which comprised 15 species (lineages) and 12 species respectively. The other diverse family was Actinomycetaceae which comprised of seven species. *Fusobacterium* and *Leptotrichia* genera were under Fusobacteriaceae family which comprised seven species. Less diverse families were Neisseriaceae (six species), Pasteurellaceae (five species), Flavobacteriaceae (four species), Veillonellaceae (four species), Micrococcaceae (three species) and Campylobacteraceae (two species).

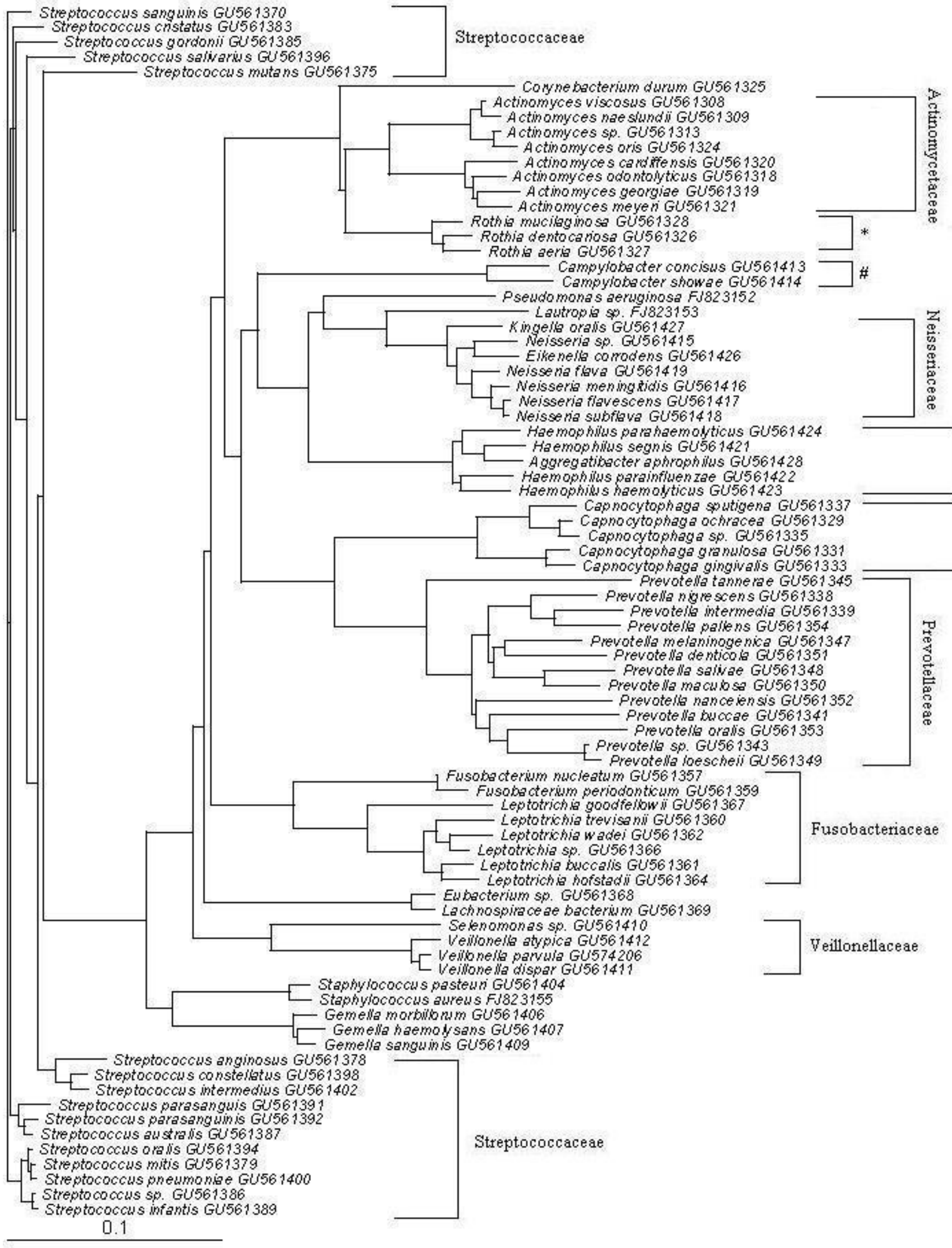


Figure 4.4 Phylogram of isolated bacterial species. Bar represents a 10% difference in nucleotide sequence. Each of families has been labeled. GenBank accession numbers are provided.
* = Micrococcaceae; # = Campylobacteraceae

4.8 Antibiotic susceptibility tests

4.8.1 Clindamycin susceptibility test

Table 4.10 shows that two out of nine *Capnocytophaga gingivalis* isolates were resistant to clindamycin. Seven out of 10 *Eikenella corrodens* isolates were resistant to clindamycin. The minimum concentration of clindamycin to inhibit 90% of the *E. corrodens* isolates was 128μg/ml. Two out of 15 *Haemophilus parainfluenzae* isolates were resistant to clindamycin. Two of the *Lautropia* sp. isolates were resistant to clindamycin. All six species of the *Neisseria* genus were resistant to clindamycin. Isolated *Neisseria meningitidis* and *Pseudomonas aeruginosa* were resistant to clindamycin. One out of five *Neisseria flavescens* isolates was resistant to clindamycin. One out of 11 *Neisseria subflava* isolates was resistant to clindamycin. One out of 15 *Streptococcus sanguinis* isolates was resistant to clindamycin. One out of 20 *Streptococcus mitis* was resistant to clindamycin.

Table 4.10 Susceptibility of oral bacterial isolates to clindamycin

Bacteria (No. of isolates)	**MIC range** (μg/ml)	**MIC50** (μg/ml)	**MIC90** (μg/ml)	**No. of resistant isolates**
Actinomyces naeslundii (29)	<0.016 – 0.125	0.032	0.094	0
Actinomyces odontolyticus (22)	<0.016 – 0.19	0.032	0.094	0
Actinomyces georgiae (6)	<0.016 – 0.064	<0.016	0.047	0
Actinomyces viscosus (4)	0.064 – 0.094	-	-	0
Actinomyces sp. (6)	<0.016 – 0.094	<0.016	0.064	0
Actinomyces meyeri (1)	<0.016	-	-	0
Actinomyces cardiffensis (1)	0.094	-	-	0
Actinomyces oris (9)	0.016 – 0.25	0.047	0.064	0
Capnocytophaga gingivalis (9)	<0.016 – >256	<0.016	0.016	2
Capnocytophaga granulosa (8)	<0.016	<0.016	<0.016	0
Capnocytophaga sputigena (1)	<0.016	-	-	0
Capnocytophaga ochracea (5)	<0.016	<0.016	<0.016	0
Capnocytophaga sp. (3)	<0.016	-	-	0
Campylobacter showae (4)	<0.016 – 0.032	-	-	0
Campylobacter concisus (1)	0.023	-	-	0
Corynebacterium durum (1)	0.047	-	-	0
Eikenella corrodens (10)	2 – >256	32	128	7
Eubacterium sp. (1)	0.094	-	-	0
Fusobacterium nucleatum (9)	0.032 – 0.25	0.064	0.125	0

Table 4.10, continued

Fusobacterium periodonticum (2)	0.5	-	-	0
Gemella morbillorum (10)	<0.016 – 0.064	0.016	0.032	0
Gemella haemolysans (6)	<0.016 – 0.38	0.032	0.25	0
Gemella sanguinis (11)	<0.016 – 0.016	<0.016	0.016	0
Aggregatibacter aphrophilus (4)	0.016 – 0.094	-	-	0
Haemophilus sp. (2)	2	-	-	0
Haemophilus parainfluenzae (15)	<0.016 – 16	3	8	2
Haemophilus haemolyticus (3)	<0.016	-	-	0
Haemophilus segnis (6)	<0.016 – 0.5	0.047	0.25	0
Haemophilus parahaemolyticus (2)	<0.016 – 0.023	-	-	0
Kingella oralis (2)	4	-	-	0
Lachnospiraceae bacterium (3)	<0.016	-	-	0
Lautropia sp. (2)	12	-	-	2
Leptotrichia hofstadii (5)	<0.016	<0.016	<0.016	0
Leptotrichia buccalis (5)	<0.016 – 0.016	<0.016	0.016	0
Leptotrichia sp. (4)	<0.016	-	-	0
Leptotrichia wadei (2)	<0.016	-	-	0
Leptotrichia goodfellowii (1)	<0.016	-	-	0
Leptotrichia trevisanii (1)	<0.016	-	-	0
Neisseria sp. (6)	8 – >256	24	48	6
Neisseria flavescens (5)	2 – 8	3	4	1
Neisseria subflava (11)	2 – 8	3	4	1
Neisseria meningitidis (1)	8	-	-	1
Neisseria flava (1)	4	-	-	0
Prevotella buccae (3)	<0.016	-	-	0
Prevotella sp. (6)	<0.016 – 0.016	<0.016	<0.016	0
Prevotella oralis (1)	<0.016	-	-	0
Prevotella melaninogenica (7)	<0.016	<0.016	<0.016	0
Prevotella nigrescens (3)	<0.016 – 0.064	-	-	0
Prevotella loescheii (1)	<0.016	-	-	0
Prevotella denticola (1)	<0.016	-	-	0
Prevotella maculosa (3)	<0.016	-	-	0
Prevotella tannerae (1)	<0.016	-	-	0
Prevotella nanceiensis (1)	<0.016	-	-	0
Prevotella salivae (1)	<0.016	-	-	0
Prevotella pallens (1)	<0.016	-	-	0
Prevotella intermedia (6)	<0.016 – 1	0.023	0.094	0
Pseudomonas aeruginosa (1)	>256	-	-	1
Rothia mucilaginosa (6)	0.064 – 3	0.38	0.75	0
Rothia dentocariosa (10)	0.047 – 1	0.5	0.75	0
Rothia aeria (8)	0.125 – 3	1	3	0
Streptococcus sanguinis (15)	0.016 – 16	0.047	0.094	1
Streptococcus mutans (7)	0.016 – 0.032	0.016	0.016	0
Streptococcus parasanguinis (7)	0.023 – 0.064	0.023	0.047	0
Streptococcus parasanguis (3)	0.016 – 0.064	-	-	0
Streptococcus gordonii (5)	0.047 – 0.094	0.094	0.094	0
Streptococcus sp. (8)	0.016 – 0.094	0.047	0.064	0
Streptococcus cristatus (3)	0.032 – 0.125	-	-	0
Streptococcus mitis (20)	0.016 – >256	0.032	0.064	1

Table 4.10, continued

Streptococcus oralis (11)	0.016 – 0.064	0.032	0.064	0
Streptococcus anginosus (4)	0.047 – 0.19	-	-	0
Streptococcus infantis (5)	<0.016 – 0.032	0.016	0.023	0
Streptococcus constellatus (2)	0.032 – 0.064	-	-	0
Streptococcus australis (2)	0.016 – 0.032	-	-	0
Streptococcus pneumoniae (4)	0.016 – 0.25	0.016	0.094	0
Streptococcus intermedius (1)	0.047	-	-	0
Streptococcus salivarius (3)	0.032 – 0.064	-	-	0
Staphylococcus aureus (1)	6	-	-	0
Staphylococcus pasteuri (2)	0.047 – 0.094	-	-	0
Selenomonas sp. (1)	0.032	-	-	0
Veillonella parvula (5)	<0.016 – 0.016	<0.016	<0.016	0
Veillonella dispar (1)	0.016	-	-	0
Veillonella atypica (1)	0.016	-	-	0

MIC50 and MIC90 only applicable for species that has at least five isolates
Clindamycin - Susceptible ≤ 2 μg/ml; resistant ≥ 8 μg/ml

4.8.2 Amoxicillin-clavulanic acid susceptibility test

Table 4.11 shows that all isolates were susceptible to amoxicillin-clavulanic acid except *Pseudomonas aeruginosa*.

Table 4.11 Susceptibility of oral bacterial isolates to amoxicillin-clavulanic acid

Bacteria (No. of isolates)	**MIC range** (μg/ml)	**MIC50** (μg/ml)	**MIC90** (μg/ml)	**No. of resistant isolates**
Actinomyces naeslundii (29)	<0.016 – 0.032	<0.016	0.016	0
Actinomyces odontolyticus (22)	<0.016 – 0.064	0.016	0.032	0
Actinomyces georgiae (6)	<0.016 – 0.032	<0.016	0.016	0
Actinomyces viscosus (4)	<0.016 – 0.016	-	-	0
Actinomyces sp. (6)	<0.016 – 0.016	<0.016	0.016	0
Actinomyces meyeri (1)	<0.016	-	-	0
Actinomyces cardiffensis (1)	<0.016	-	-	0
Actinomyces oris (9)	<0.016 – 0.016	<0.016	0.016	0
Capnocytophaga gingivalis (9)	0.016 – 0.064	0.016	0.047	0
Capnocytophaga granulosa (8)	<0.016 – 0.016	<0.016	<0.016	0
Capnocytophaga sputigena (1)	0.016	-	-	0
Capnocytophaga ochracea (5)	<0.016 – 0.032	0.016	0.032	0
Capnocytophaga sp. (3)	0.016 – 0.023	-	-	0
Campylobacter showae (4)	0.023 – 0.047	-	-	0
Campylobacter concisus (1)	0.047	-	-	0
Corynebacterium durum (1)	<0.016	-	-	0
Eikenella corrodens (10)	<0.016 – 0.38	0.125	0.25	0
Eubacterium sp. (1)	0.125	-	-	0
Fusobacterium nucleatum (9)	0.016 – 0.5	0.016	0.25	0

Table 4.11, continued

Fusobacterium periodonticum (2)	2	-	-	0
Gemella morbillorum (10)	<0.016	<0.016	<0.016	0
Gemella haemolysans (6)	<0.016 – 0.032	<0.016	<0.016	0
Gemella sanguinis (11)	<0.016 – 0.094	0.023	0.032	0
Aggregatibacter aphrophilus (4)	<0.016 – 0.047	-	-	0
Haemophilus sp. (2)	0.032	-	-	0
Haemophilus parainfluenzae (15)	<0.016 – 0.75	0.25	0.125	0
Haemophilus haemolyticus (3)	<0.016	-	-	0
Haemophilus segnis (6)	<0.016 – 0.125	0.047	0.125	0
Haemophilus parahaemolyticus (2)	0.016 – 0.023	-	-	0
Kingella oralis (2)	0.047	-	-	0
Lachnospiraceae bacterium (3)	<0.016	-	-	0
Lautropia sp. (2)	0.016	-	-	0
Leptotrichia hofstadii (5)	<0.016 – 0.023	0.016	0.016	0
Leptotrichia buccalis (5)	0.032 – 0.047	0.032	0.032	0
Leptotrichia sp. (4)	<0.016 – 0.032	-	-	0
Leptotrichia wadei (2)	<0.016	-	-	0
Leptotrichia goodfellowii (1)	<0.016	-	-	0
Leptotrichia trevisanii (1)	<0.016	-	-	0
Neisseria sp. (6)	0.094 – 1.5	0.125	0.5	0
Neisseria flavescens (5)	0.125 – 1	0.5	1	0
Neisseria subflava (11)	0.19 – 0.38	0.19	0.38	0
Neisseria meningitidis (1)	0.064	-	-	0
Neisseria flava (1)	0.38	-	-	0
Prevotella buccae (3)	0.023	-	-	0
Prevotella sp. (6)	<0.016 – 0.023	<0.016	0.023	0
Prevotella oralis (1)	<0.016	-	-	0
Prevotella melaninogenica (7)	<0.016	<0.016	<0.016	0
Prevotella nigrescens (3)	0.016 – 0.064	-	-	0
Prevotella loescheii (1)	<0.016	-	-	0
Prevotella denticola (1)	<0.016	-	-	0
Prevotella maculosa (3)	<0.016	-	-	0
Prevotella tannerae (1)	<0.016	-	-	0
Prevotella nanceiensis (1)	<0.016	-	-	0
Prevotella salivae (1)	<0.016	-	-	0
Prevotella pallens (1)	<0.016	-	-	0
Prevotella intermedia (6)	0.016 – 0.25	0.094	0.125	0
Pseudomonas aeruginosa (1)	>256	-	-	1
Rothia mucilaginosa (6)	<0.016 – 0.125	0.016	0.032	0
Rothia dentocariosa (10)	<0.016	<0.016	<0.016	0
Rothia aeria (8)	<0.016 – 0.016	<0.016	0.016	0
Streptococcus sanguinis (15)	0.023 – 0.094	0.064	0.094	0
Streptococcus mutans (7)	0.016 – 0.032	0.016	0.016	0
Streptococcus parasanguinis (7)	0.023 – 0.047	0.023	0.032	0
Streptococcus parasanguis (3)	0.032 – 0.5	-	-	0
Streptococcus gordonii (5)	0.016 – 0.032	0.023	0.023	0
Streptococcus sp. (8)	<0.016 – 0.032	0.016	0.016	0
Streptococcus cristatus (3)	0.064 – 0.094	-	-	0
Streptococcus mitis (20)	<0.016 – 0.064	0.016	0.023	0

Table 4.11, continued

Streptococcus oralis (11)	<0.016 – 0.032	0.016	0.032	0
Streptococcus anginosus (4)	0.023 – 0.064	-	-	0
Streptococcus infantis (5)	<0.016 – 0.016	<0.016	0.016	0
Streptococcus constellatus (2)	0.047 – 0.094	-	-	0
Streptococcus australis (2)	0.016 – 0.032	-	-	0
Streptococcus pneumoniae (4)	<0.016	-	-	0
Streptococcus intermedius (1)	0.047	-	-	0
Streptococcus salivarius (3)	<0.016 – 0.064	-	-	0
Staphylococcus aureus (1)	0.25	-	-	0
Staphylococcus pasteuri (2)	0.047	-	-	0
Selenomonas sp. (1)	0.016	-	-	0
Veillonella parvula (5)	<0.016 – 0.016	0.016	0.016	0
Veillonella dispar (1)	0.023	-	-	0
Veillonella atypica (1)	0.016	-	-	0

MIC50 and MIC90 only applicable for species that has at least five isolates
Amoxicillin-clavulanic acid - Susceptible ≤ 4/2 μg/ml; resistant ≥ 16/8 μg/ml

4.9 Identification of isolated aerobic bacteria using conventional methods

According to Bergey's manual, the results in table 4.12 confirm the identity of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The identification of the *S. aureus* was supported by the ability of the microorganism to grow well on mannitol salt agar (selective medium for *Staphylococcus* sp.). *P. aeruginosa* identification was supported by the present of diffusible green pigment in the medium. The identity of both cultures was confirmed by 16S rDNA sequence analysis.

Table 4.12 Biochemical tests of the aerobic pure cultures isolated from oral cavity

Tests		
Fermentation :		
Glucose	+	-
Lactose	+	-
Sucrose	+	-
Mannitol	+	-
Trehalose	NA	-
Sorbitol	NA	-
OF medium	Oxidative and fermentation	Oxidative
Gelatin hydrolysis	+	+
Starch hydrolysis	-	-
Esculin hydrolysis	+	+
Citrate utilization	-	+
Indole production	-	-

Table 4.12, continued

H_2S production	-	-
Nitrate reduction	+	N_2
Oxidase	-	+
Catalase	+	+
Arginine dihyrolase Lysine decarboxylase Ornithine decarboxylase	+ - -	+ - -
MR VP	+ +	- -
Motility	non-motile	motile
Gram staining	+	-
Morphology		
Microscopic morphology	cocci	bacillus
Colonial morphology	round, smooth, yellow	wrinkled, smooth
Identity	*Staphylococcus aureus*	*Pseudomonas aeruginosa*

NA = not available
N_2 = nitrogen gas production

Figure 4.5 *P. aeruginosa*

Figure 4.6 *S. aureus*

4.10 Isolation and identification of aerobically cultured bacteria by 16S rDNA sequence analysis

Aerobic cultures were identified by 16S rDNA sequencing. Total of 17 species were identified from the three sampling sites (Table 4.13).

Table 4.13 Aerobically cultured bacteria isolated and identified by 16S rDNA sequencing from oral cavity sites

Gingival crevices	**N**	**Teeth surfaces**	**N**	**Tongue dorsa**	**N**
Actinomyces naeslundii	3	*Actinomyces naeslundii*	1	*Lautropia* sp.	1
Capnocytophaga granulosa	1	*Actinomyces viscosus*	1	*Neisseria subflava*	2
Neisseria subflava	2	*Actinomyces oris*	5	*Streptococcus mitis*	3
Rothia mucilaginosa	1	*Kingella oralis*	1	*Streptococcus infantis*	1
Pseudomonas aeruginosa	1	*Lautropia* sp.	1	*Streptococcus pneumoniae*	1
Streptococcus mitis	3	*Neisseria flavescens*	1	*Streptococcus parasanguinis*	1
Streptococcus oralis	1	*Neisseria subflava*	1		
Staphylococcus aureus	1	*Rothia mucilaginosa*	1		
		Streptococcus sanguinis	1		
T = 6 X 10^5 CFU/ml		T = 4.7 X 10^4 CFU/ml		T = 1 X 10^6 CFU/ml	

N = number of isolates
T = total CFU/ml

4.11 Characterization of *Streptococcus salivarius* with electron microscopy

4.11.1 Transmission Electron Microscopy of *S. salivarius*

Figure 4.7(a) shows the image of *S. salivarius* under TEM. The measurements were done based on the thickness of the cell wall. Figure 4.7(b) shows the image of *S. salivarius* under TEM. The diameter of the bacterium is shown. The fuzzy coat on the surface of the cell is the fibrils.

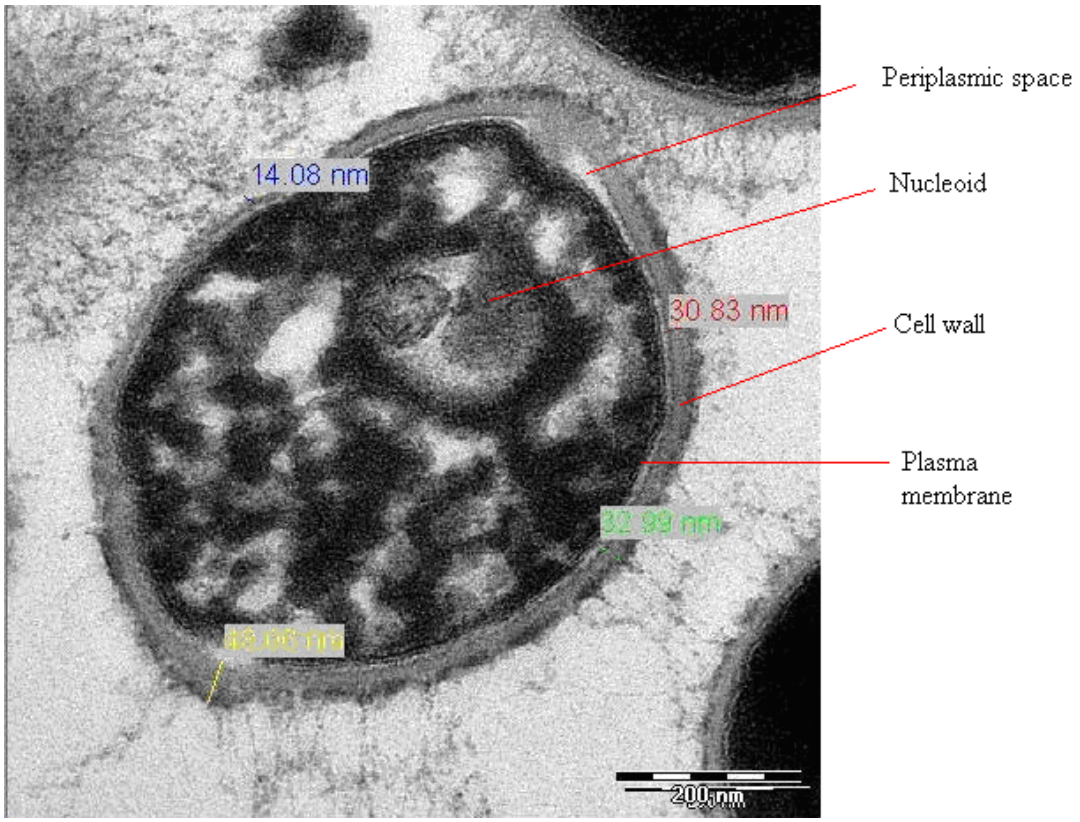


Figure 4.7(a) Transmission Electron Micrograph (TEM) of *Streptococcus salivarius*. The thickness of the cell wall was measured.

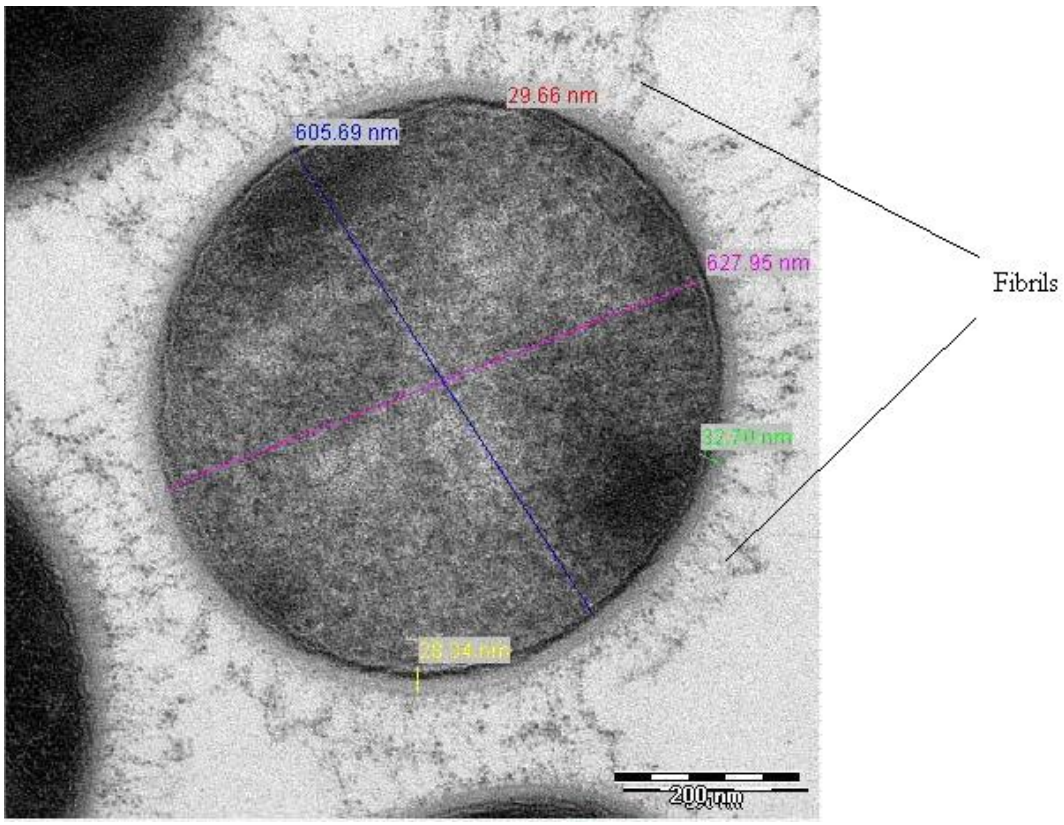


Figure 4.7(b) TEM of *S. salivarius*. The diameter of the cell was measured.

4.11.2 Scanning Electron Microscopy of *S. salivarius*

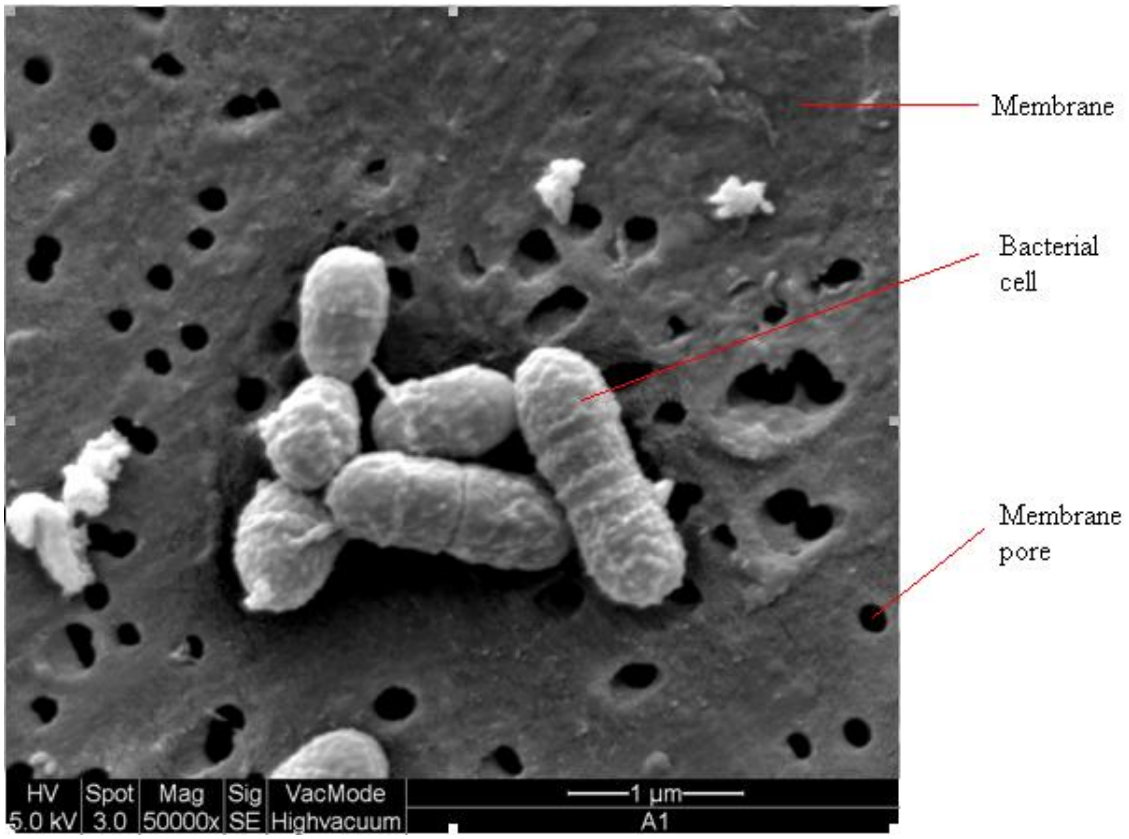


Figure 4.8(a) SEM of *S. salivarius* on nucleopore membrane. SEM was done at 5kV.

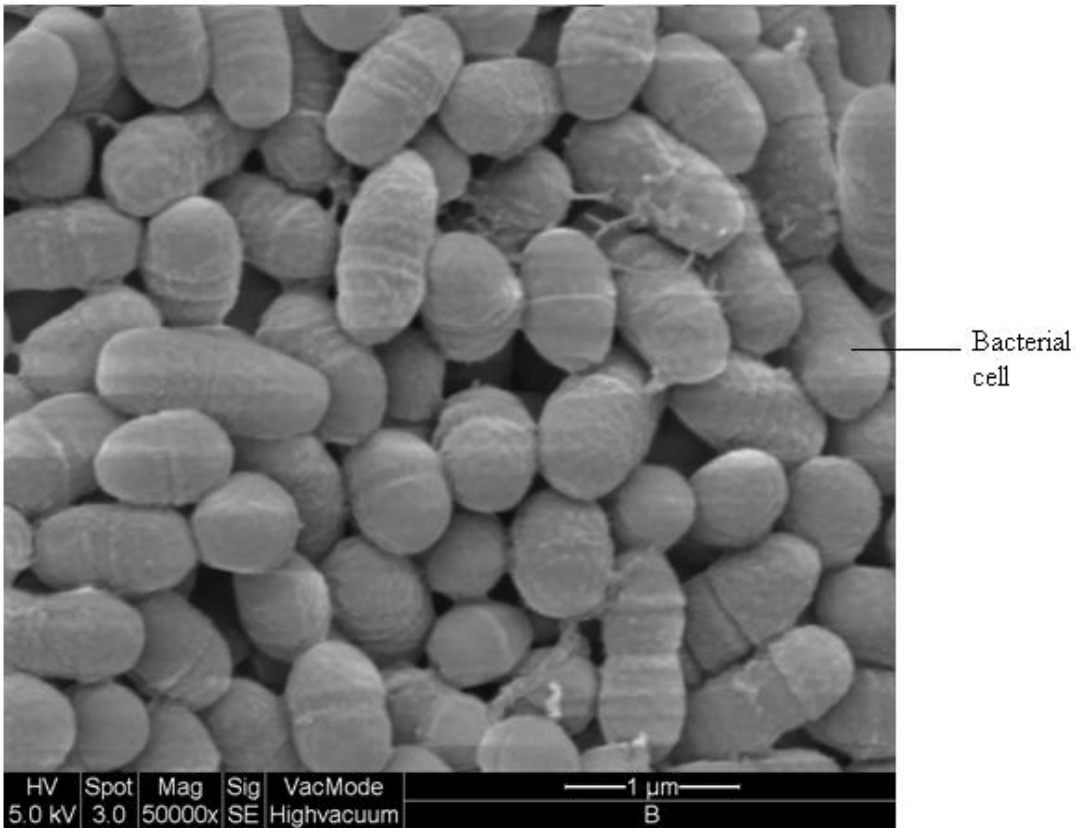
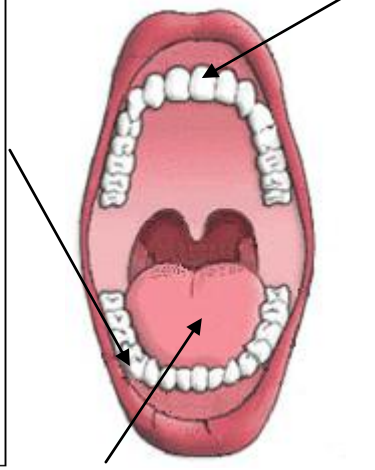


Figure 4.8(b) SEM of *S. salivarius* by microcarrier method. SEM was done at 5kV.

Figure 4.8(a) shows the SEM image of *S. salivarius* on nucleopore Track-Etch membrane. *S. salivarius* are Gram-positive coccoid bacteria. Figure 4.8(b) shows the SEM image of *S. salivarius* in pellet. In pellet state, the cells are in compact conformation.

Gingival crevice

Actinomyces naeslundii	*Prevotella melaninogenica*
Gemella morbillorum	*Lachnospiraceae bacterium*
Prevotella intermedia	*Streptococcus anginosus*
Leptotrichia buccalis	*Streptococcus cristatus*
Eikenella corrodens	*Streptococcus constellatus*
Capnocytophaga gingivalis	*Gemella haemolysans*
Prevotella sp.	*Neisseria* sp.
Campylobacter showae	*Haemophilus segnis*
Aggregatibacter aphrophilus	*Haemophilus parainfluenzae*
Capnocytophaga ochracea	*Haemophilus* sp.
Capnocytophaga granulosa	*Actinomyces cardiffensis*
Fusobacterium nucleatum	*Prevotella tannerae*
Leptotrichia hofstadii	*Prevotella salivae*
Streptococcus sanguinis	*Prevotella loescheii*
Streptococcus mitis	*Prevotella maculosa*
Streptococcus oralis	*Leptotrichia trevisanii*
Actinomyces viscosus	*Leptotrichia wadei*
Actinomyces sp.	*Leptotrichia* sp.
Actinomyces odontolyticus	*Leptotrichia goodfellowii*
Actinomyces georgiae	*Eubacterium* sp.
Actinomyces oris	*Streptococcus mutans*
Rothia dentocariosa	*Streptococcus intermedius*
Capnocytophaga sp.	*Campylobacter concisus*
Prevotella nigrescens	*Haemophilus haemolyticus*
Prevotella buccae	*Kingella oralis*
Prevotella sp.	



Teeth surface

Actinomyces naeslundii	*Actinomyces meyeri*
Rothia dentocariosa	*Corynebacterium durum*
Streptococcus sanguinis	*Capnocytophaga ochracea*
Actinomyces odontolyticus	*Capnocytophaga sputigena*
Rothia aeria	*Prevotella nigrescens*
Capnocytophaga granulosa	*Prevotella intermedia*
Fusobacterium nucleatum	*Prevotella melaninogenica*
Streptococcus mutans	*Prevotella denticola*
Haemophilus parainfluenzae	*Leptotrichia* sp.
Actinomyces sp.	*Streptococcus anginosus*
Actinomyces georgiae	*Streptococcus parasanguinis*
Capnocytophaga gingivalis	*Gemella morbillorum*
Streptococcus gordonii	*Gemella haemolysans*
Streptococcus oralis	*Gemella sanguinis*
Actinomyces oris	*Selenomonas* sp.
Leptotrichia hofstadii	*Neisseria meningitidis*
Streptococcus mitis	*Neisseria flavescens*
Streptococcus sp.	*Neisseria flava*
Neisseria sp.	*Haemophilus haemolyticus*
Eikenella corrodens	*Haemophilus parahaemolyticus*
Actinomyces viscosus	

Tongue dorsum

Actinomyces odontolyticus	*Fusobacterium periodonticum*	*Fusobacterium nucleatum*
Gemella sanguinis	*Streptococcus* sp.	*Leptotrichia wadei*
Streptococcus mitis	*Streptococcus australis*	*Leptotrichia* sp.
Haemophilus parainfluenzae	*Gemella haemolysans*	*Lachnospiraceae bacterium*
Rothia mucilaginosa	*Actinomyces naeslundii*	*Streptococcus parasanguis*
Streptococcus infantis	*Actinomyces* sp.	*Streptococcus oralis*
Streptococcus parasanguinis	*Rothia dentocariosa*	*Staphylococcus pasteuris*
Veillonella parvula	*Rothia aeria*	*Gemella morbillorum*
Prevotella melaninogenica	*Capnocytophaga gingivalis*	*Veillonella dispar*
Streptococcus salivarius	*Capnocytophaga* sp.	*Veillonella atypica*
Streptococcus pneumoniae	*Prevotella nanceiensis*	*Neisseria* sp.
Neisseria flavescens	*Prevotella oralis*	*Haemophilus haemolyticus*
Neisseria subflava	*Prevotella pallens*	*Haemophilus parahaemolyticus*
Haemophilus segnis	*Eikenella corrodens*	

Figure 4.9 Bacterial species from the oral cavity.
Prevalent species are highlighted and resistant species are shown in red.

Chapter 5

Discussion

Chapter 5: Discussion

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The objectives of this study were to characterize the diversity of oral bacteria in Malaysian subjects and to evaluate their resistance to selected antibiotics. Based on the current review of literature, the present study represents a first report of its kind in describing oral bacterial diversity and antibiotics resistance or susceptibility patterns of oral bacteria among Malaysian subjects.

5.1 Colonization pattern of the bacterial species in the oral cavity

The detected bacteria species have shown some site specificity in their colonization. Comparison of the bacteria species from the three sampling sites indicated presence of characteristic bacteria in each site. *Rothia mucilaginosa*, *Fusobacterium periodonticum*, *Streptococcus australis, S. infantis, S. salivarius, S. pneumoniae*, *Veillonella parvula* and *Neisseria subflava* are associated with tongue flora only (Table 4.1). *R. mucilaginosa* and *S. salivarius* are usually associated with healthy tongue microflora (Aas *et al*., 2005; Kazor *et al*., 2003; Preza *et al*., 2009). The bacteriocins of *S. salivarius* have been reported to inhibit pathogenic *Streptococcus pyogenes* and cariogenic *Streptococcus mutans* (Simmonds *et al*., 1995; Tagg, 2004). *F. periodonticum* has been mostly implicated in halitosis (Kazor *et al*., 2003). *S. australis* has been detected only on soft tissue like tongue dorsum (Aas *et al*., 2005). *S. pneumoniae* is the major causative agent for pneumonia, meningitis and bacteremia. Many serotypes of *S. pneumoniae* have been identified. Some serotypes were invasive and some were not (Lambertsen *et al*., 2010). From Denmark, it was reported that invasive isolates were less likely to be multiresistant compared with non-invasive isolates (Lambertsen *et al*., 2010). *N. subflava* can cause meningitis, bacteremia, and endocarditis (Furuya *et al*., 2007). It is important to realize that these opportunistic pathogens are part of our normal oral flora. However, the level of such oral microflora

should be kept minimal at all times to prevent opportunistic diseases. Hence oral hygiene can be maintained for good general health and well-being.

Streptococcus gordonii were isolated on teeth surfaces only (Table 4.1). *S. gordonii* are typically detected on teeth surfaces (Aas *et al*., 2005). *Actinomyces georgiae, A. oris, Capnocytophaga granulosa, Leptotrichia hofstadii*, and *Streptococcus sanguinis* were isolated only from gingival crevices and teeth surfaces in this study (Table 4.1). Another study obtained the same result for *S. sanguinis* (Aas *et al*., 2005). *Prevotella buccae, Leptotrichia buccalis, Streptococcus cristatus, Campylobacter showae* and *Aggregatibacter aphrophilus* were detected only in gingival crevices in the current study. While *A. georgiae*, *L. buccalis* and *C. showae* were found prevalent in the subgingival microbiota of Mexican subjects (Ximenez-Fyvie *et al*., 2006). In contrast, Preza *et al*. (2009) showed that *S. cristatus* were associated with subgingiva and also supragingiva. *L. buccalis* were commonly detected in healthy and diseased subjects (Paster *et al*., 2001).

Actinomyces odontolyticus and *Haemophilus parainfluenzae* have been isolated from all the three sampling sites (Table 4.1). *H. parainfluenzae* had also been found at all of the sampling sites in the study by Preza *et al*. (2009). *H. parainfluenzae* had been associated with chronic obstructive lung disease and endocarditis (Mitchell and Hill, 2000).

Streptococcus gordonii and *S. sanguinis* are pioneer teeth colonizers. These microorganisms have interesting antagonistic interaction with *Streptococcus mutans*, their competitor. *S. gordonii* and *S. sanguinis* are able to produce H_2O_2 to inhibit *S. mutans* under aerobic conditions only. However, high levels of glucose will reduce the

production of H_2O_2 from *S. gordonii* and *S. sanguinis*. *S. mutans* can produce mutacins to inhibit *S. gordonii* and *S. sanguinis* under any condition. This enables *S. mutans* to have competitive advantage under aerobic conditions in the presence of glucose and under anaerobic conditions (Kreth *et al*., 2008).

5.2 The distribution of bacterial species in the oral cavity

In this study, five bacterial phyla were found instead of six bacterial phyla previously reported as cultivable oral microflora (Paster *et al*., 2001 and 2006). Spirochaetes were not found in this study presumably because the bacterial species from this phylum are strict anaerobes. *Treponema denticola* was one of the cultivable species of Spirochaetes which is commonly detected in both healthy and diseased subjects (Paster *et al*., 2001). In the present study, no attempt was made to conduct isolation and subculture in strict anaerobic conditions.

The gingival crevice was dominated by members of Bacteroidetes and Firmicutes which accounted for 26% and 24% respectively of the 146 identified isolates (Figure 4.1). At the species level, of the 146 identified isolates (Table 4.1), the predominant species were *Actinomyces naeslundii* (8.9%), *Gemella morbillorum* (5.5%), *Eikenella corrodens* (4.8%), *Prevotella* sp. (4.1%), *Streptococcus oralis* (3.4%), *Leptotrichia buccalis* (3.4%), *Prevotella intermedia* (3.4%), and *Capnocytophaga gingivalis* (3.4%). In comparison, in Mexican subjects using culture independent approach, the proportion of the 40 test species in subgingival microbiota in descending order included *A. naeslundii, L. buccalis, P. intermedia, E. corrodens, C. gingivalis, G. morbillorum, S. oralis* and so on (Ximenez-Fyvie *et al*., 2006).

It is evident that the proportion of oral bacterial species varies between different countries and ethnic groups (Botero *et al*., 2007; Colombo *et al*., 2002; Darout *et al*., 2003; Haffajee *et al*., 2005; Mayorga-Fayad *et al*., 2007). *Gemella morbillorum* from the gingival crevice is the causative agent for endocarditis and was found to be resistant to β-lactams and aminoglycosides (Kofteridis *et al*., 2006). *Eikenella corrodens* was mostly indicated in bite wounds, head and neck infections (Oztoprak *et al*., 2009).

The teeth surface was dominated by Actinobacteria (37%) and Firmicutes (31%). Of 112 isolates from teeth surface, the predominant isolated species were *Actinomyces naeslundii* (9.8%), *Streptococcus sanguinis* (9.8%), *Rothia dentocariosa* (6.3%), *R. aeria* (6.3%), *Streptococcus mutans* (5.4%), *Actinomyces odontolyticus* (4.5%), *Fusobacterium nucleatum* (4.5%), *Streptococcus gordonii* (4.5%), and *Haemophilus parainfluenzae* (4.5%). In a study using pyrosequencing to study the dental plaque microflora, it was shown that Actinobacteria, Fusobacteria, and Spirochaetes were the dominant phyla (Keijser *et al*., 2008). However, in the present study, the Fusobacteria accounted for the lowest number of isolates (Figure 4.2) and this was in agreement with Aas *et al*. (2005) and Preza *et al*. (2009). *Actinomyces* species and *Streptococcus* species are saccharolytic bacteria that convert carbohydrate to lactic acid. These species have been associated with dental caries (Mikx and van der Hoeven, 1975). *S. mutans* are the putative cariogenic bacteria. *F. nucleatum* has been mostly detected in endodontic infection by culture dependent and independent methods (Siqueira *et al*., 2007). *R. dentocariosa* has been associated with periodontal disease and has also been associated with infective endocarditis and bacteremia (Yang *et al*., 2007). *A. odontolyticus* have been reported to cause empyema thoracis, a systemic disease (Mohan *et al*., 2009).

On dorsum of the tongue, Firmicutes were predominant contributing 51% of the 118 identified isolates (Figure 4.3). The predominant isolated species were *Actinomyces odontolyticus* (11%), *Gemella sanguinis* (8.5%), *Streptococcus mitis* (7.6%), *Haemophilus parainfluenzae* (5.9%), and *Neisseria subflava* (5.1%) (Table 4.1). *Streptococcus* species contributed the highest number of isolates on dorsum of the tongue which was 37 of 60 Firmicutes isolates. This result was consistent with study by Aas *et al*. (2005) and Kazor *et al*. (2003). *G. sanguinis* were detectable only on the tongue dorsum of healthy subjects compared with halitosis subjects (Kazor *et al*., 2003). In the study of Washio *et al*. (2005), *Veillonella dispar* and *A. odontolyticus* were isolated in high numbers on the tongue of no or low odour and odour groups as compared with other H_2S producing bacteria. In present study, only one *V. dispar* isolate was detected.

5.3 Subject-specificity of the oral bacterial species

Some species seem to be subject-specific in this study. Subject-specificity is attributed to host factors that affect the colonization of the oral cavity. These factors may be associated with diet and genetics or oral hygiene. Certain sets of species are able to adapt to each other and the host so that the microbial community is able to persist in the oral cavity of the host. Some bacterial species were commonly detected in the hosts. These species were highly adaptable to different hosts.

Based on table 4.2, 4.3 and 4.4, *Prevotella salivae*, *P. nanceiensis*, and *P. oralis* were detected on the tongue dorsum of M 1 subject only. *Prevotella loescheii* were detected in the gingival crevices of M 2 subject only. *Prevotella denticola* were detected on the teeth surfaces of M 2 subject only. *Staphylococcus pasteuri* were detected on tongue dorsum of M 2 subject only. *Prevotella tannerae*, *Campylobacter*

concisus, and *Kingella oralis* were detected in the gingival crevices of M 3 subject only. *Neisseria flava* were detected on the teeth surfaces of C 1 subject only. *Capnocytophaga sputigena* were detected on the teeth surfaces of C 2 subject only. *Leptotrichia trevisanii* and *Streptococcus intermedius* were detected in the gingival crevices of C 3 subject only. *Actinomyces meyeri*, *Corynebacterium durum*, and *Neisseria meningitidis* were detected on teeth surfaces of C 3 subject only. *Prevotella pallens* and *Streptococcus parasanguis* were detected on the tongue dorsum of I 1 subject only. *Leptotrichia goodfellowii* were detected in the gingival crevices of I 2 subject only. *Veillonella dispar* and *V. atypica* were detected on the tongue dorsum of I 2 subject only. *Actinomyces cardiffensis* were detected in gingival crevices of I 3 subject only.

5.4 Role of oral bacteria in systemic infections

A case study cited from literature is of interest to indicate the role of oral bacteria in systemic infections. *Prevotella oralis*, *P. denticola*, and *Streptococcus parasanguis* have been implicated in endocarditis (Dominguez-Castellano *et al*., 2001; Quaglio *et al*., 1999). Rats immunized with FimA protein of *S. parasanguis* could protect the rats from endocarditis caused by other oral *Streptococcus* species (Kitten *et al*., 2002). *Prevotella tannerae* was reported as potential pathogen of endodontic infections (Xia *et al*., 2000). *Campylobacter concisus* were reported to be prevalent in fecal samples of Crohn's disease patients (Guslandi *et al*., 2009). *Veillonella parvula*, *Capnocytophaga sputigena*, *C. gingivalis*, *Leptotrichia buccalis*, and *L. trevisanii* were able to spread into bloodstreams which cause bacteremia (Garcia-Cia *et al*., 2004; Mantadakis *et al*., 2003; Strach *et al*., 2006; Tee *et al*., 2001; Ulstrup and Hartzen, 2006). *Streptococcus intermedius* have been reported to cause infective endocarditis and abscess (Tran *et al*., 2008). *Actinomyces meyeri* have been reported to cause splenic

abscess. *Actinomyces* species are opportunistic pathogen that can lead to diseases in immuno-compromised individual (Garduno *et al*., 2000). *Neisseria meningitidis* can cause invasive meningococcal disease. Four serotypes have been identified in *N. meningitidis*, mostly serotypes B and C are the causative agents (Woodard and Berman, 2006). *Veillonella dispar* was reported to cause prosthetic joint infection (Marchandin *et al*., 2001). A 75 year old man developed septic arthritis of his knee after dental root extraction without antibiotic prophylaxis due to *Prevotella loescheii*. The patient was cured after taking amoxicillin-clavulanic acid (Fe-Marques *et al*., 2008). Many of the oral bacteria should not be regarded as non-pathogenic especially for immuno-compromised individuals.

5.5 Bacterial density and diversity of the oral cavity

A total of 72 anaerobic cultured bacterial species had been isolated and identified from the oral cavities of the nine Malaysian subjects investigated in this study. On average, each subject harbored 27 species from the three sampling sites of the oral cavity (Table 4.5). From this study results, gingival crevice had higher bacterial diversity compared with teeth surface and dorsum of the tongue. The bacterial diversity of teeth surface and tongue are similar. Many different types of bacteria species are able to adapt and inhabit within the gingival crevice. In culture independent approach, teeth surfaces are showed to harbor 52 species, subgingival areas harbored 47 species and tongue dorsa 40 species (Aas *et al*., 2005). Based on table 4.8, total CFU from gingival crevice and dorsum of the tongue were significantly higher than teeth surface. For aerobic cultivation, the total CFU on gingival crevice and dorsum of the tongue were shown to be higher than teeth surface (Table 4.13). But it is difficult to draw clear comparison between the CFU/ml of bacteria present in the specimens from the three sites because this will so greatly be influenced by the method of sampling in terms of

the amount picked up by the curette. Nevertheless it is showed that cultivable bacteria were dominant in gingival crevice and dorsum of the tongue. Both sites have higher tendencies to retain thick biofilm compared with teeth surface. This tells us that tongue scrapping is also very important in oral hygiene measures. Shear force from chewing may cause the teeth surface to have lower bacterial density on it. Variation in bacterial diversity among the subjects showed that oral microbial population is dynamic.

5.6 The prevalence of the bacterial species in gingival crevice, teeth surface and dorsum of the tongue

5.6.1 Teeth surface

Four of the subjects had *Streptococcus mutans* (Table 4.9). This showed that healthy subjects could harbor *S. mutans* but the proportion of this species in the total bacteria load was not high. *S. mutans* accounted for 5.4% of 112 isolates. Other bacteria like *Lactobacillus*, *Bifidobacterium*, *Propionibacterium*, *Atopobium* and *Pseudoramibacter alactolyticus* that are usually associated with dental caries were not detected in this study. This may be due to limitations of the cultivation methods used. Lactobacilli and *Bifidobacterium* readily grow on MRS medium which had been traditionally formulated to facilitate the growth of *Lactobacillus* (deMan *et al*., 1960). *Propionibacterium* species, *Atopobium* species and *Pseudoramibacter alactolyticus* are obligate anaerobes. These bacteria were not present amongst the isolates recovered from the subjects of this current study as only a relatively small number of isolates were obtained from each subject. Many other species are likely to be present but not obtained in this small subset of the population. Similarly, Aas *et al*. (2005) did not detect *Lactobacillus* species, *Bifidobacterium* species and *Atopobium* species in healthy oral cavity. In addition, they did not find *S. mutans*.

Actinomyces naeslundii and *Streptococcus sanguinis* were detected in seven out of nine subjects (78%) while five of the nine subjects (56%) had *Rothia dentocariosa* (Table 4.9). Hence, these species can be considered common inhabitant of the teeth surface. *S. sanguinis* were detected on the lateral aspect of tongue, teeth surface and subgingival areas (Paster *et al*., 2006). In these sites, *S. sanguinis* were more prevalent on teeth surface than the other sites. In our study, *S. sanguinis* were detected on teeth surface and gingival crevice (three subjects) but not tongue dorsum. Paster *et al*. (2006) had detected *S. sanguinis* on the lateral aspect of tongue perhaps due to proximity of the region to the teeth for collecting the bacteria. According to Aas *et al*. (2005), *R. dentocariosa* were detected from teeth and subgingival areas. Hence, *R. dentocariosa* were more prevalent on teeth than subgingiva. Their results were similar to that of our study. However, in our study one of the subject's tongue dorsum had *R. dentocariosa*.

5.6.2 Gingival crevice

Of nine subjects, six (67%) had *Actinomyces naeslundii* and *Gemella morbillorum* (Table 4.9). Five subjects (56%) had *Leptotrichia buccalis*, *Eikenella corrodens* and *Prevotella intermedia*, a species associated with periodontal disease (Matto *et al*., 1999). These five were the most prevalent species. Paster *et al*. (2006) showed that *Streptococcus intermedius* were prevalent in the subgingiva. In contrast, in our study these bacteria were only detected in one of the subjects. The cultivable putative periodontal pathogens *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* were not detected in the current study. This is presumably due to strict anaerobic cultivation requirement for these species. Similarly, investigators did not detect *P. gingivalis* and *A. actinomycetemcomitans* in healthy oral cavity (Aas *et al*., 2005).

5.6.3 Dorsum of the tongue

Seven of the nine subjects (78%) had *Actinomyces odontolyticus* and *Gemella sanguinis* (Table 4.9). Six of the nine subjects (67%) were detected with *Streptococcus mitis* and *Haemophilus parainfluenzae*. Hence, these four species may have been the commonly occurring bacteria. *Streptococcus parasanguinis* was detected on teeth surface and tongue dorsum but more often from the tongue dorsum (Table 4.9). Four subjects had *S. parasanguinis* on their tongue dorsum and only one subject had these bacteria on his teeth surface. The results are generally in agreement with Paster *et al.* (2006). In our study *G. sanguinis* was found on one of the subject's teeth surface. However, Aas *et al*. (2005) did not find *G. sanguinis* on the teeth of their subjects.

In current study, *Prevotella melaninogenica*, a species associated with halitosis was detected at all the sites but at low frequency (on average, two subjects per site). Oral microflora studies in the elderly (73-93 years) by Preza *et al*. (2009) and infants (1-7 months) by Kononen *et al*. (1992) showed different results. *P. melaninogenica* were prevalent on dorsum of the tongue and absent at subgingiva in elderly while 70% of the infant were detected with *P. melaninogenica* from their tongue (Kononen *et al*., 1992). This showed that the age of the host played a role in the oral microflora composition and distribution.

Overall, *Actinomyces odontolyticus*, *Streptococcus mitis*, and *Haemophilus parainfluenzae* were the most prevalent species present at all three sampling sites (Table 4.9 and figure 4.9).

5.7 Phylogenetic relationship between bacterial species

Figure 4.4 shows an overview of the bacterial diversity of the healthy oral cavity. The phylogenetic tree showed eight main families which included Streptococcaceae, Prevotellaceae, Actinomycetaceae, Fusobacteriaceae, Neisseriaceae, Pasteurellaceae, Flavobacteriaceae, and Veillonellaceae. *Gemella* genus has not yet been grouped as a family in the latest NCBI taxonomy and they are related to *Staphylococcus*. *Gemella* and *Staphylococcus* are sharing the same clade. *Staphylococcus aureus* and *S. pasteuri* are in Staphylococcaceae. *Corynebacterium durum*, *Eubacterium* sp., Lachnospiraceae bacterium, *Pseudomonas aeruginosa* and *Lautropia* sp. are grouped under Corynebacteriaceae, Eubacteriaceae, and Lachnospiraceae, Pseudomonadaceae and Burkholderiaceae families respectively.

5.8 Antibiotic susceptibility tests

Susceptible testing methods traditionally use disk diffusion, and agar or broth dilution. These methods often provide inconsistent results for slow growing bacteria and anaerobes and are very laborious and time consuming to obtain quantitative results (Sanchez *et al*., 1992). However, these problems can be overcome by using Etest® strips from AB Biodisk.

Molecular methods to study the resistance of the oral bacteria are not effective tools because many genes are involved in resistance. About 150 genes are involved for β-lactamase enzymes (Jacoby, 2006) while tetracycline resistance involves about 37 genes (Roberts, 2005). Detection of these resistance genes alone may not tell us whether the bacteria are resistant to the given antibiotics. For example, *Prevotella* strains which tested β-lactamase-positive were susceptible to amoxicillin (Kuriyama *et*

al., 2007) while some β-lactamase-negative isolates were resistant to penicillin (Wybo *et al*., 2007).

5.8.1 Clindamycin resistance

Clindamycin is a bacteriostatic antibiotic. Clindamycin is in the group of macrolides. Macrolides are well absorbed by gastrointestinal tract and diffuse easily into various tissues. Clindamycin is very effective in penetrating bones. Clindamycin inhibits protein synthesis by targeting the ribosomes. Clindamycin is inactivated by the liver and eliminated through bile (Seymour and Hogg, 2008). Clindamycin has been associated with severe colitis caused by *Clostridium difficile*. The intake of clindamycin alters the normal flora of colon leading to overgrowth of *C. difficile* and cause infection. However, the activity of clindamycin against anaerobes is generally considered to be effective (Brook *et al*., 2005; Chan and Chan, 2003).

In the current study, some strains of *Capnocytophaga gingivalis*, *Eikenella corrodens*, *Haemophilus parainfluenzae*, *Lautropia* sp., *Neisseria* sp., *N. meningitidis*, *N. flavescens*, *N. subflava*, *Pseudomonas aeruginosa*, *Streptococcus sanguinis*, and *S. mitis* were resistant to clindamycin (Table 4.10). Of these, *E. corrodens* showed high resistance rate (70%). The resistant strains of *E. corrodens* exhibited high resistance with the MIC range from 16 to >256μg/ml. The resistant strains of *C. gingivalis* were highly resistant as the MIC was >256μg/ml. Of 15 strains of *H. parainfluenzae*, two resistant strains showed MIC of 8μg/ml and 16μg/ml, respectively. Two of the resistant strains of *Lautropia* sp. showed MIC of 12μg/ml. All of the *Neisseria* sp. isolates were resistant and two of them have MIC >256μg/ml. *P. aeruginosa* and one of the *S. mitis* isolates were highly resistant and exhibited MIC >256μg/ml.

In this study (Table 4.10), 24 out of 411 isolates (5.8%) were resistant to clindamycin. It is known that clindamycin is not effective against some non-major putative pathogens like *Eikenella corrodens* and *Haemophilus* species (Brook *et al*., 2005). A study by Khemaleelakul *et al*. (2002) in Thailand has shown that 11% of their 118 endodontic infections isolates were resistant to clindamycin while Baumgartner and Xia (2003) in US reported that 4% of 98 isolates of endodontic infections were resistant to clindamycin. Merriam *et al*. (2006) using the agar dilution method, showed that some strains of *Prevotella melaninogenica* and *Veillonella* species were resistant to clindamycin but the rate is low. The same study showed that all 31 of the *Eikenella corrodens* strains were resistant to clindamycin. In Spain, a study by Maestre *et al*. (2007), 10.3% of 29 *Prevotella buccae* strains, 21.1% of 19 *P. denticola* strains, 11.8% of 17 *P. intermedia* strains, and 9.1% of 11 *P. melaninogenica* strains were resistant to clindamycin. A study by Kulik *et al*. (2008) in Switzerland, showed that 99.1% of 259 *P. intermedia* strains were susceptible to clindamycin. *Prevotella* species are commonly found in infected root canal and endodontic abscesses. It is interesting to note that in our study, none of the *Prevotella* strains were resistant to clindamycin.

5.8.2 Amoxicillin-clavulanic acid resistance

Amoxicillin-clavulanic acid is a combination bacteriocidal antibiotic. It is the combination of amoxicillin trihydrate, a β-lactam antibiotic and potassium clavulanate, a β-lactamase inhibitor (Davies *et al*., 1988). *Streptomyces clavuligerus* produce clavulanic acid, a β-lactam which acts as competitive inhibitor to β-lactamases (Tahlan *et al*., 2004). Amoxicillin-clavulanic acid is a member of penicillin group. This drug diffuses in body fluids but does not enter cells. β-lactam antibiotics inhibit the activity of penicillin-binding proteins (PBPs) in bacterial cell wall synthesis. Disrupted cell wall integrity will cause the bacterial cell to lyse under osmostic pressure. The presence of

β-lactamases, production of low affinity PBPs, antibiotic impermeability and antibiotic efflux are associated with resistance towards β-lactam antibiotics (Falagas and Siakavellas, 2000; Seymour and Hogg, 2008).

β-lactamases are β-lactam hydrolyzing enzymes and are structurally related to PBPs. β-lactamases have been divided into four classes (A to D) based on their molecular structure (Poole, 2004). Classes A, C and D are metal-independent enzymes which have an active site serine. These are the extended spectrum β-lactamases which target wide range of β-lactam antibiotics. Class B are metal-dependent (Zn^{2+}) enzymes (Poole, 2004). Frequently, β-lactamases are plasmid encoded but chromosomal encoded β-lactamases are also present and often associated with integrons (Poole, 2004).

Hydrophilic β-lactam antibiotics enter the cell via porins. Porin deficiency results in antibiotic impermeability and may lead to resistance. There are five families of bacterial drug efflux systems: the major facilitator superfamily; the ATP-binding cassette family; the resistance-nodulation-division family; the multidrug and toxic compound extrusion family; and the small multidrug resistance family (Poole, 2004).

Low affinity PBPs play an important role in resistance particularly in methicillin resistant *S. aureus* (Poole, 2004). These low affinity PBPs have reduced affinity towards β-lactam antibiotics. To overcome this resistance, the binding of the antibiotic to the low affinity PBPs needs to be improved.

In this study, all isolates were susceptible to amoxicillin-clavulanic acid with the exception of *Pseudomonas aeruginosa*, which exhibited MIC >256μg/ml (Table 4.11). A study by Khemaleelakul *et al*. (2002) in Thailand showed that all 118 of their

isolates from endodontic infections were susceptible to amoxicillin-clavulanic acid. Baumgartner and Xia (2003) in US reported that all 98 isolates from endodontic infections were also susceptible to amoxicillin-clavulanic acid while Kulik *et al*. (2008) showed all of 259 *Prevotella intermedia* strains from periodontal diseases were susceptible to amoxicillin-clavulanic acid. In contrast, a study by Lewis *et al*. (1995) in UK, showed that 5% of their isolates from acute suppurative oral infection were resistant to amoxicillin-clavulanic acid. Merriam *et al*. (2006) using the agar dilution method showed that some *Campylobacter* species were resistant to amoxicillin-clavulanic acid but the rate of resistance is low.

Resistance pattern generated by Etest may be used as reference in selection of the type of antibiotic and their appropriate dosage. The dosage is an important factor because insufficient antibiotic dosages could not eradicate the targeted pathogen. Insufficient dosage or sub-optimal dosage can predispose to emergence of antibiotic resistance (Ball *et al*., 2002). When there is allergy to penicillin, clindamycin can be recommended. Both antibiotics should be reserved for severe or unresolved infection especially periodontal disease.

5.9 Identification of oral bacteria by molecular methods

The identification of the isolated oral bacteria was initially done by using biochemical tests (conventional method), but this is very laborious and time consuming. The identities of the bacteria were not always conclusive and usually ambiguous. Of all the isolated aerobic oral bacteria, only *Staphylococcus aureus* and *Pseudomonas aeruginosa* were identified successfully. To overcome this problem, Biolog® bacterial identification kits (phenotype arrays) were used. The results produced did not allow identity probability above 98%. Usually, many replicates were

needed to get conclusive results. Phenotypic analysis in bacterial identification is known to lead to misidentification of the strains (Tang *et al*., 1998), since phenotypic characteristics can change under different circumstances. Many studies have highlighted these problems especially in identification of *Streptococcus* (Hillman *et al*., 1989; Tardiff *et al*., 1989). Even different strains of the same species may have variation in the phenotypic trait (Beighton *et al*., 1991; Kilian *et al*., 1989).

In view of the above short comings, molecular methods have been used to identify the bacteria. For oral bacterial identification, 16S rDNA sequence analysis is a rapid and reliable method. However, some bacteria which are closely related may show high identity in their 16S rDNA sequences. For example, *Streptococcus mitis, S. oralis* and *S. pneumoniae* share > 99% homology in their 16S rDNA sequence. Many other gene sequences have been used in the taxonomic study of *Streptococcus* in addition to 16S rDNA sequence (Kawamura *et al*., 1995). The gene sequences were *groESL* (Teng *et al*., 2002), $sodA_{int}$ (Poyart *et al*., 2002), intergenic spacer (ITS) region (Chen *et al*., 2004), and *rnpB* (Tapp *et al*., 2003). ITS and *rnpB* gene sequences were better in differentiating the closely related *Streptococcus* species. In this study, the *rnpB* gene sequence was used to identify the *Streptococcus* species without ambiguity.

5.10 Electron microscopy of *Streptococcus salivarius*

TEM and SEM were done with the isolated *S. salivarius* from dorsum of the tongue (Figure 4.7(a), 4.7(b), 4.8(a), 4.8(b)) to examine their morphology. In this study, three strains of *S. salivarius* were examined. *S. salivarius* consist of two serological subgroups: Lancefield group K^+ which carries fibrils on the cell surface and Lancefield group K^- which carries fimbriae on the cell surface (Gibbons *et al*., 1972; Handley *et al*., 1984). The flexible fibrils do not have consistent width due to clumping but

fimbriae have consistent width because they do not clump. Fibrils mediate saliva-induced aggregation, hemagglutination, coaggregation and adherence to buccal epithelial cells. Fimbriae do not mediate saliva-induced aggregation and hemagglutination. The adherence to buccal epithelial cells is reduced for K^- strains (Handley *et al*., 1984; Levesque *et al*., 2001). In the current study, the isolated *S. salivarius* were K^+ strains which showed fibrils on their cell surface. To confirm this result, Lancefield group K serum can be used to test the strains. K^+ strains will react with the serum while K^- does not react based on the cited reference (Handley *et al*., 1984).

Studies have shown that *S. salivarius* are able to inhibit the putative pathogenic *S. mutans* and *S. pyogenes* by the production of a variety of broad spectrum bacteriocin-like inhibitory substances (BLIS). *S. salivarius* strain K12 has been used as a commercial probiotic in halitosis treatment and for throat health (Horz *et al*., 2007). Oral volatile sulfur compound levels were reduced after replacing some of the oral bacteria of the halitosis patient with *S. salivarius* strain K12 (Burton *et al*., 2006). Children harboring *S. salivarius* have lower chance in acquiring *S. pyogenes* which cause pharyngitis, tonsillitis, suppurative and non-suppurative complications (Tagg, 2004).

Chapter 6

Conclusion

The bacteria that inhabit the oral cavity are diverse and form a dynamic community. Some bacterial species show a pattern of site-specificity and subject-specificity while some are common in all sites of the oral cavity and in all subjects. In this study, no novel species of oral bacteria were identified. All bacterial species were susceptible to amoxicillin-clavulanic acid except *Pseudomonas aeruginosa* which had high resistance to amoxicillin-clavulanic acid. Clindamycin also showed high activity against most of the isolates except some strains of *Capnocytophaga gingivalis*, *Eikenella corrodens*, *Haemophilus parainfluenzae*, *Lautropia* sp., *Neisseria* sp., *N. meningitidis*, *N. flavescens*, *N. subflava*, *Streptococcus sanguinis*, *S. mitis* and *P. aeruginosa*.

Chapter 7

Suggestion for further study

In the future, oral bacterial profile of Malaysian subjects with specific diseases can be studied. The results can be used to compare with the current study which involved only healthy subjects. Additional study of metronidazole resistance of the oral bacteria can be done to evaluate the effectiveness of this commonly used antibiotic. The evaluation of metronidazole will be useful to local clinicians. More subjects need to be screened for the presence of *S. salivarius* in the Malaysian population and their numbers correlated with specific oral diseases.

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Appendices

Appendix 1
Animal ethics approval letter

UNIVERSITY OF MALAYA
KUALA LUMPUR

يونيورسيتي مالايا

The Leader In Research and Innovation

PEJABAT KETUA

6 Ogos 2009

Teoh Wuen Yew
Institut Sains Biologi
Fakulti Sains
Universiti Malaya

Tuan,

STUDY OF BACTERIAL DIVERSITY IN THE ORAL CAVITY OF MALAYSIAN SUBJECTS

Dengan sukacitanya Jawatankuasa Etika Penjagaan dan Penggunaan Haiwan, Fakulti Perubatan, Universiti Malaya telah meluluskan permohonan untuk penyelidikan tersebut di atas.

No rujukan etika: **ISB/05/08/2009/TWY (R)**

Sila ambil perhatian bahawa nombor rujukan etika yang diberi adalah sah bermula dari **5 Ogos 2009 sehingga 4 Ogos 2011**.

Sila lengkapkan borang yang dilampirkan bersama dengan surat ini (Animal Traffic Record) dan hendaklah dikembalikan kepada pihak kami setelah penyelidikan tamat.

Sekian, terima kasih.

Yang benar,

b/p

Dr. Haji Azizuddin Bin Haji Kamaruddin
Ketua
Pusat Haiwan Makmal
Fakulti Perubatan
Merangkap Setiausaha Jawatankuasa Etika Penjagaan dan Penggunaan Haiwan

SK : Puan Zura Syazleena Hamizan
Setiausaha MCRC
Pejabat Dekan
Fakulti Perubatan

MS ISO 9001:2000 REG. NO. AR 2760

Ketua, Pusat Haiwan Makmal
Fakulti Perubatan, Universiti Malaya, 50603 Kuala Lumpur, Malaysia
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Faculty of Medicine, University of Malaya, 50603, Kuala Lumpur, Malaysia
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E-mail: azizud@um.edu.my Website: http://www.um.edu.my

Appendix 2

23-4-2008

Volunteer information sheet

Study of bacterial diversity in the oral cavity of Malaysian subjects

Dear volunteer,

We would like to invite you to take part in a research study. In this study, we wish to obtain information about the bacterial diversity of the human oral cavity. The oral bacterial diversity of the three Malaysian ethnic groups namely Malays, Chinese and Indians will be studied.

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

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

Thank you.

.....

Teoh Wuen Yew
M.S candidate
Microbiology Division
Institute of Biological Sciences
Faculty of Science
Universiti Malaya
Kuala Lumpur

Appendix 3

Consent form

Study of bacterial diversity in the oral cavity of Malaysian subjects

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

Signature of volunteer : …….

Name : …….

Appendix 4
16S rDNA sequences of GumJ 1 bacteria from Macrogen


File: GumJ_1_PCR-27f.ab1 Run Ended: 2008/11/23 17:35:54 Signal G:280 A:219 C:206 T:217
Sample: GumJ_1_PCR_27f Lane: 74 Base spacing: 15.905454 950 bases in 11259 scans Page 1 of 2
File: GumJ_1_PCR-27f.ab1 Run Ended: 2008/11/23 17:35:54 Signal G:280 A:219 C:206 T:217
Sample: GumJ_1_PCR_27f Lane: 74 Base spacing: 15.905454 950 bases in 11259 scans Page 2 of 2

16S rDNA sequence of GumJ 1

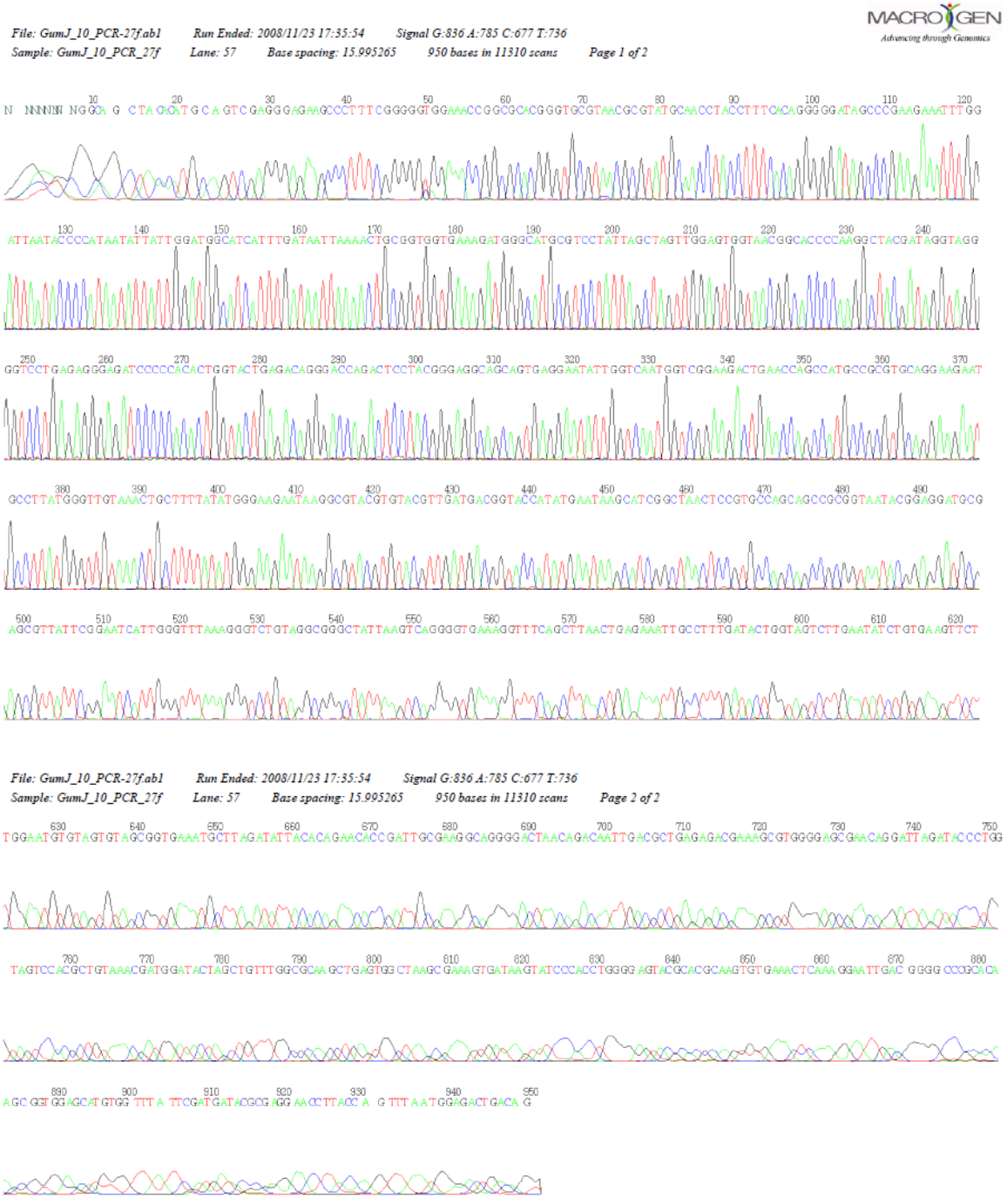
GGGCTTAACACATGCAGTCGAGGGGAAACGGCATTATGTGCTTGCACATTTTGGACGTCGA
CCGGCGCACGGGTGAGTATCGCGTATCCAACCTTCCCTCCACTCGGGGATACCCCGTTGAAA
GACGGCCTAATACCCGATGTTGTCCACATATGGCATCTGACGTGGACCAAAGATTCATCGGT
GGAGGATGGGGATGCGTCTGATTAGCTTGTTGGTGCGGGTAACGGCCCACCAAGGCGACGA
TCAGTAGGGGTTCTGAGAGGAAGGTCCCCCACATTGGAACTGAGACACGGTCCAAACTCCT
ACGGGAGGCAGCAGTGAGGAATATTGGTCAATGGACGTAAGTCTGAACCAGCCAAGTAGC
GTGCAGGATTGACGGCCCTATGGGTTGTAAACTGCTTTTGTTGGGGAGTAAAGTGGGGCAC
GCGTGCCTTTTTGCATTTACCCTTCGAATAAGGACCGGCTAATTCCGTGCCAGCAGCCGCGG
TAATACGGAAGGTCCAGGCGTTATCCGGATTTATTGGGTTTAAAGGGAGTGTAGGCGGTCT
GTTAAGCGTGTTGTGAAATTTAAGTGCTCAACATCTACCTTGCAGCGCGAACTGGCGGACTT
GAGTGCACGCAACGTATGCGGAATTCATGGTGTAGCGGTGAAATGCTTAGATATCATGACG
AACTCCGATTGCGAAGGCAGCGTACGGGAGTGTTACTGACGCTTAAGCTCGAAGGTGCGGG
TATCGAACAGGATTAGATACCCTGGTAGTCCGCACGGTAAACGATGGATGCCCGCTGTTAG
CGCCTGGCGTTAGCGGCTAAGCGAAAGCATTAAGCATCCCACCTGGGGAGTACGCCGGCAA
CGGTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGAGGAACATGTGGTTTAATTC
GATGATACGCGAGGAACCTTACCCGGGCTTGAATTGCAGACGTAGGATACAGAGATGTTGA
CTCCCTTCGGGGCGTCTGTGAAGGTGCTGCATGGTTGTCGTCAGCTCGTGCCGTGAGGTGTC
GGCTTAAGTGCCATAACGAGCGCAACCCCTTTCCTTAGTTGCCATCAGGTGATGCTGGGCAC
TCTGGGGACACTGCCACCGCAAGGTGTGAGGAAGGTGGGGATGACGTCAAATCAGCACGG
CCCTTACGTCCGGGGCTACACACGTGTTACAATGGCCGGTACAGAGGGACGGTGCAATGCA
AATTGCATCCAATCTTGAAAGCCGGTCCCAGTTCGGACTGAGGTCTGCAACCCGACCTCACG
AAGCTGGATTCGCTAGTAATCGCGCATCAGCCATGGCGCGGTGAATACGTTCCCGGGCCTT
GTACACACCGCCCGTCAAGCCATGAAAGCCGGGGGTGCCTGAAGTTCGTAACCGCGAGGAG
CGACCTAGGGCAAAAA

BLAST result

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
FJ983029.1	Uncultured bacterium clone OPEN_PLAQUE_2 16S ribosomal RNA ge	2577	2577	98%	0.0	99%
AY689225.1	Prevotella intermedia strain ChDC KB29 16S ribosomal RNA gene, p	2571	2571	98%	0.0	99%
FJ983035.1	Uncultured bacterium clone OPEN_PLAQUE_9 16S ribosomal RNA ge	2569	2569	98%	0.0	99%
AY689223.1	Prevotella intermedia strain ChDC KB18 16S ribosomal RNA gene, p	2566	2566	98%	0.0	99%
AY689222.1	Prevotella intermedia strain ChDC KB14 16S ribosomal RNA gene, p	2566	2566	98%	0.0	99%
AY689221.1	Prevotella intermedia strain ChDC KB3 16S ribosomal RNA gene, pa	2566	2566	98%	0.0	99%

Appendix 5
16S rDNA sequences of GumJ 10 bacteria from Macrogen


File: GumJ_10_PCR-27f.ab1 Run Ended: 2008/11/23 17:35:54 Signal G:836 A:785 C:677 T:736
Sample: GumJ_10_PCR_27f Lane: 57 Base spacing: 15.995265 950 bases in 11310 scans Page 1 of 2
File: GumJ_10_PCR-27f.ab1 Run Ended: 2008/11/23 17:35:54 Signal G:836 A:785 C:677 T:736
Sample: GumJ_10_PCR_27f Lane: 57 Base spacing: 15.995265 950 bases in 11310 scans Page 2 of 2

16S rDNA of GumJ 10

GGCAGCTACACATGCAGTCGAGGGAGAAGCCCTTTCGGGGGTGGAAACCGGCGCACGGGT
GCGTAACGCGTATGCAACCTACCTTTCACAGGGGGATAGCCCGAAGAAATTTGGATTAATA
CCCCATAATATTATTGGATGGCATCATTTGATAATTAAAACTGCGGTGGTGAAAGATGGGC
ATGCGTCCTATTAGCTAGTTGGAGTGGTAACGGCACCCCAAGGCTACGATAGGTAGGGGTC
CTGAGAGGGAGATCCCCCACACTGGTACTGAGACAGGGACCAGACTCCTACGGGAGGCAG
CAGTGAGGAATATTGGTCAATGGTCGGAAGACTGAACCAGCCATGCCGCGTGCAGGAAGA
ATGCCTTATGGGTTGTAAACTGCTTTTATATGGGAAGAATAAGGCGTACGTGTACGTTGATG
ACGGTACCATATGAATAAGCATCGGCTAACTCCGTGCCAGCAGCCGCGGTATACGGAGGAT
GCGAGCGTTATTCGGAATCATTGGGTTTAAAGGGTCTGTAGGCGGGCTATTAAGTCAGGGG
TGAAAGGTTTCAGCTTAACTGAGAAATTGCCTTTGATACTGGTAGTCTTGAATATCTGTGAA
GTTCTTGGAATGTGTAGTGTAGCGGTGAAATGCTTAGATATTACACAGAACACCGATTGCG
AAGGCAGGGGACTAACAGACAATTGACGCTGAGAGACGAAAGCGTGGGGAGCGAACAGGA
TTAGATACCCTGGTAGTCCACGCTGTAAACGATGGATACTAGCTGTTTGGCGCAAGCTGAGT
GGCTAAGCGAAAGTGATAAGTATCCCACCTGGGGAGTACGCACGCAAGTGTGAAACTCAAA
GGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGATACGCGAGG
AACCTTACCAAGGTTTAAATGGAGACTGACAGGTGTAGAGATACGCCCTTCTTCGGACAGT
TTTCAAGGTGCTGCATGGTTGTCGTCAGCTCGTGCCGTGAGGTGTCAGGTTAAGTCCTATAA
CGAGCGCAACCCCTATTGTTAGTTACCAGCAAGTAAAGTTGGGGACTCTAGCAAGACTGCC
GGTGTAAACCGTGAGGAAGGTGGGGATGACGTCAAATCATCACGGCCCTTACATCTTGGGC
TACACACGTGCTACAATGGTCGTTACAGAGAGCAGCCACTGCGCGAGCAGGAGCGAATCTA
TAAAGACGATCACAGTTCGGATCGGAGTCTGCAACTCGACTCCGTGAAGCTGGAATCGCTA
GTAATCGGATATCAGCCATGATCCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTC
AAGCCATGGAAGCTGGGAGTACCTGAAGTCGGTCACCGCAAGGAGCTGCCTAGGTTAAGCC
C

BLAST result

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
NR_026094.1	Capnocytophaga gingivalis strain ATCC 33624 16S ribosomal RNA,	2516	2516	99%	0.0	99%
L14639.1	Capnocytophaga gingivalis 16S ribosomal RNA	2481	2481	99%	0.0	98%
AF543295.1	Capnocytophaga gingivalis 16S ribosomal RNA gene, partial sequen	2477	2477	99%	0.0	98%
AM420187.1	Uncultured Capnocytophaga sp. partial 16S rRNA gene, clone 502B(	2449	2449	99%	0.0	98%
AM420112.1	Uncultured Capnocytophaga sp. partial 16S rRNA gene, clone 303G(	2431	2431	99%	0.0	98%
AY005073.1	Capnocytophaga sp. oral strain S3 16S ribosomal RNA gene, partial	2422	2422	99%	0.0	97%

Appendix 6
BLAST results of isolates

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AB062278.1	Actinomyces naeslundii gene for 16S rRNA, partial sequence	2551	2551	99%	0.0	98%
AF543282.1	Actinomyces naeslundii 16S ribosomal RNA gene, partial sequence	2540	2540	99%	0.0	98%
AY008315.1	Actinomyces sp. oral clone EP011 16S ribosomal RNA gene, partial	2505	2505	99%	0.0	97%
AJ234045.1	Actinomyces naeslundii 16S rRNA gene, strain CCUG 33519	2505	2505	99%	0.0	97%
X81062.1	A.naeslundii 16S rRNA gene	2484	2484	96%	0.0	98%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
NR_025521.1	Actinomyces cardiffensis strain CCUG 44997 16S ribosomal RNA, pa	2627	2627	99%	0.0	99%
AF355192.1	Actinomyces sp. VA20732_01 16S ribosomal RNA gene, partial sequ	2303	2303	96%	0.0	96%
X78720.1	Actinomyces turicensis 16S rRNA gene, strain APL10	2289	2289	96%	0.0	96%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
FJ821296.1	Actinomyces georgiae strain 600681/2009 16S ribosomal RNA gene	2564	2564	97%	0.0	99%
NR_026182.1	Actinomyces georgiae strain 6843 DSM 16S ribosomal RNA, partial	2534	2534	96%	0.0	99%
AF287750.1	Actinomyces sp. oral strain B27SC 16S ribosomal RNA gene, partial	2462	2462	99%	0.0	97%
FJ976388.1	Uncultured Actinomyces sp. clone 22BB48 16S ribosomal RNA gene	2455	2455	92%	0.0	99%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EU071474.1	Uncultured Actinomyces sp. clone EHFS1_S032 16S ribosomal RNA	2547	2547	98%	0.0	99%
GQ131411.1	Actinomyces odontolyticus strain F0309 16S ribosomal RNA gene, p	2545	2545	98%	0.0	99%
GQ131415.1	Actinomyces sp. oral taxon 180 strain F0310 16S ribosomal RNA ge	2531	2531	98%	0.0	98%
AF385522.1	Actinomyces sp. oral strain Hal-1083 16S ribosomal RNA gene, part	2512	2512	98%	0.0	98%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AF287749.1	Actinomyces sp. oral clone AP064 16S ribosomal RNA gene, partial	2619	2619	98%	0.0	99%
GQ421316.1	Actinomyces oris strain S24V 16S ribososmal RNA gene, partial seq	2564	2564	98%	0.0	98%
GQ421320.1	Actinomyces oris strain S64C 16S ribososmal RNA gene, partial seq	2542	2542	97%	0.0	98%
AF385553.1	Actinomyces sp. oral clone BL008 16S ribosomal RNA gene, partial	2525	2525	98%	0.0	98%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
NR_029286.1	Actinomyces meyeri strain Prevot 2477B 16S ribosomal RNA, partia	2540	2540	98%	0.0	99%
FJ983028.1	Uncultured bacterium clone OPEN_PLAQUE_1 16S ribosomal RNA ge	2431	2431	94%	0.0	99%
GQ073205.1	Uncultured bacterium clone nbw206c09c1 16S ribosomal RNA gene,	2405	2405	93%	0.0	99%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
FM874123.1	Uncultured bacterium partial 16S rRNA gene, clone MC01H07	2632	2632	99%	0.0	99%
AF543275.1	Actinomyces sp. ChDC B197 16S ribosomal RNA gene, partial sequ	2614	2614	99%	0.0	99%
AF287747.1	Actinomyces sp. oral clone AG004 16S ribosomal RNA gene, partial	2610	2610	99%	0.0	99%
FM874888.1	Uncultured bacterium partial 16S rRNA gene, clone SA04F10	2604	2604	99%	0.0	99%
AM697350.1	Uncultured bacterium partial 16S rRNA gene, isolate BF0002C047	2604	2604	99%	0.0	99%
AF543297.1	Actinomyces sp. ChDC OS47 16S ribosomal RNA gene, partial sequ	2599	2599	98%	0.0	99%
FM873522.1	Uncultured bacterium partial 16S rRNA gene, clone MA01B02	2593	2593	99%	0.0	99%
AM420034.1	Uncultured Actinomyces sp. partial 16S rRNA gene, clone 201G06(o	2579	2579	99%	0.0	99%
AF543286.1	Actinomyces viscosus 16S ribosomal RNA gene, partial sequence	2564	2564	99%	0.0	98%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EU083529.1	Aggregatibacter aphrophilus strain CIP 70.73 16S ribosomal RNA ge	2518	2518	99%	0.0	98%
CP001607.1	Aggregatibacter aphrophilus NJ8700, complete genome	2507	1.504e+04	96%	0.0	99%
EF605278.1	Aggregatibacter aphrophilus strain NJ8700 16S ribosomal RNA gene	2507	2507	96%	0.0	99%
AM419971.1	Uncultured Haemophilus sp. partial 16S rRNA gene, clone 101G02(c	2507	2507	96%	0.0	99%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AM419984.1	Uncultured Campylobacter sp. partial 16S rRNA gene, clone 102B05	2547	2547	99%	0.0	99%
AF550655.1	Campylobacter showae strain LMG 12636 16S ribosomal RNA gene,	2547	2547	99%	0.0	99%
FJ470450.1	Uncultured bacterium clone A_D_02_02 16S ribosomal RNA gene, p	2529	2529	99%	0.0	99%
GQ167660.1	Campylobacter showae strain UNSWCD 16S ribosomal RNA gene, p	2519	2519	97%	0.0	99%
L06975.1	Campylobacter showae 16S ribosomal RNA sequence	2486	2486	99%	0.0	98%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AF550653.1	Campylobacter concisus strain LMG 7545 16S ribosomal RNA gene,	2560	2560	99%	0.0	99%
CP000792.1	Campylobacter concisus 13826, complete genome	2549	7648	99%	0.0	99%
AM697210.1	Uncultured bacterium partial 16S rRNA gene, isolate BF0001C051	2543	2543	99%	0.0	99%
AF550654.1	Campylobacter concisus strain LMG 13937 16S ribosomal RNA gene	2543	2543	99%	0.0	99%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AY429469.1	Capnocytophaga sp. P2 oral strain P4P_12 16S ribosomal RNA gene	2460	2460	99%	0.0	98%
U41353.1	Capnocytophaga ochracea LMG 12115 16S ribosomal RNA gene, pa	2446	2446	99%	0.0	97%
AY005080.1	Capnocytophaga sp. oral clone X089 16S ribosomal RNA gene, part	2444	2444	99%	0.0	98%
AF538853.1	Capnocytophaga sp. oral strain P4G_35_P4 16S ribosomal RNA gene	2438	2438	99%	0.0	97%
GU227184.1	Uncultured Capnocytophaga sp. clone 03_2_B05 16S ribosomal RNA	2433	2433	99%	0.0	97%
CP001632.1	Capnocytophaga ochracea DSM 7271, complete genome	2427	9710	99%	0.0	97%
FJ470418.1	Uncultured bacterium clone A_D_01_31 16S ribosomal RNA gene, p	2423	2423	99%	0.0	97%
NR_027581.1	Capnocytophaga ochracea DSM 7271 16S ribosomal RNA, partial se	2416	2416	99%	0.0	97%
U41354.1	Capnocytophaga ochracea FDC 7b 16S ribosomal RNA gene, partial	2414	2414	99%	0.0	97%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
U41347.1	Capnocytophaga granulosa LMG16022 T 16S ribosomal RNA gene, r	2560	2560	99%	0.0	99%
AM419994.1	Uncultured Capnocytophaga sp. partial 16S rRNA gene, clone 102E0	2547	2547	99%	0.0	99%
X97248.1	C.granulosa 16S rRNA gene	2542	2542	99%	0.0	99%
AM420112.1	Uncultured Capnocytophaga sp. partial 16S rRNA gene, clone 303G0	2514	2514	99%	0.0	99%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AB508950.1	Capnocytophaga sputigena gene for 16S ribosomal RNA, partial sec	2532	2532	99%	0.0	99%
AF133536.1	Capnocytophaga sputigena 16S ribosomal RNA gene, partial sequer	2527	2527	99%	0.0	99%
NR_026095.1	Capnocytophaga sputigena strain ATCC 33612 16S ribosomal RNA,	2516	2516	99%	0.0	99%
L14636.1	Capnocytophaga sputigena 16S ribosomal RNA	2486	2486	99%	0.0	99%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AY005078.1	Capnocytophaga sp. oral clone X066 16S ribosomal RNA gene, part	2446	2446	100%	0.0	98%
GU227184.1	Uncultured Capnocytophaga sp. clone 03_2_B05 16S ribosomal RNA	2444	2444	100%	0.0	98%
FJ470418.1	Uncultured bacterium clone A_D_01_31 16S ribosomal RNA gene, p	2425	2425	100%	0.0	97%
AY429469.1	Capnocytophaga sp. P2 oral strain P4P_12 16S ribosomal RNA gene	2422	2422	100%	0.0	97%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AF537593.1	Corynebacterium durum isolate 99-0047 16S ribosomal RNA gene,	2564	2564	98%	0.0	99%
AM697536.1	Uncultured bacterium partial 16S rRNA gene, isolate BF0002D047	2560	2560	98%	0.0	99%
AF543285.1	Corynebacterium durum 16S ribosomal RNA gene, partial sequence	2532	2532	98%	0.0	99%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EF511948.1	Uncultured bacterium clone P1D1-485 16S ribosomal RNA gene, par	2621	2621	99%	0.0	99%
AB525415.1	Eikenella corrodens gene for 16S ribosomal RNA, paertial sequence	2615	2615	99%	0.0	99%
EF511989.1	Uncultured bacterium clone P1D1-574 16S ribosomal RNA gene, par	2615	2615	99%	0.0	99%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AB525414.1	Eubacterium saburreum gene for 16S ribosomal RNA, paertial sequ	2547	2547	98%	0.0	99%
FJ470430.1	Uncultured bacterium clone A_D_01_58 16S ribosomal RNA gene, p	2532	2532	98%	0.0	98%
AY349376.1	Eubacterium sp. oral clone IR009 16S ribosomal RNA gene, partial s	2494	2494	98%	0.0	98%
AF201981.1	Human oral bacterium C73 16S ribosomal RNA gene, partial sequer	2484	2484	98%	0.0	98%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
FJ471656.1	Fusobacterium nucleatum strain ChDC F313 16S ribosomal RNA ger	2553	2553	99%	0.0	99%
FJ471651.1	Fusobacterium nucleatum strain ChDC F305 16S ribosomal RNA ger	2553	2553	99%	0.0	99%
FJ471654.1	Fusobacterium nucleatum strain ChDC F310 16S ribosomal RNA ger	2547	2547	99%	0.0	99%
FJ471653.1	Fusobacterium nucleatum strain ChDC F309 16S ribosomal RNA ger	2547	2547	99%	0.0	99%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
FJ471670.2	Fusobacterium periodonticum strain ChDC F314 16S ribosomal RNA	2538	2538	100%	0.0	99%
FJ471673.1	Fusobacterium periodonticum strain ChDC F334 16S ribosomal RNA	2532	2532	100%	0.0	99%
FJ471672.1	Fusobacterium periodonticum strain ChDC F321 16S ribosomal RNA	2532	2532	100%	0.0	99%
EU647516.1	Uncultured Fusobacterium sp. clone 1614 16S ribosomal RNA gene,	2529	2529	99%	0.0	99%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EU071516.1	Uncultured Gemella sp. clone EHFS1_S13g 16S ribosomal RNA gene	2641	2641	98%	0.0	99%
AY959147.1	Uncultured bacterium clone rRNA374 16S ribosomal RNA gene, part	2641	2641	98%	0.0	99%
EF509672.1	Uncultured bacterium clone P4D1-750 16S ribosomal RNA gene, par	2636	2636	98%	0.0	99%
EF509654.1	Uncultured bacterium clone P4D1-506 16S ribosomal RNA gene, par	2636	2636	98%	0.0	99%
NR_025903.1	Gemella haemolysans strain ATCC 10379 16S ribosomal RNA, parti	2636	2636	98%	0.0	99%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
NR_025904.1	Gemella morbillorum strain 2917B 16S ribosomal RNA, partial sequ	2615	2615	99%	0.0	99%
AM697440.1	Uncultured bacterium partial 16S rRNA gene, isolate BF0001D055	2603	2603	99%	0.0	99%
AM420005.1	Uncultured Gemella sp. partial 16S rRNA gene, clone 102G10(oral)	2599	2599	99%	0.0	99%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EU071472.1	Uncultured Gemella sp. clone EHFS1_S01h 16S ribosomal RNA gene	2599	2599	98%	0.0	99%
NR_026419.1	Gemella sanguinis strain 2045-94 16S ribosomal RNA, partial seque	2593	2593	98%	0.0	98%
EF509667.1	Uncultured bacterium clone P4D1-536 16S ribosomal RNA gene, par	2586	2586	98%	0.0	98%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AF300472.1	Haemophilus segnis 16S ribosomal RNA gene, partial sequence	2597	2597	98%	0.0	99%
DQ003621.1	Haemophilus genomosp. P2 oral clone MB3 C24 16S ribosomal RNA	2558	2558	98%	0.0	99%
AF224299.1	Haemophilus segnis 16S ribosomal RNA gene, partial sequence	2542	2542	98%	0.0	98%
M75043.1	Haemophilus segnis 16S small subunit ribosomal RNA gene sequenc	2529	2529	98%	0.0	98%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AJ295746.3	Haemophilus parahaemolyticus partial 16S rRNA gene, strain NCTC	2584	2584	99%	0.0	99%
AJ290758.2	Haemophilus parahaemolyticus partial 16S rRNA gene, strain HIM57	2556	2556	99%	0.0	99%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EF511968.1	Uncultured bacterium clone P1D1-680 16S ribosomal RNA gene, par	2604	2604	98%	0.0	99%
EF511190.1	Uncultured bacterium clone P5D1-413 16S ribosomal RNA gene, par	2604	2604	98%	0.0	99%
EF511161.1	Uncultured bacterium clone P5D1-589 16S ribosomal RNA gene, par	2604	2604	98%	0.0	99%
EF511101.1	Uncultured bacterium clone P5D1-431 16S ribosomal RNA gene, par	2604	2604	98%	0.0	99%
EF511063.1	Uncultured bacterium clone P5D1-466 16S ribosomal RNA gene, par	2604	2604	98%	0.0	99%
EU083530.1	Haemophilus parainfluenzae strain CIP 102513 16S ribosomal RNA	2599	2599	98%	0.0	99%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EU185458.1	Haemophilus haemolyticus strain 82P14H1 16S ribosomal RNA gene	2540	2540	95%	0.0	99%
EU185452.1	Haemophilus haemolyticus strain 74P33H1 16S ribosomal RNA gene	2540	2540	95%	0.0	99%
EU185447.1	Haemophilus haemolyticus strain 70P28H 16S ribosomal RNA gene,	2536	2536	95%	0.0	99%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
FJ823141.1	Kingella oralis strain TeTP 16S ribosomal RNA gene, partial sequenc	2571	2571	98%	0.0	99%
NR_025891.1	Kingella oralis strain UB-38 16S ribosomal RNA, partial sequence >(	2545	2545	98%	0.0	98%
FJ976419.1	Uncultured Kingella sp. clone 25B127 16S ribosomal RNA gene, part	2447	2447	93%	0.0	99%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
FJ577253.2	Lachnospiraceae bacterium 'Oral Taxon 107' strain F0167 16S ribos	2571	2571	99%	0.0	99%
AF287777.1	Eubacterium cf. saburreum oral strain C27KA 16S ribosomal RNA g	2564	2564	99%	0.0	99%
AM277332.2	Uncultured bacterium partial 16S rRNA gene, clone AP07S.29	2532	2532	99%	0.0	98%
EU644462.1	Uncultured Clostridiales bacterium clone 328a 16S ribosomal RNA g	2527	2527	99%	0.0	98%
AM420023.1	Uncultured Clostridiales bacterium partial 16S rRNA gene, clone 201	2510	2510	99%	0.0	98%
AY230771.1	Eubacterium sp. 'Smarlab BioMol-2301231' 16S ribosomal RNA gene	2497	2497	99%	0.0	98%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AF287816.1	Leptotrichia sp. oral isolate A39FD 16S ribosomal RNA gene, partial	2532	2532	99%	0.0	99%
AM420043.1	Uncultured Leptotrichia sp. partial 16S rRNA gene, clone 202D05(or	2523	2523	99%	0.0	99%
FJ717336.2	Leptotrichia wadei strain F0279 16S ribosomal RNA gene, partial se	2518	2518	99%	0.0	99%
AY029802.1	Leptotrichia wadeii strain LB16 16S ribosomal RNA gene, partial seq	2494	2494	99%	0.0	98%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
CP001685.1	Leptotrichia buccalis DSM 1135, complete genome	2503	1.248e+04	98%	0.0	98%
AM420173.1	Uncultured Leptotrichia sp. partial 16S rRNA gene, clone 501D10(or	2492	2492	98%	0.0	98%
L37788.1	Leptotrichia buccalis 16S ribosomal RNA gene, complete sequence	2460	2460	98%	0.0	98%
GU086178.1	Leptotrichia buccalis strain HKU27 16S ribosomal RNA gene, partial	2396	2396	92%	0.0	99%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
NR_025647.1	Leptotrichia hofstadii strain LB23 16S ribosomal RNA, partial sequer	2549	2549	98%	0.0	99%
AM420175.1	Uncultured Leptotrichia sp. partial 16S rRNA gene, clone 501E06(ora	2542	2542	98%	0.0	99%
AF287813.1	Leptotrichia sp. oral strain FAC5 16S ribosomal RNA gene, partial se	2536	2536	98%	0.0	99%
FJ577251.2	Leptotrichia hofstadii strain F0254 16S ribosomal RNA gene, partial	2534	2534	98%	0.0	99%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EU432173.1	Uncultured bacterium clone MB86 16S ribosomal RNA gene, partial s	2551	2551	98%	0.0	99%
FJ470411.1	Uncultured bacterium clone A_D_01_15 16S ribosomal RNA gene, p	2547	2547	98%	0.0	99%
AY349387.1	Leptotrichia sp. oral clone IK040 16S ribosomal RNA gene, partial se	2545	2545	98%	0.0	99%
GU227197.1	Uncultured Leptotrichia sp. clone 03_5_B09 16S ribosomal RNA gen	2523	2523	98%	0.0	99%
AM420193.1	Uncultured Leptotrichia sp. partial 16S rRNA gene, clone 502D06(or	2523	2523	98%	0.0	99%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
FJ577259.2	Leptotrichia goodfellowii strain F0264 16S ribosomal RNA gene, part	2492	2492	98%	0.0	98%
NR_025649.1	Leptotrichia goodfellowii strain LB57 16S ribosomal RNA, partial seq	2479	2479	98%	0.0	98%
FJ976432.1	Uncultured Leptotrichia sp. clone S085 16S ribosomal RNA gene, pa	2311	2311	93%	0.0	98%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AM420220.1	Uncultured Leptotrichia sp. partial 16S rRNA gene, clone 601D06(or	2534	2534	99%	0.0	99%
AY029801.1	Leptotrichia trevisanii strain LB11 16S ribosomal RNA gene, partial s	2531	2531	99%	0.0	99%
NR_028769.1	Leptotrichia trevisanii strain 'WeeTee' 1999 16S ribosomal RNA, par	2525	2525	99%	0.0	99%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
FJ470436.1	Uncultured bacterium clone A_D_01_67 16S ribosomal RNA gene, p	2610	2610	99%	0.0	99%
AY005023.1	Neisseria sp. oral clone AP085 16S ribosomal RNA gene, partial seq	2603	2603	99%	0.0	99%
GQ131417.1	Neisseria sp. oral taxon 014 str. F0314 16S ribosomal RNA gene, pa	2597	2597	99%	0.0	99%
AM420020.1	Uncultured Neisseria sp. partial 16S rRNA gene, clone 201C11(oral)	2582	2582	99%	0.0	99%

1	Query seq [2]	Query length (nt)	Links [3]	Hit HOMD files [4]	HOMD clone name	Identities (%)	Mismatch [5]	Identities (%) [6]	Mismatch [6]	Score (bits)	Query start [7]	Sbjct start [7]
☐	Seq1	1395		609_9301	Neisseria flava Oral Taxon 609 (AJ239301)	99.5*	7/1379	99.9*	2/1374	2121	14	43
				764_9292	Neisseria sicca Oral Taxon 764 (AJ239292)	99.4*	8/1379	99.8*	3/1374	2118	14	43
				682_9282	Neisseria mucosa Oral Taxon 682 (AJ239282)	99.4*	8/1379	99.8*	3/1374	2118	14	43
				729_9281	Neisseria pharyngis Oral Taxon 729 (AJ239281)	99.3*	10/1379	99.6*	5/1374	2111	14	43

1	Query seq [2]	Query length (nt)	Links [3]	Hit HOMD files [4]	HOMD clone name	Identities (%)	Mismatch [5]	Identities (%) [6]	Mismatch [6]	Score (bits)	Query start [7]	Sbjct start [7]
☐	Seq1	1440	/	610_9280	Neisseria flavescens Oral Taxon 610 {AJ239280}	99.3*	10/1439	99.5*	7/1436	2203	5	11
				476_9291	Neisseria subflava Oral Taxon 476 {AJ239291}	99*	15/1440	99.3*	10/1435	2183	5	11
				669_2273	Neisseria meningitidis Oral Taxon 669 {AF382273}	98.3	25/1440	98.6*	20/1435	2148	5	11
				092_0738	Neisseria weaveri Oral Taxon 092 {L10738}	98.1	28/1439	98.3	25/1436	2140	5	11

1	Query seq [2]	Query length (nt)	Links [3]	Hit HOMD files [4]	HOMD clone name	Identities (%)	Mismatch [5]	Identities (%) [6]	Mismatch [6]	Score (bits)	Query start [7]	Sbjct start [7]
☐	Seq1	1438	/	476_9291	Neisseria subflava Oral Taxon 476 {AJ239291}	99.4*	8/1436	99.8*	3/1431	2205	6	15
				610_9280	Neisseria flavescens Oral Taxon 610 {AJ239280}	98.5*	22/1438	99.1*	13/1429	2151	6	15
				729_9281	Neisseria pharyngis Oral Taxon 729 {AJ239281}	98.1	27/1436	98.5*	22/1431	2138	6	15
				682_9282	Neisseria mucosa Oral Taxon 682 {AJ239282}	98.1	27/1436	98.5*	22/1431	2138	6	15

1	Query seq [2]	Query length (nt)	Links [3]	Hit HOMD files [4]	HOMD clone name	Identities (%)	Mismatch [5]	Identities (%) [6]	Mismatch [6]	Score (bits)	Query start [7]	Sbjct start [7]
☐	Seq1	1441	/	669_2273	Neisseria meningitidis Oral Taxon 669 {AF382273}	98.9*	16/1432	99*	14/1430	2176	8	18
				737_6167	Neisseria polysaccharea Oral Taxon 737 {L06167}	98.8*	17/1432	99*	14/1429	2174	8	18
				610_9280	Neisseria flavescens Oral Taxon 610 {AJ239280}	98.1	27/1433	98.4	23/1429	2136	8	18
				476_9291	Neisseria subflava Oral Taxon 476 {AJ239291}	97.8	31/1433	98.1	27/1429	2121	8	18

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EF534314.1	Prevotella maculosa strain W1609 16S ribosomal RNA gene, partial	2532	2532	98%	0.0	98%
EF534315.1	Prevotella maculosa strain W1609 16S ribosomal RNA gene, partial	2527	2527	98%	0.0	98%
AM420018.1	Uncultured Prevotellaceae bacterium partial 16S rRNA gene, clone 2	2510	2510	98%	0.0	98%
AY005057.1	Prevotella sp. oral clone AA020 16S ribosomal RNA gene, partial se	2468	2468	98%	0.0	97%
AY005058.1	Prevotella sp. oral clone AA016 16S ribosomal RNA gene, partial se	2462	2462	98%	0.0	97%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AM420031.1	Uncultured Prevotella sp. partial 16S rRNA gene, clone 201F12(oral)	2377	2377	98%	0.0	98%
L16478.1	Prevotella buccae ATCC 33690 16S ribosomal RNA gene, complete	2372	2372	98%	0.0	98%
L16477.1	Prevotella buccae ATCC 33574 16S ribosomal RNA gene, complete	2355	2355	98%	0.0	98%
FJ940881.1	Prevotella baroniae strain LBP 19 16S ribosomal RNA gene, partial s	1807	1807	98%	0.0	90%
EF510565.1	Uncultured bacterium clone P2D11-462 16S ribosomal RNA gene, pa	2543	2543	98%	0.0	99%
EF510538.1	Uncultured bacterium clone P2D11-454 16S ribosomal RNA gene, pa	2543	2543	98%	0.0	99%
EF510483.1	Uncultured bacterium clone P2D1-513 16S ribosomal RNA gene, par	2543	2543	98%	0.0	99%
EF510457.1	Uncultured bacterium clone P2D1-722 16S ribosomal RNA gene, par	2543	2543	98%	0.0	99%
EF510497.1	Uncultured bacterium clone P2D1-733 16S ribosomal RNA gene, par	2540	2540	98%	0.0	99%
EF510473.1	Uncultured bacterium clone P2D1-717 16S ribosomal RNA gene, par	2540	2540	98%	0.0	98%
AY323525.1	Prevotella melaninogenica 16S ribosomal RNA gene, partial sequen	2540	2540	98%	0.0	98%
EF510855.1	Uncultured bacterium clone P2D15-541 16S ribosomal RNA gene, pa	2538	2538	98%	0.0	98%
EF510841.1	Uncultured bacterium clone P2D15-694 16S ribosomal RNA gene, pa	2538	2538	98%	0.0	98%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AJ005634.2	Prevotella tannerae 16S rRNA gene, strain ATCC 51259	2558	2558	98%	0.0	99%
AY093466.1	Uncultured bacterium clone MB-A2-118 16S ribosomal RNA, partial s	2538	2538	98%	0.0	99%
AF183404.1	Prevotella tannerae strain 83-10-2 16S ribosomal RNA, complete se	2538	2538	98%	0.0	99%
AF183402.1	Prevotella tannerae strain 131-9-1 16S ribosomal RNA, complete se	2527	2527	98%	0.0	98%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EF509216.1	Uncultured bacterium clone P3D1-415 16S ribosomal RNA gene, par	2579	2579	98%	0.0	99%
AY689227.1	Prevotella nigrescens strain ChDC KB5 16S ribosomal RNA gene, pa	2573	2573	98%	0.0	99%
AM420084.1	Uncultured Prevotella sp. partial 16S rRNA gene, clone 302B11(oral	2562	2562	98%	0.0	99%
AY689229.1	Prevotella nigrescens strain ChDC KB50 16S ribosomal RNA gene, p	2549	2549	98%	0.0	99%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AM420056.1	Uncultured Prevotella sp. partial 16S rRNA gene, clone 203003(oral'	2398	2398	98%	0.0	99%
AY005061.1	Prevotella sp. oral strain B31FD 16S ribosomal RNA gene, partial se	2398	2398	98%	0.0	99%
FJ470487.1	Uncultured bacterium clone A_D_03_07 16S ribosomal RNA gene, p	2390	2390	98%	0.0	99%
AM420039.1	Uncultured Prevotella sp. partial 16S rRNA gene, clone 202A12(oral	2388	2388	98%	0.0	99%
FJ577255.2	Prevotella sp. 'Oral Taxon 317' 16S ribosomal RNA gene, partial sec	2386	2386	98%	0.0	99%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EF511895.1	Uncultured bacterium clone P1D1-568 16S ribosomal RNA gene, par	2580	2580	98%	0.0	99%
EF511975.1	Uncultured bacterium clone P1D1-551 16S ribosomal RNA gene, par	2577	2577	98%	0.0	99%
EF511957.1	Uncultured bacterium clone P1D1-510 16S ribosomal RNA gene, par	2577	2577	98%	0.0	99%
EF511929.1	Uncultured bacterium clone P1D1-764 16S ribosomal RNA gene, par	2575	2575	98%	0.0	99%
EF511999.1	Uncultured bacterium clone P1D1-678 16S ribosomal RNA gene, par	2571	2571	98%	0.0	99%
EF511976.1	Uncultured bacterium clone P1D1-703 16S ribosomal RNA gene, par	2571	2571	98%	0.0	99%
EF511966.1	Uncultured bacterium clone P1D1-536 16S ribosomal RNA gene, par	2571	2571	97%	0.0	99%
EF511900.1	Uncultured bacterium clone P1D1-528 16S ribosomal RNA gene, par	2571	2571	98%	0.0	99%
EF511886.1	Uncultured bacterium clone P1D1-740 16S ribosomal RNA gene, par	2571	2571	98%	0.0	99%
EF511906.1	Uncultured bacterium clone P1D1-533 16S ribosomal RNA gene, par	2569	2569	98%	0.0	99%
FJ577257.2	Prevotella sp. 'Oral Taxon 299' 16S ribosomal RNA gene, partial sec	2566	2566	98%	0.0	99%
EF511962.1	Uncultured bacterium clone P1D1-767 16S ribosomal RNA gene, par	2566	2566	98%	0.0	99%
EF511944.1	Uncultured bacterium clone P1D1-563 16S ribosomal RNA gene, par	2566	2566	98%	0.0	99%
FJ470590.1	Uncultured bacterium clone A_S_01_85 16S ribosomal RNA gene, p	2555	2555	98%	0.0	99%
EF512000.1	Uncultured bacterium clone P1D1-743 16S ribosomal RNA gene, par	2555	2555	98%	0.0	99%
AM420038.1	Uncultured Prevotella sp. partial 16S rRNA gene, clone 202A10(oral	2553	2553	96%	0.0	99%
AY957555.1	Prevotella nanceiensis strain AIP 261.03 16S ribosomal RNA gene,	2553	2553	96%	0.0	99%
EF405529.1	Prevotella nanceiensis strain LBN 297 16S ribosomal RNA gene, par	2543	2543	95%	0.0	99%
AM696784.1	Uncultured bacterium partial 16S rRNA gene, isolate BF0001A071	2536	2536	98%	0.0	98%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EF508938.1	Uncultured bacterium clone P3D5-486 16S ribosomal RNA gene, par	2497	2497	98%	0.0	98%
Y13106.1	Prevotella pallens 16S rRNA gene, strain 9423	2488	2488	98%	0.0	98%
NR_026417.1	Prevotella pallens strain 10371 16S ribosomal RNA, partial sequenc	2484	2484	98%	0.0	98%
AM420128.1	Uncultured Prevotella sp. partial 16S rRNA gene, clone 401G10(oral	2481	2481	98%	0.0	98%
Y13107.1	Prevotella pallens 16S rRNA gene, strain 8792	2466	2466	98%	0.0	98%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
FJ983003.1	Uncultured bacterium clone OPEN_ROOT_48 16S ribosomal RNA ger	2591	2591	98%	0.0	99%
FJ982959.1	Uncultured bacterium clone OPEN_ROOT_2 16S ribosomal RNA gene	2591	2591	98%	0.0	99%
AY323522.1	Prevotella oralis 16S ribosomal RNA gene, partial sequence	2591	2591	98%	0.0	99%
L16480.1	Prevotella oralis ATCC 33269) 16S ribosomal RNA gene, complete s	2514	2514	98%	0.0	98%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
NR_024816.1	Prevotella salivae strain EPSA11 16S ribosomal RNA, partial sequen	2492	2492	98%	0.0	98%
AF385512.1	Prevotella sp. oral clone DO033 16S ribosomal RNA gene, partial se	2481	2481	98%	0.0	98%
AY349396.1	Prevotella sp. oral clone GI032 16S ribosomal RNA gene, partial sec	2403	2403	98%	0.0	97%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
FJ577255.2	Prevotella sp. 'Oral Taxon 317' 16S ribosomal RNA gene, partial sec	2547	2547	98%	0.0	99%
FJ470487.1	Uncultured bacterium clone A_D_03_07 16S ribosomal RNA gene, p	2521	2521	98%	0.0	99%
AM420039.1	Uncultured Prevotella sp. partial 16S rRNA gene, clone 202A12(oral	2521	2521	98%	0.0	99%
EU663611.1	Uncultured Prevotella sp. clone BA1 16S ribosomal RNA gene, partia	2519	2519	98%	0.0	99%
AM420056.1	Uncultured Prevotella sp. partial 16S rRNA gene, clone 203003(oral	2519	2519	98%	0.0	99%
AY005061.1	Prevotella sp. oral strain B31FD 16S ribosomal RNA gene, partial se	2519	2519	98%	0.0	99%
FJ983102.1	Uncultured bacterium clone CLOSED_PLAQUE_22 16S ribosomal RN	2508	2508	98%	0.0	99%
AY278624.1	Prevotella genomosp. C1 16S ribosomal RNA gene, partial sequenc	2508	2508	98%	0.0	99%
DQ139963.1	Candidatus Prevotella conceptionensis 16S ribosomal RNA gene, pa	2503	2503	98%	0.0	98%
FJ717335.2	Prevotella sp. F0295 16S ribosomal RNA gene, partial sequence	2436	2436	98%	0.0	98%
AY836508.1	Prevotella loescheii strain NCTC 11321 16S ribosomal RNA gene, pa	2435	2435	98%	0.0	98%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
GQ050145.1	Uncultured bacterium clone nbu484a02c1 16S ribosomal RNA gene,	2222	2222	96%	0.0	98%
EF509226.1	Uncultured bacterium clone P3D1-660 16S ribosomal RNA gene, par	2220	2220	98%	0.0	97%
EF509179.1	Uncultured bacterium clone P3D1-459 16S ribosomal RNA gene, par	2220	2220	98%	0.0	97%
EF509155.1	Uncultured bacterium clone P3D1-471 16S ribosomal RNA gene, par	2220	2220	98%	0.0	97%
AY323524.1	Prevotella denticola 16S ribosomal RNA gene, partial sequence	2218	2218	98%	0.0	97%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AY546095.1	Rothia dentocariosa ATCC BAA-907 16S ribosomal RNA gene, partia	2529	2529	98%	0.0	99%
AJ717364.1	Rothia dentocariosa 16S rRNA gene, isolate CV54	2529	2529	98%	0.0	99%
EU071468.1	Uncultured Rothia sp. clone EHFS1_S01d 16S ribosomal RNA gene,	2512	2512	98%	0.0	99%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ide
DQ409140.1	Rothia mucilaginosa 16S ribosomal RNA gene, partial sequence	2562	2562	99%	0.0	99%
FJ470589.1	Uncultured bacterium clone A_S_01_77 16S ribosomal RNA gene, p	2556	2556	99%	0.0	99%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AM420244.1	Uncultured Rothia sp. partial 16S rRNA gene, clone 603015(oral)	2588	2588	98%	0.0	99%
AY538697.1	Rothia sp. Smarlab 3302411 16S ribosomal RNA gene, partial seque	2588	2588	98%	0.0	99%
AJ131122.1	Rothia sp. CCUG 25688 16S rRNA gene	2582	2582	98%	0.0	99%
Y13025.1	Actynomyces sp. 16S rRNA gene	2582	2582	98%	0.0	99%
FM872928.1	Uncultured bacterium partial 16S rRNA gene, clone FB04G03	2579	2579	98%	0.0	99%
EU293888.1	Rothia aeria strain Lan 16S ribosomal RNA gene, partial sequence	2577	2577	98%	0.0	99%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
FJ435675.1	Staphylococcus pasteuri strain NJ-1 16S ribosomal RNA gene, partia	2641	2641	98%	0.0	99%
FJ773995.1	Staphylococcus sp. dv8 16S ribosomal RNA gene, partial sequence	2636	2636	98%	0.0	99%
EF127830.1	Staphylococcus pasteuri strain LF-2 16S ribosomal RNA gene, partia	2636	2636	98%	0.0	99%
EU026429.1	Uncultured Staphylococcus sp. clone 4 16S ribosomal RNA gene, pa	2634	2634	98%	0.0	99%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
FJ976251.1	Uncultured Streptococcus sp. clone 6BB311 16S ribosomal RNA gen	1676	2476	92%	0.0	99%
GQ113851.1	Uncultured bacterium clone nbw636h03c1 16S ribosomal RNA gene,	1676	2449	92%	0.0	98%
GQ113647.1	Uncultured bacterium clone nbw634b12c1 16S ribosomal RNA gene,	1676	2471	92%	0.0	99%
GQ105185.1	Uncultured bacterium clone nbw527b05c1 16S ribosomal RNA gene,	1676	2476	92%	0.0	99%
GQ105074.1	Uncultured bacterium clone nbw525q05c1 16S ribosomal RNA gene,	1676	2476	92%	0.0	99%
GQ102819.1	Uncultured bacterium clone nbw706d07c1 16S ribosomal RNA gene,	1676	2476	92%	0.0	99%
GQ101559.1	Uncultured bacterium clone nbw496e06c1 16S ribosomal RNA gene,	1676	2476	92%	0.0	99%
GQ100324.1	Uncultured bacterium clone nbw480f07c1 16S ribosomal RNA gene,	1676	2465	92%	0.0	99%
GQ080932.1	Uncultured bacterium clone nbw1141c03c1 16S ribosomal RNA gene	1676	2476	92%	0.0	99%
GQ078141.1	Uncultured bacterium clone nbw1164e04c1 16S ribosomal RNA gene	1676	2465	92%	0.0	99%
GQ078140.1	Uncultured bacterium clone nbw1164e02c1 16S ribosomal RNA gene	1676	2465	92%	0.0	99%
GQ073556.1	Uncultured bacterium clone nbw210a07c1 16S ribosomal RNA gene,	1676	2465	92%	0.0	99%
GQ073072.1	Uncultured bacterium clone nbw204q04c1 16S ribosomal RNA gene,	1676	2471	92%	0.0	99%
GQ067747.1	Uncultured bacterium clone nbw142c05c1 16S ribosomal RNA gene,	1676	2476	92%	0.0	99%
GQ032246.1	Uncultured bacterium clone nbw904c05c1 16S ribosomal RNA gene,	1676	2465	92%	0.0	99%
GQ000417.1	Uncultured bacterium clone nbu103d03c1 16S ribosomal RNA gene,	1676	2476	92%	0.0	99%
GQ000003.1	Uncultured bacterium clone nbu101a04c1 16S ribosomal RNA gene,	1676	2471	92%	0.0	99%
EU189961.1	Streptococcus sanguinis 16S ribosomal RNA gene, partial sequence	1676	2624	98%	0.0	99%
CP000387.1	Streptococcus sanguinis SK36, complete genome	1676	1.041e+04	98%	0.0	99%
AY981825.1	Uncultured bacterium clone NV20 16S ribosomal RNA gene, partial s	1676	2471	92%	0.0	99%
AY691542.1	Streptococcus sanguinis strain ChDC YSA1 16S ribosomal RNA gene	1676	2606	98%	0.0	99%
NR_024841.1	Streptococcus sanguinis strain ATCC 10556 16S ribosomal RNA, par	1676	2618	98%	0.0	99%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AY584476.1	Streptococcus cristatus strain ATCC 51100 16S ribosomal RNA gene	2580	2580	97%	0.0	99%
AY188347.1	Streptococcus cristatus strain ATCC 51100 16S ribosomal RNA gene	2580	2580	97%	0.0	99%
FJ983033.1	Uncultured bacterium clone OPEN_PLAQUE_7 16S ribosomal RNA ge	2575	2575	97%	0.0	99%
AM420202.1	Uncultured Streptococcus sp. partial 16S rRNA gene, clone 502H08(	2569	2569	97%	0.0	99%
AY005047.1	Streptococcus sp. oral strain B5SC 16S ribosomal RNA gene, partia	2558	2558	97%	0.0	99%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
DQ677784.1	Streptococcus mutans strain ChDC YM25 16S ribosomal RNA gene,	2625	2625	98%	0.0	99%
DQ677775.1	Streptococcus mutans strain ChDC YM44 16S ribosomal RNA gene,	2625	2625	98%	0.0	99%
DQ677774.1	Streptococcus mutans strain ChDC YM40 16S ribosomal RNA gene,	2625	2625	98%	0.0	99%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EF509005.1	Uncultured bacterium clone P3D5-717 16S ribosomal RNA gene, par	2641	2641	99%	0.0	99%
AY691536.1	Streptococcus anginosus strain ChDC YA4 16S ribosomal RNA gene	2641	2641	99%	0.0	99%
AY986762.1	Streptococcus anginosus strain ChDC YA5 16S ribosomal RNA gene	2641	2641	99%	0.0	99%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AY278631.1	Streptococcus genomosp. C3 16S ribosomal RNA gene, partial sequ	2595	2595	98%	0.0	99%
FM873723.1	Uncultured bacterium partial 16S rRNA gene, clone MA03E11	2590	2590	98%	0.0	99%
EF509769.1	Uncultured bacterium clone P4D1-551 16S ribosomal RNA gene, par	2584	2584	98%	0.0	99%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EU071471.1	Uncultured Streptococcus sp. clone EHFS1_S01g 16S ribosomal RNA	2614	2614	98%	0.0	99%
DQ303191.1	Streptococcus parasanguinis strain ATCC 15912 16S ribosomal RNA	2603	2603	98%	0.0	99%
AY485605.1	Streptococcus parasanguinis strain ATCC 15912 16S small subunit r	2599	2599	98%	0.0	99%
NR_024842.1	Streptococcus parasanguinis strain ATCC 15912 16S ribosomal RNA	2599	2599	98%	0.0	99%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AM697392.1	Uncultured bacterium partial 16S rRNA gene, isolate BF0001D007	2645	2645	99%	0.0	99%
AM697167.1	Uncultured bacterium partial 16S rRNA gene, isolate BF0001C008	2639	2639	99%	0.0	99%
EF682883.1	Uncultured Firmicutes bacterium clone F14 4F FL 16S ribosomal RN	2634	2634	99%	0.0	99%
AY485606.1	Streptococcus gordonii strain ATCC 10558 16S small subunit riboso	2634	2634	99%	0.0	99%
NR_028666.1	Streptococcus gordonii strain SK3 16S ribosomal RNA, complete sec	2634	2634	99%	0.0	99%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EF510374.1	Uncultured bacterium clone P2D1-692 16S ribosomal RNA gene, par	2612	2612	99%	0.0	99%
EF510460.1	Uncultured bacterium clone P2D1-560 16S ribosomal RNA gene, par	2608	2608	98%	0.0	99%
EF510466.1	Uncultured bacterium clone P2D1-730 16S ribosomal RNA gene, par	2606	2606	99%	0.0	99%
EF510371.1	Uncultured bacterium clone P2D1-569 16S ribosomal RNA gene, par	2606	2606	99%	0.0	99%
EF510500.1	Uncultured bacterium clone P2D1-505 16S ribosomal RNA gene, par	2601	2601	99%	0.0	99%
EF510406.1	Uncultured bacterium clone P2D1-570 16S ribosomal RNA gene, par	2601	2601	99%	0.0	99%
AM696961.1	Uncultured bacterium partial 16S rRNA gene, isolate BF0002A146	2597	2597	99%	0.0	99%
GQ898532.1	Uncultured bacterium clone S3-54 16S ribosomal RNA gene, partial	2595	2595	98%	0.0	99%
EF510387.1	Uncultured bacterium clone P2D1-735 16S ribosomal RNA gene, par	2595	2595	99%	0.0	99%
AM157441.1	Streptococcus parasanguis 16S rRNA gene, clone 3V4	2595	2595	99%	0.0	99%
EF510412.1	Uncultured bacterium clone P2D1-574 16S ribosomal RNA gene, par	2590	2590	99%	0.0	99%
AM157421.1	Streptococcus parasanguis 16S rRNA gene, clone 5C3	2590	2590	99%	0.0	99%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
DQ346432.2	Uncultured Streptococcus sp. clone 2.27 16S ribosomal RNA gene, p	2614	2614	98%	0.0	99%
EU137418.1	Uncultured bacterium clone Oh 3137A7C 16S ribosomal RNA gene,	2608	2608	98%	0.0	99%
AY485604.1	Streptococcus australis strain ATCC 700641 16S small subunit ribos	2599	2599	98%	0.0	99%
AF385525.1	Streptococcus sp. oral strain T1-E5 16S ribosomal RNA gene, partia	2593	2593	97%	0.0	99%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
FJ154802.1	Streptococcus salivarius strain CCUG 32452 16S ribosomal RNA ger	2527	2527	97%	0.0	99%
FJ154799.1	Streptococcus salivarius strain CCRI 17393 16S ribosomal RNA gen	2527	2527	97%	0.0	99%
AM696917.1	Uncultured bacterium partial 16S rRNA gene, isolate BF0002A102	2527	2527	97%	0.0	99%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AM420116.1	Uncultured Streptococcus sp. partial 16S rRNA gene, clone 401B03(	2604	2604	98%	0.0	99%
EF511098.1	Uncultured bacterium clone P5D1-391 16S ribosomal RNA gene, par	2588	2588	98%	0.0	99%
FJ405281.1	Streptococcus sp. F1 16S ribosomal RNA gene, partial sequence	2582	2582	98%	0.0	99%
EF511072.1	Uncultured bacterium clone P5D1-651 16S ribosomal RNA gene, par	2582	2582	98%	0.0	99%
DQ016729.1	Uncultured Streptococcus sp. clone 1.23 16S ribosomal RNA gene, p	2582	2582	98%	0.0	99%
AF385523.1	Streptococcus sp. oral strain H3-M2 16S ribosomal RNA gene, partia	2580	2580	98%	0.0	99%
EF511193.1	Uncultured bacterium clone P5D1-417 16S ribosomal RNA gene, par	2577	2577	98%	0.0	99%
EF511129.1	Uncultured bacterium clone P5D1-427 16S ribosomal RNA gene, par	2577	2577	98%	0.0	99%
EF511070.1	Uncultured bacterium clone P5D1-660 16S ribosomal RNA gene, par	2577	2577	98%	0.0	99%
EF511208.1	Uncultured bacterium clone P5D4-525 16S ribosomal RNA gene, par	2571	2571	98%	0.0	99%
EF511191.1	Uncultured bacterium clone P5D1-388 16S ribosomal RNA gene, par	2571	2571	98%	0.0	99%
EF511134.1	Uncultured bacterium clone P5D1-645 16S ribosomal RNA gene, par	2571	2571	98%	0.0	99%
EF511121.1	Uncultured bacterium clone P5D1-443 16S ribosomal RNA gene, par	2571	2571	98%	0.0	99%
EF511118.1	Uncultured bacterium clone P5D1-450 16S ribosomal RNA gene, par	2571	2571	98%	0.0	99%
EF511102.1	Uncultured bacterium clone P5D1-411 16S ribosomal RNA gene, par	2571	2571	98%	0.0	99%
FM873074.1	Uncultured bacterium partial 16S rRNA gene, clone FC01G09	2569	2569	98%	0.0	99%
FJ192921.1	Uncultured Streptococcus sp. clone GI5-006-B07 16S ribosomal RN/	2569	2569	98%	0.0	99%
AY485603.1	Streptococcus infantis strain ATCC 700779 16S small subunit riboso	2569	2569	98%	0.0	99%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AY349403.1	Selenomonas sp. oral clone FT050 16S ribosomal RNA gene, partial	2459	2459	98%	0.0	98%
AF385514.1	Selenomonas sp. oral clone DO042 16S ribosomal RNA gene, partia	2422	2422	97%	0.0	98%
AY349404.1	Selenomonas sp. oral clone GI064 16S ribosomal RNA gene, partial	2403	2403	97%	0.0	97%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
CP001820.1	Veillonella parvula DSM 2008, complete genome	2628	1.043e+04	99%	0.0	99%
AF366266.1	Uncultured Veillonella sp. clone BU083 16S ribosomal RNA gene, pa	2608	2608	99%	0.0	99%
AY995767.1	Veillonella parvula strain ATCC 10790 16S ribosomal RNA gene, par	2606	2606	99%	0.0	98%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
FM873417.1	Uncultured bacterium partial 16S rRNA gene, clone FD03C07	2652	2652	99%	0.0	99%
AY995770.1	Veillonella dispar strain ATCC 17748 16S ribosomal RNA gene, part	2645	2645	99%	0.0	99%
EU670057.1	Uncultured bacterium clone FIU_KM_MD_005 16S ribosomal RNA ge	2641	2641	99%	0.0	99%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EU670064.1	Uncultured bacterium clone FIU_KM_MD_012 16S ribosomal RNA ge	2590	2590	99%	0.0	99%
EU071469.1	Uncultured Veillonella sp. clone EHFS1_S01e 16S ribosomal RNA ge	2590	2590	99%	0.0	98%
AM697393.1	Uncultured bacterium partial 16S rRNA gene, isolate BF0001D008	2582	2582	99%	0.0	98%
EU670054.1	Uncultured bacterium clone FIU_KM_MD_002 16S ribosomal RNA ge	2573	2573	99%	0.0	98%
AY995768.1	Veillonella atypica strain ATCC 17744 16S ribosomal RNA gene, par	2573	2573	99%	0.0	98%
GQ179688.1	Uncultured Veillonella sp. clone VE4A07 16S ribosomal RNA gene, p	2569	2569	99%	0.0	98%
X84007.1	V.atypica 16S rRNA gene	2566	2566	99%	0.0	98%

Appendix 7
BLAST results of *rnpB* gene

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AJ511698.1	Streptococcus oralis partial rnpB gene for ribonuclease P RNA, strai	595	595	91%	4e-167	99%
EU340865.1	Streptococcus oralis strain CCUG 24891 ribonuclease P RNA (rnpB)	584	584	89%	1e-163	99%
AJ511694.1	Streptococcus mitis partial rnpB gene for ribonuclease P RNA, strair	523	523	91%	2e-145	95%
CP000920.1	Streptococcus pneumoniae P1031, complete genome	507	507	91%	2e-140	94%
CP000919.1	Streptococcus pneumoniae JJA, complete genome	507	507	91%	2e-140	94%
CP000918.1	Streptococcus pneumoniae 70585, complete genome	507	507	91%	2e-140	94%
AE007317.1	Streptococcus pneumoniae R6, complete genome	507	507	91%	2e-140	94%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
CP000920.1	Streptococcus pneumoniae P1031, complete genome	628	628	94%	4e-177	99%
CP000919.1	Streptococcus pneumoniae JJA, complete genome	628	628	94%	4e-177	99%
CP000918.1	Streptococcus pneumoniae 70585, complete genome	628	628	94%	4e-177	99%
AE007317.1	Streptococcus pneumoniae R6, complete genome	628	628	94%	4e-177	99%
CP001015.1	Streptococcus pneumoniae G54, complete genome	628	628	94%	4e-177	99%
AE005672.3	Streptococcus pneumoniae TIGR4, complete genome	628	628	94%	4e-177	99%
CP000410.1	Streptococcus pneumoniae D39, complete genome	628	628	94%	4e-177	99%
AJ511703.1	Streptococcus pneumoniae partial rnpB gene for ribonuclease P RN/	627	627	94%	2e-176	99%
FM211187.1	Streptococcus pneumoniae ATCC 700669 complete genome	623	623	94%	2e-175	99%
CP001033.1	Streptococcus pneumoniae CGSP14, complete genome	623	623	94%	2e-175	99%
CP000921.1	Streptococcus pneumoniae Taiwan19F-14, complete genome	617	617	94%	1e-173	99%
CP000936.1	Streptococcus pneumoniae Hungary19A-6, complete genome	617	617	94%	1e-173	99%
AJ511694.1	Streptococcus mitis partial rnpB gene for ribonuclease P RNA, strair	571	571	94%	8e-160	96%
AJ511698.1	Streptococcus oralis partial rnpB gene for ribonuclease P RNA, strai	549	549	94%	4e-153	95%
EU340865.1	Streptococcus oralis strain CCUG 24891 ribonuclease P RNA (rnpB)	538	538	92%	8e-150	95%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AJ511737.1	Streptococcus intermedius partial rnpB gene for ribonuclease P RNA	593	593	93%	2e-166	99%
AJ511743.1	Streptococcus intermedius partial rnpB gene for ribonuclease P RNA	588	588	93%	7e-165	98%
AJ511738.1	Streptococcus intermedius partial rnpB gene for ribonuclease P RNA	588	588	93%	7e-165	98%
AJ511735.1	Streptococcus anginosus partial rnpB gene for ribonuclease P RNA,	514	514	93%	1e-142	94%
AJ511736.1	Streptococcus anginosus partial rnpB gene for ribonuclease P RNA,	503	503	93%	3e-139	94%
AJ511732.1	Streptococcus anginosus partial rnpB gene for ribonuclease P RNA,	492	492	93%	6e-136	93%
AJ511739.1	Streptococcus anginosus partial rnpB gene for ribonuclease P RNA,	486	486	93%	3e-134	93%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AJ511694.1	Streptococcus mitis partial rnpB gene for ribonuclease P RNA, strair	580	580	93%	1e-162	97%
CP000920.1	Streptococcus pneumoniae P1031, complete genome	568	568	93%	1e-158	96%
CP000919.1	Streptococcus pneumoniae JJA, complete genome	568	568	93%	1e-158	96%
CP000918.1	Streptococcus pneumoniae 70585, complete genome	568	568	93%	1e-158	96%
AE007317.1	Streptococcus pneumoniae R6, complete genome	568	568	93%	1e-158	96%
CP001015.1	Streptococcus pneumoniae G54, complete genome	568	568	93%	1e-158	96%
CP000410.1	Streptococcus pneumoniae D39, complete genome	568	568	93%	1e-158	96%
AJ511703.1	Streptococcus pneumoniae partial rnpB gene for ribonuclease P RNA	568	568	93%	1e-158	96%
AE005672.3	Streptococcus pneumoniae TIGR4, complete genome	562	562	93%	5e-157	96%
CP000921.1	Streptococcus pneumoniae Taiwan19F-14, complete genome	560	560	93%	2e-156	96%
FM211187.1	Streptococcus pneumoniae ATCC 700669 complete genome	560	560	93%	2e-156	96%
CP001033.1	Streptococcus pneumoniae CGSP14, complete genome	560	560	93%	2e-156	96%
AJ511698.1	Streptococcus oralis partial rnpB gene for ribonuclease P RNA, strai	553	553	93%	3e-154	95%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AJ511723.1	Streptococcus salivarius partial rnpB gene for ribonuclease P RNA, s	610	610	100%	1e-171	99%
AJ511715.1	Streptococcus salivarius partial rnpB gene for ribonuclease P RNA, s	610	610	100%	1e-171	99%
AJ511726.1	Streptococcus salivarius partial rnpB gene for ribonuclease P RNA, s	604	604	100%	7e-170	99%
AJ511722.1	Streptococcus salivarius partial rnpB gene for ribonuclease P RNA, s	604	604	100%	7e-170	99%
AJ511716.1	Streptococcus salivarius partial rnpB gene for ribonuclease P RNA, s	588	588	100%	7e-165	98%
AJ511725.1	Streptococcus thermophilus partial rnpB gene for ribonuclease P RN	577	577	100%	1e-161	97%
AJ511724.1	Streptococcus vestibularis partial rnpB gene for ribonuclease P RNA	577	577	100%	1e-161	97%
AJ511721.1	Streptococcus vestibularis partial rnpB gene for ribonuclease P RNA	577	577	100%	1e-161	97%
AJ511720.1	Streptococcus vestibularis partial rnpB gene for ribonuclease P RNA	577	577	100%	1e-161	97%

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AJ511742.1	Streptococcus constellatus partial rnpB gene for ribonuclease P RNA	606	606	97%	2e-170	99%
AJ511741.1	Streptococcus constellatus partial rnpB gene for ribonuclease P RNA	606	606	97%	2e-170	99%
AJ511733.1	Streptococcus anginosus partial rnpB gene for ribonuclease P RNA,	606	606	97%	2e-170	99%
AJ511730.1	Streptococcus constellatus partial rnpB gene for ribonuclease P RNA	606	606	97%	2e-170	99%
AJ511728.1	Streptococcus constellatus partial rnpB gene for ribonuclease P RNA	606	606	97%	2e-170	99%
AJ511727.1	Streptococcus constellatus partial rnpB gene for ribonuclease P RNA	606	606	97%	2e-170	99%
AJ511740.1	Streptococcus anginosus partial rnpB gene for ribonuclease P RNA,	601	601	97%	9e-169	98%
EU340863.1	Streptococcus milleri strain NCTC 10708 ribonuclease P RNA (rnpB)	595	595	95%	4e-167	99%
AJ511734.1	Streptococcus intermedius partial rnpB gene for ribonuclease P RNA	593	593	96%	2e-166	98%
AJ511729.1	Streptococcus intermedius partial rnpB gene for ribonuclease P RNA	593	593	96%	2e-166	98%
AJ511744.1	Streptococcus anginosus partial rnpB gene for ribonuclease P RNA,	584	584	97%	9e-164	97%
AJ511731.1	Streptococcus anginosus partial rnpB gene for ribonuclease P RNA,	573	573	97%	2e-160	97%

Appendix 8
Additional statistical analysis

Kruskal-Wallis Test

Ranks

	site	N	Mean Rank
CFU	Gingival crevices	9	15.78
	Teeth surfaces	9	6.22
	Tongue dorsa	9	20.00
	Total	27	

Test Statistics[a,b]

	CFU
Chi-Square	14.249
df	2
Asymp. Sig.	.001

a. Kruskal Wallis Test

b. Grouping Variable: site

Mann-Whitney Test

Ranks

	site	N	Mean Rank	Sum of Ranks
CFU	Gingival crevices	9	12.78	115.00
	Teeth surfaces	9	6.22	56.00
	Total	18		

Test Statistics[b]

	CFU
Mann-Whitney U	11.000
Wilcoxon W	56.000
Z	-2.605
Asymp. Sig. (2-tailed)	.009
Exact Sig. [2*(1-tailed Sig.)]	.008[a]

a. Not corrected for ties.

b. Grouping Variable: site

Mann-Whitney Test

Ranks

	site	N	Mean Rank	Sum of Ranks
CFU	Gingival crevices	9	8.00	72.00
	Tongue dorsa	9	11.00	99.00
	Total	18		

Test Statistics[b]

	CFU
Mann-Whitney U	27.000
Wilcoxon W	72.000
Z	-1.194
Asymp. Sig. (2-tailed)	.233
Exact Sig. [2*(1-tailed Sig.)]	.258[a]

a. Not corrected for ties.

b. Grouping Variable: site

Mann-Whitney Test

Ranks

	site	N	Mean Rank	Sum of Ranks
CFU	Teeth surfaces	9	5.00	45.00
	Tongue dorsa	9	14.00	126.00
	Total	18		

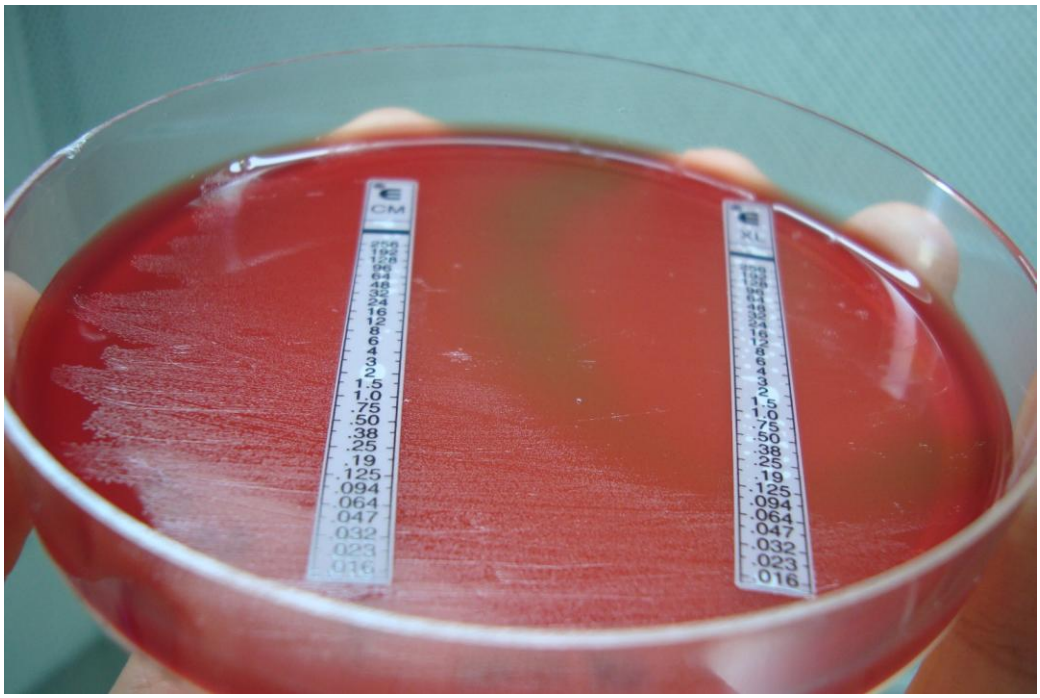
Test Statistics[b]

	CFU
Mann-Whitney U	.000
Wilcoxon W	45.000
Z	-3.578
Asymp. Sig. (2-tailed)	.000
Exact Sig. [2*(1-tailed Sig.)]	.000[a]

a. Not corrected for ties.

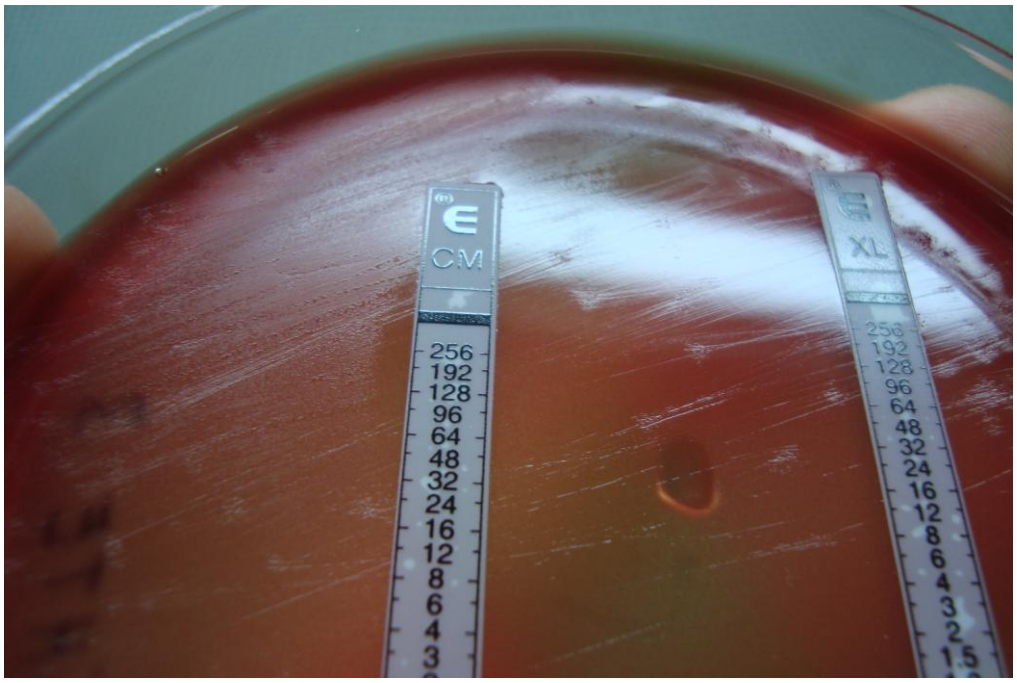
b. Grouping Variable: site

Appendix 9
Etest (CM = clindamycin; XL = amoxicillin-clavulanate)

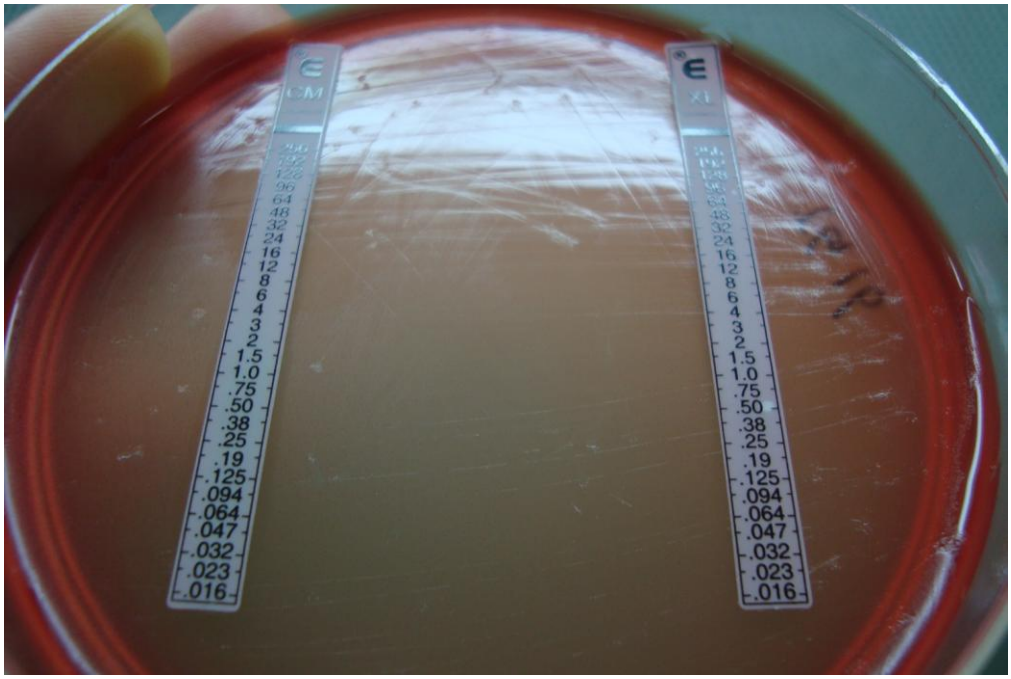


Eikenella corrodens GHG7 CM MIC = 128 µg/ml; XL MIC = 0.19 µg/ml

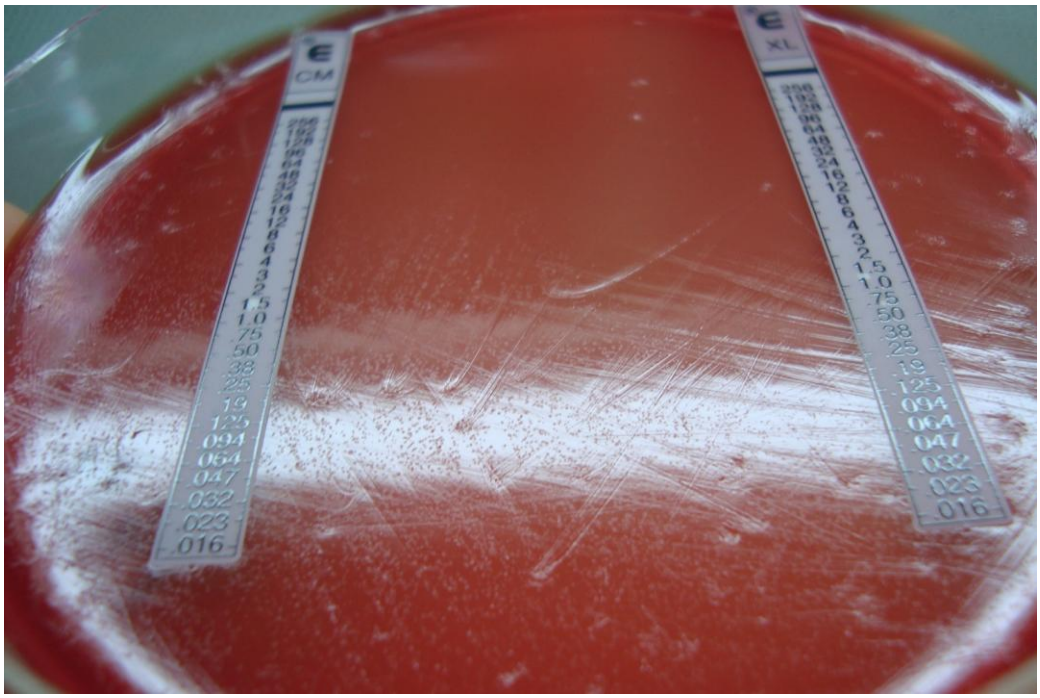
Eikenella corrodens PG2 CM MIC = 64 µg/ml; XL MIC = 0.38 µg/ml



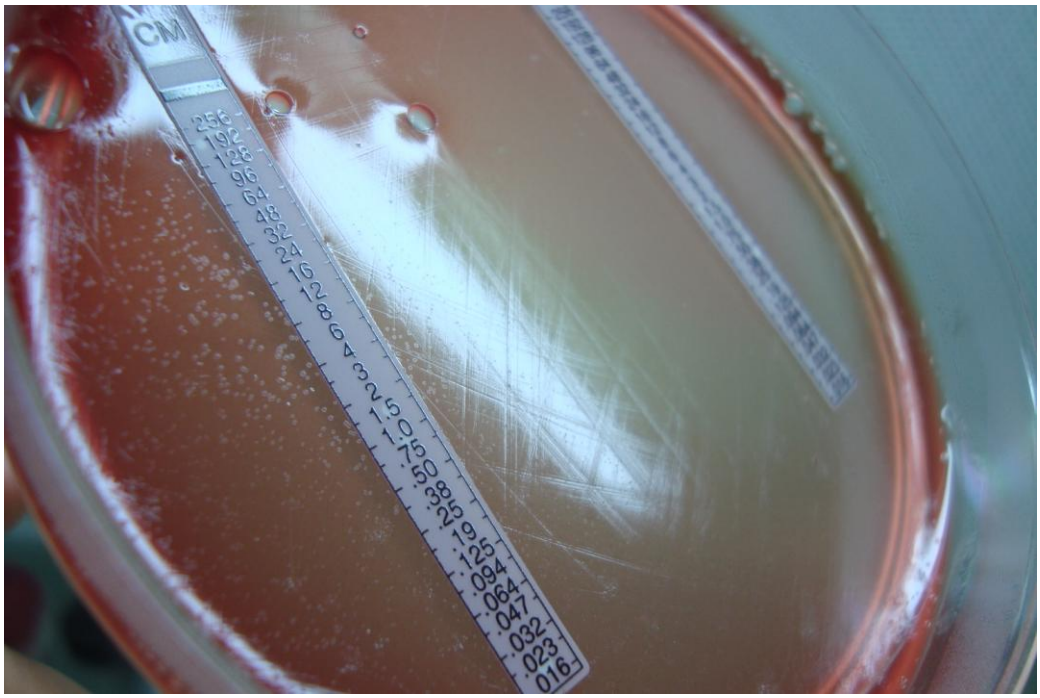
Eikenella corrodens GHTE3 CM MIC = 128 µg/ml; XL MIC =0.125 µg/ml



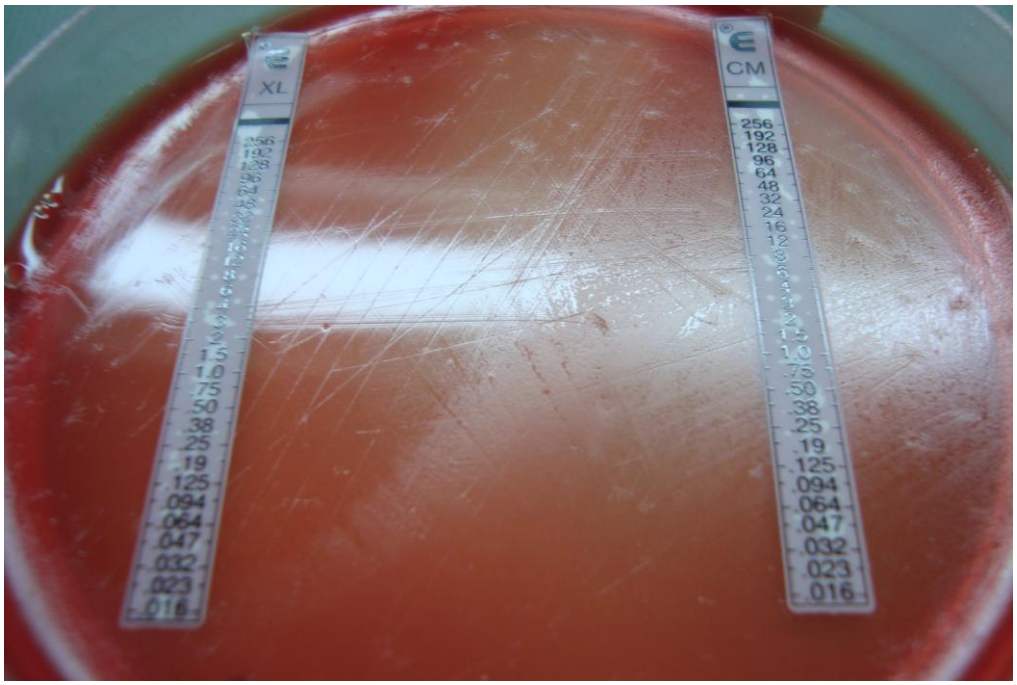
Eikenella corrodens TG16 CM MIC = 24 µg/ml; XL MIC = 0.094 µg/ml



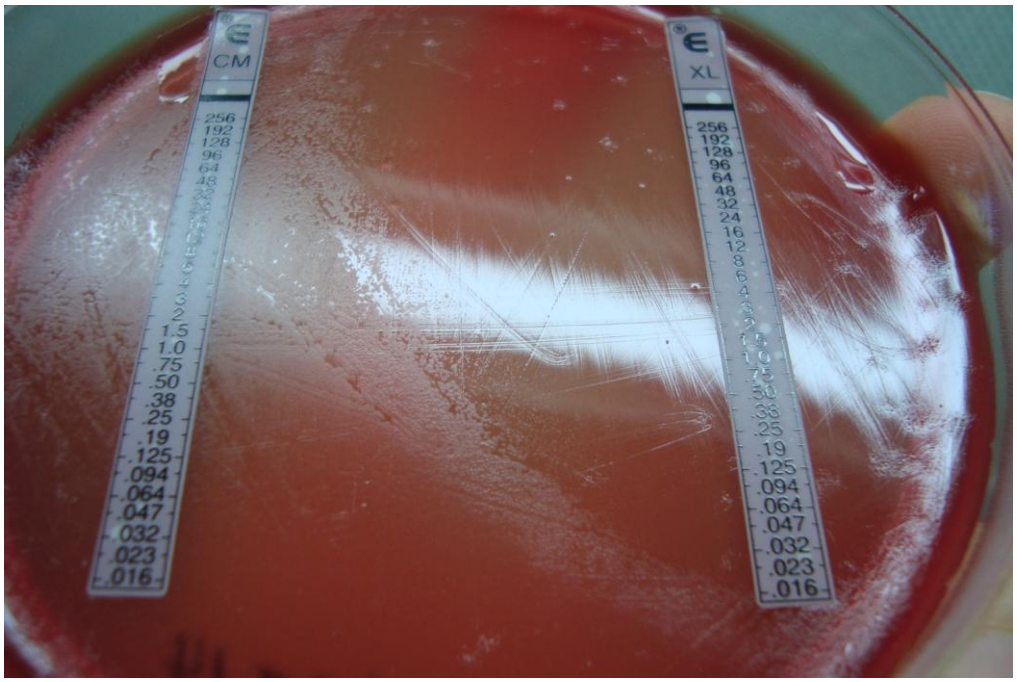
Eikenella corrodens PG14 CM MIC = 16 µg/ml; XL MIC = 0.094 µg/ml



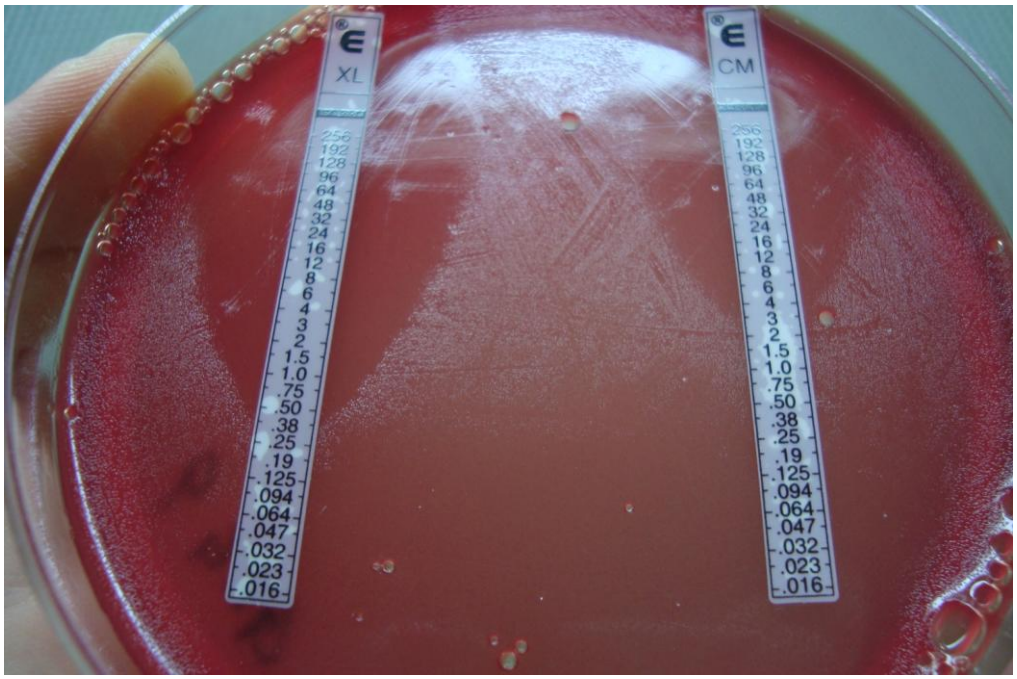
Eikenella corrodens TTO9 resistant to CM; XL MIC <0.016 µg/ml



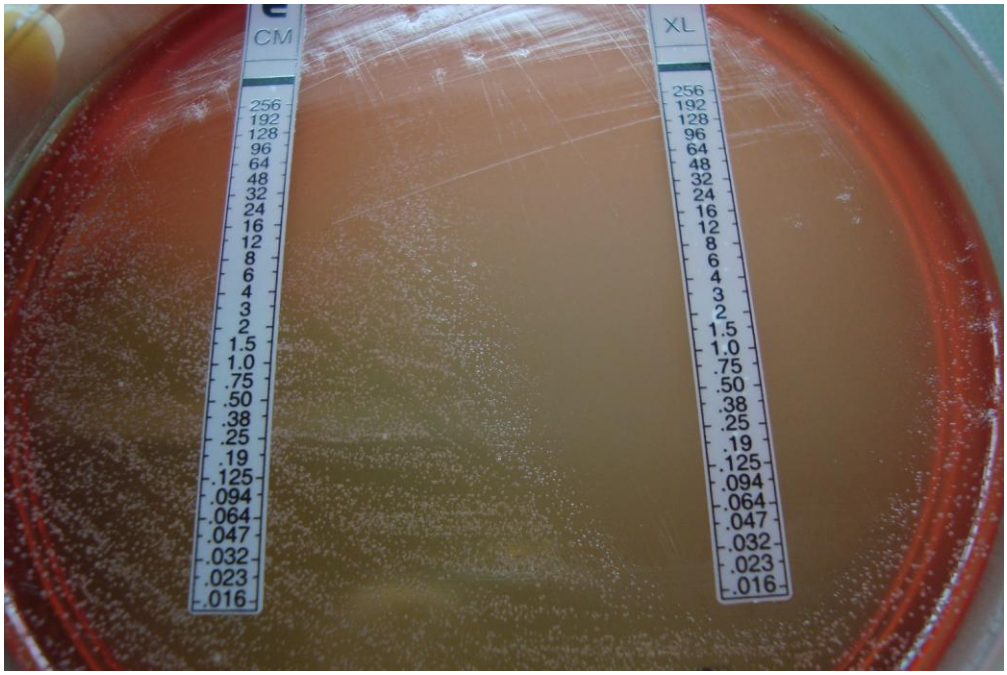
Capnocytophaga gingivalis GRTE12 resistant to CM; XL MIC = 0.023 µg/ml



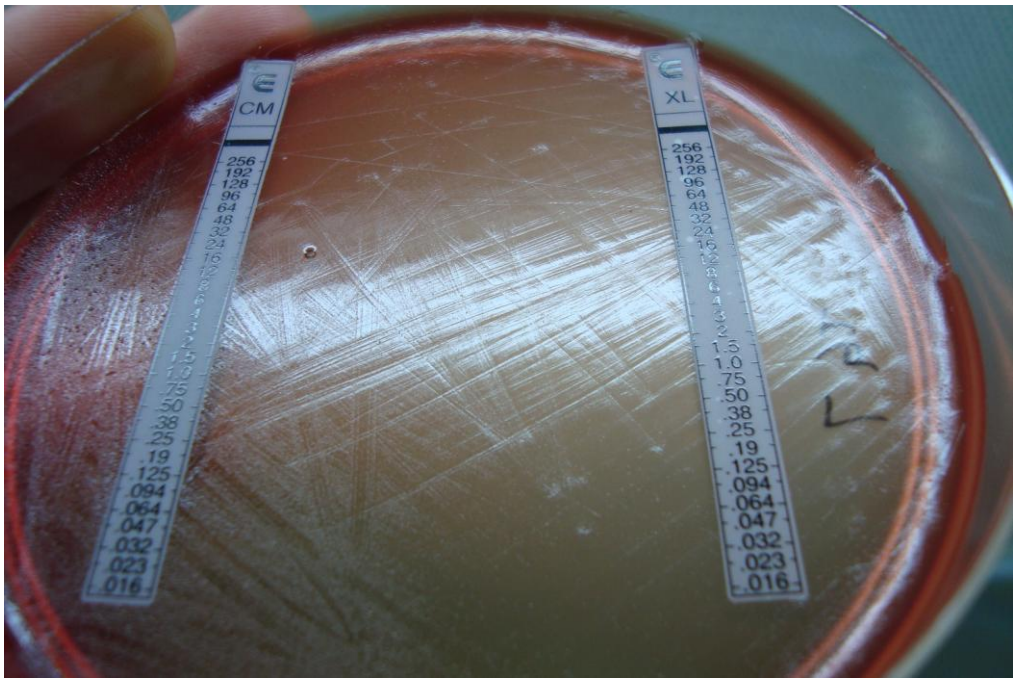
Capnocytophaga gingivalis GRT14 resistant to CM; XL MIC = 0.016 µg/ml



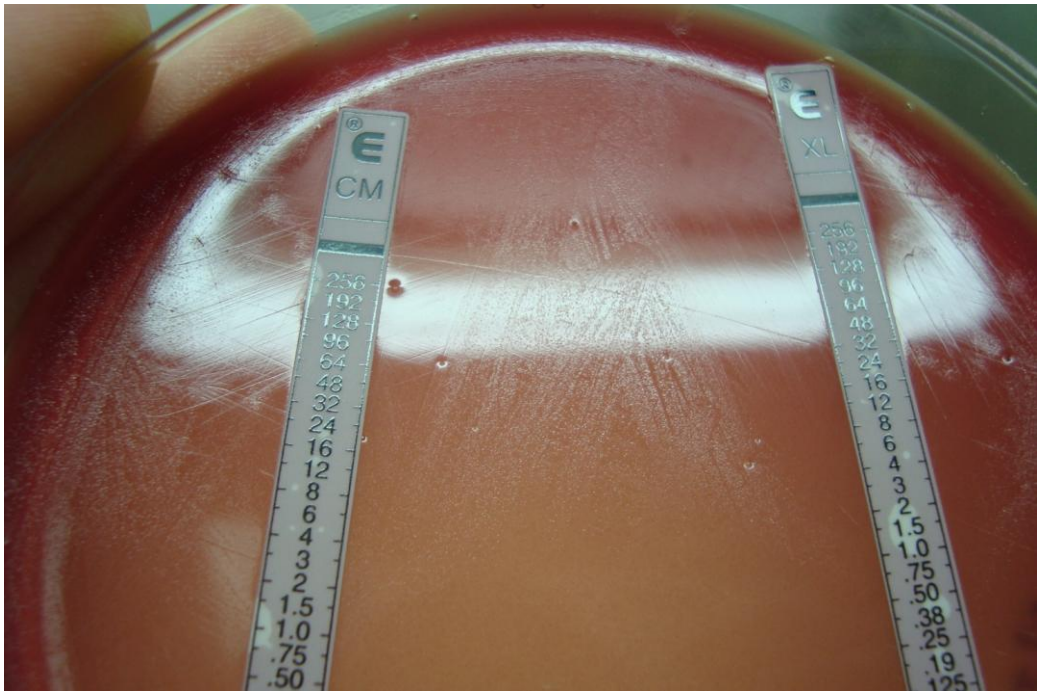
Haemophilus parainfluenzae GRG9 CM MIC = 8 µg/ml; XL MIC = 0.75 µg/ml



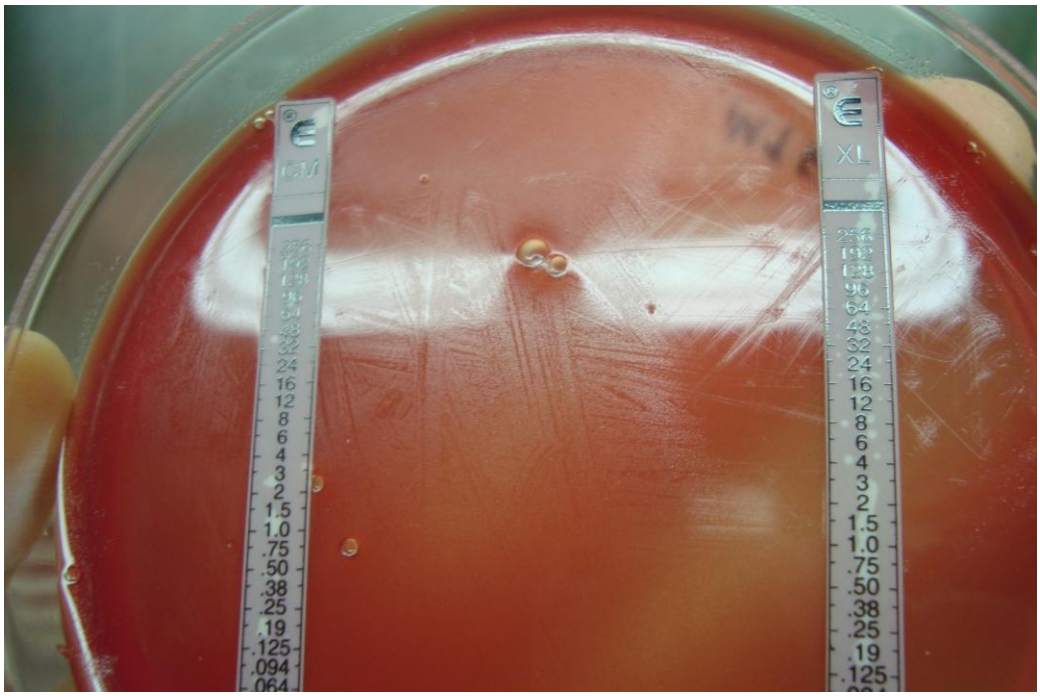
Lautropia sp. O CM MIC = 12 µg/ml; XL MIC = 0.016 µg/ml



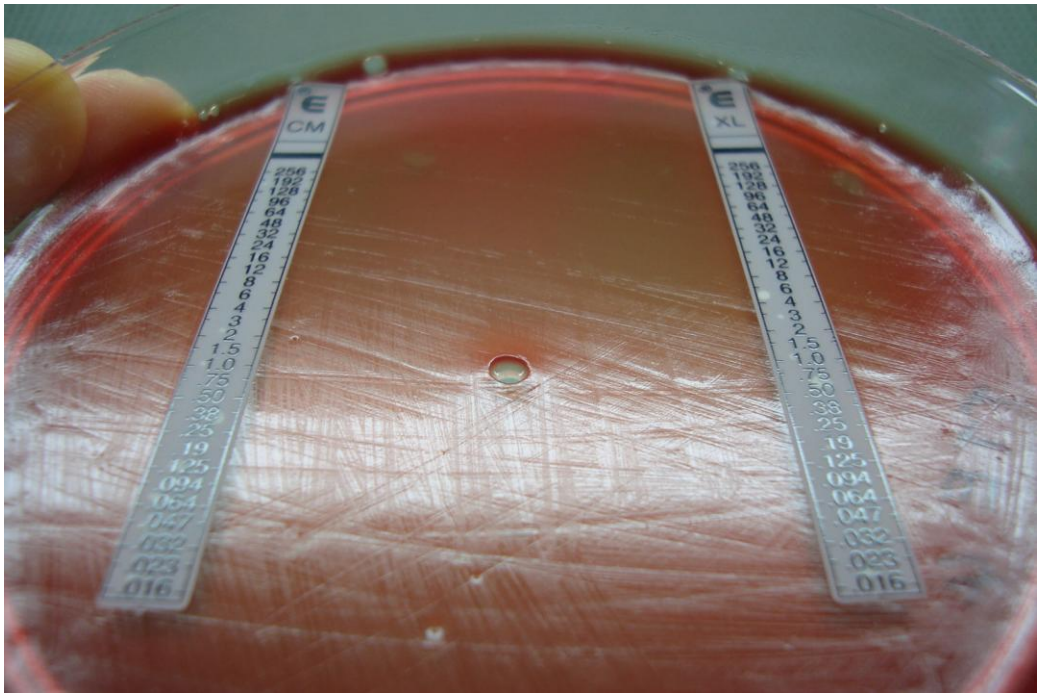
Neisseria sp. TG7 CM MIC = 48 μg/ml; XL MIC = 0.094 μg/ml



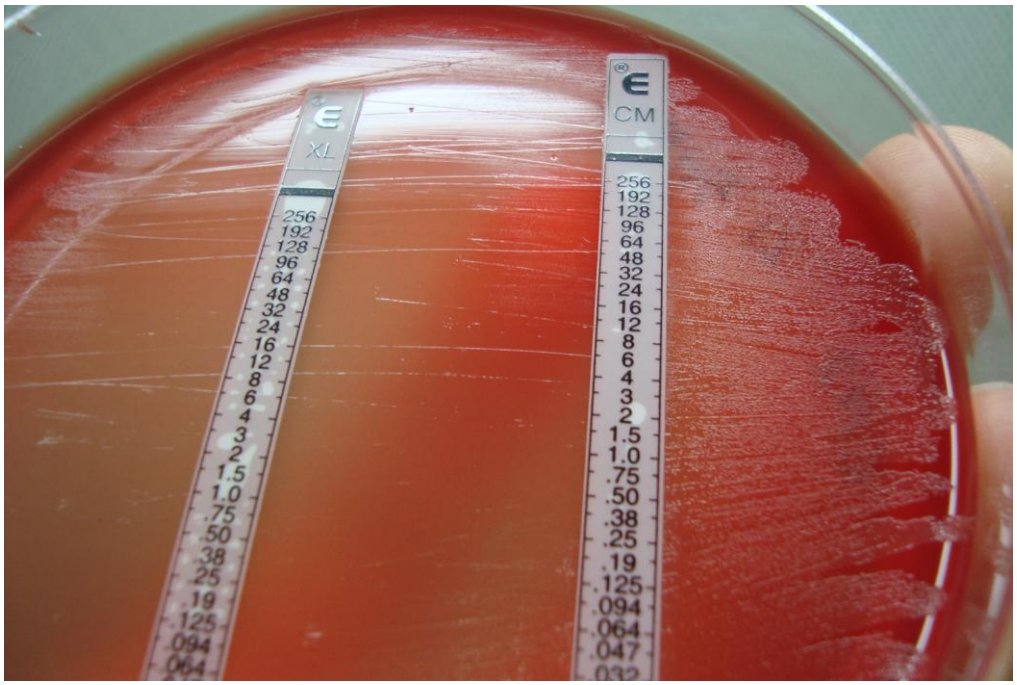
Neisseria sp. MTE1 CM MIC = 16 μg/ml; XL MIC = 1.5 μg/ml



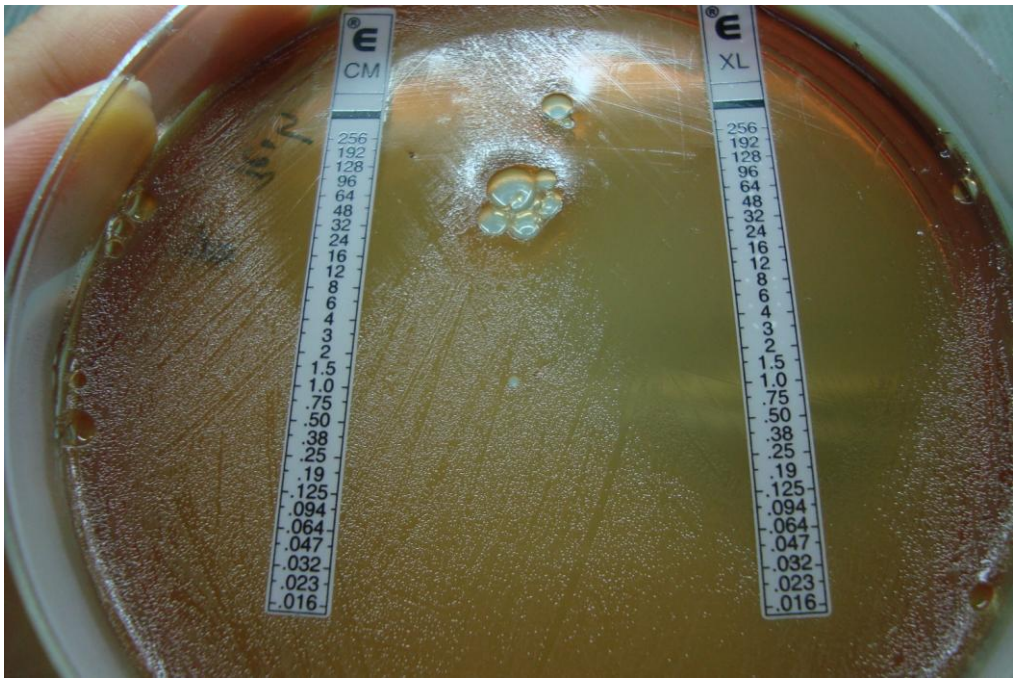
Neisseria sp. MTE16 resistant to CM; XL MIC = 0.5 µg/ml



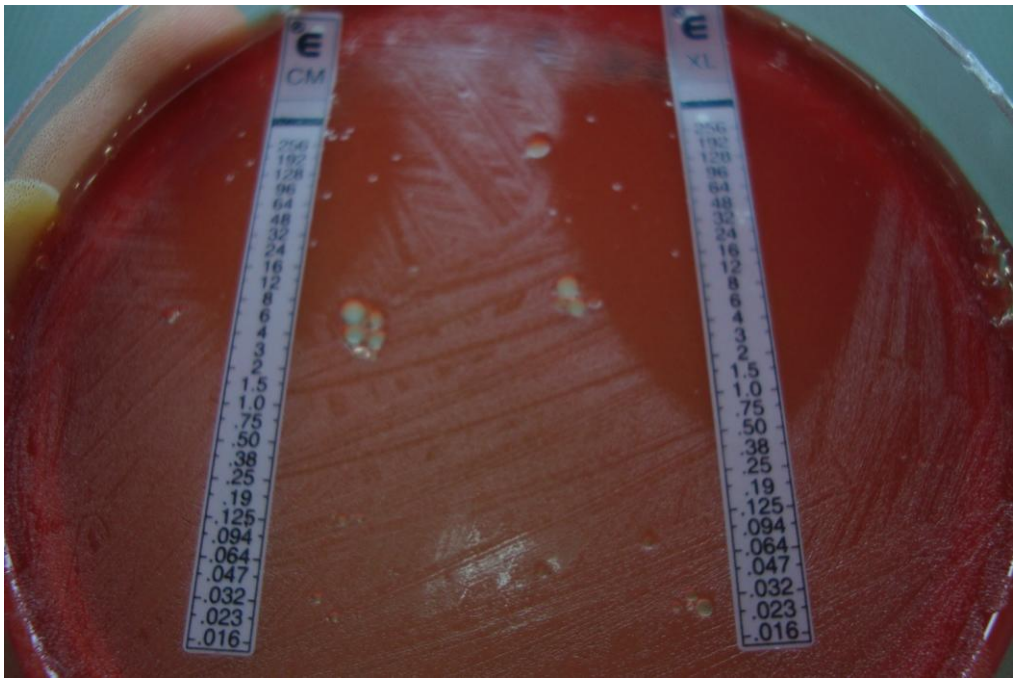
Neisseria sp. TTE4 CM MIC = 8 µg/ml; XL MIC = 0.25 µg/ml



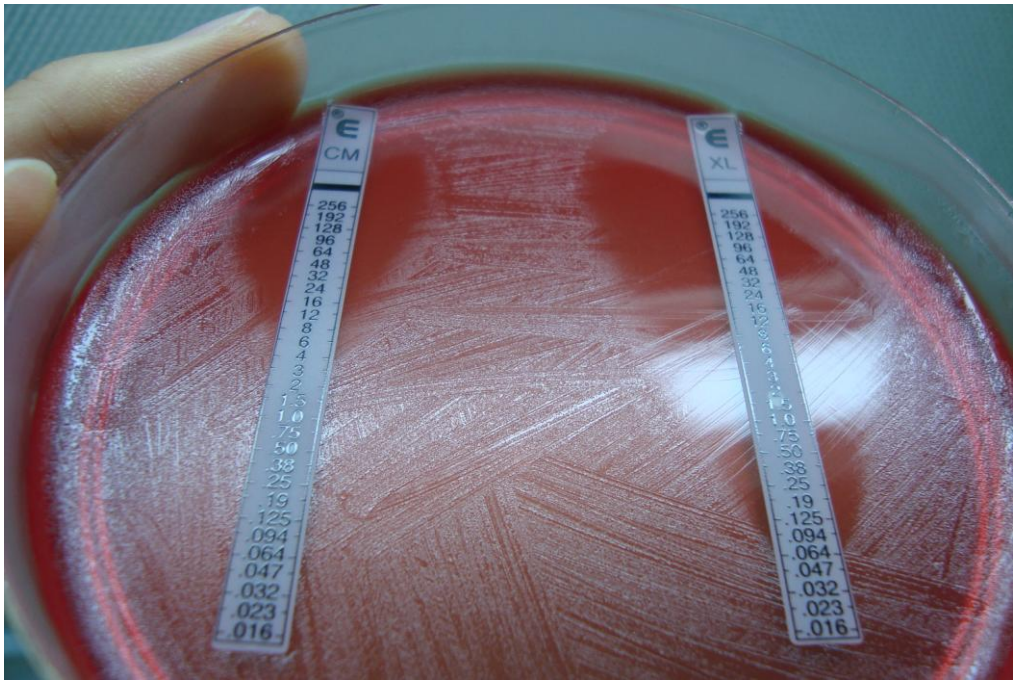
Neisseria sp. GHG17 resistant to CM; XL MIC = 0.094 µg/ml



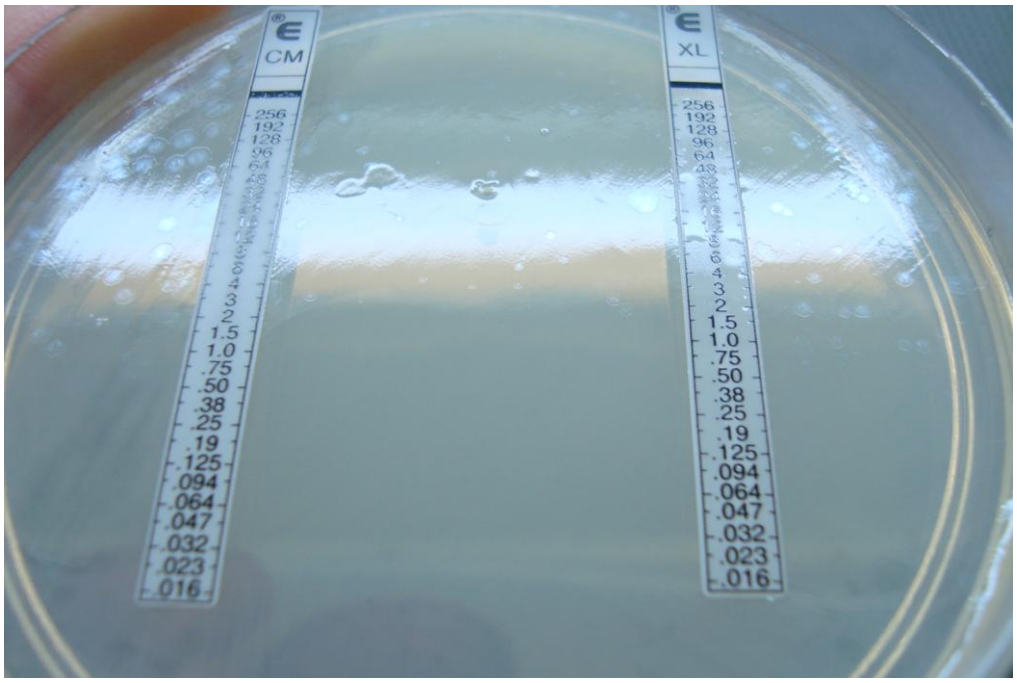
Neisseria subflava RTeoh CM MIC = 8 µg/ml; XL MIC = 0.19 µg/ml



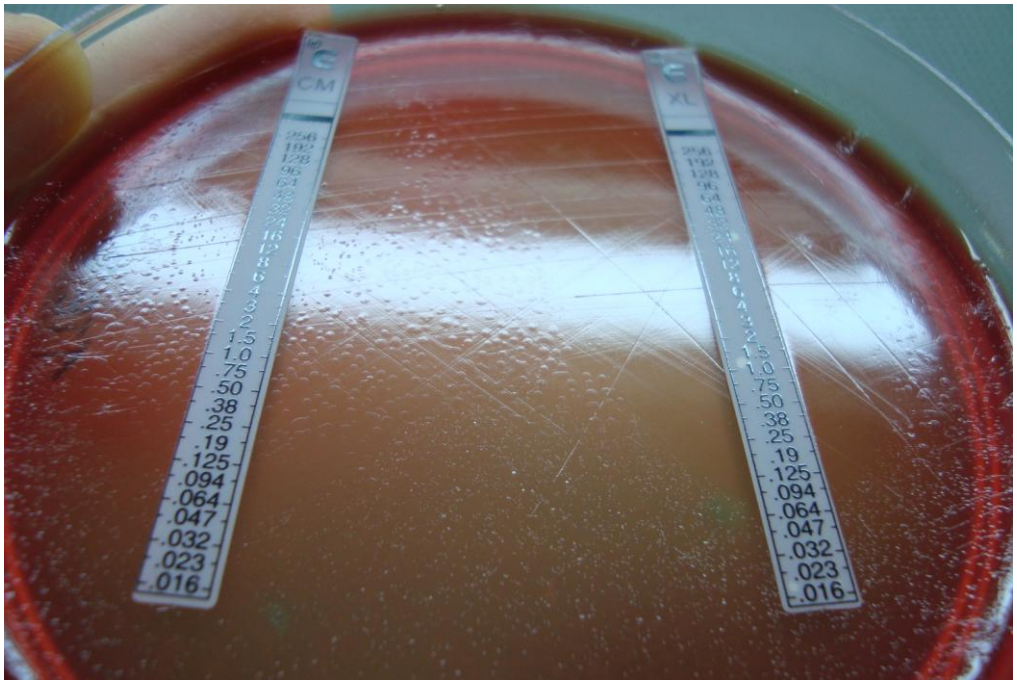
Neisseria flavescens SJeff CM MIC =8 µg/ml; XL MIC = 1 µg/ml



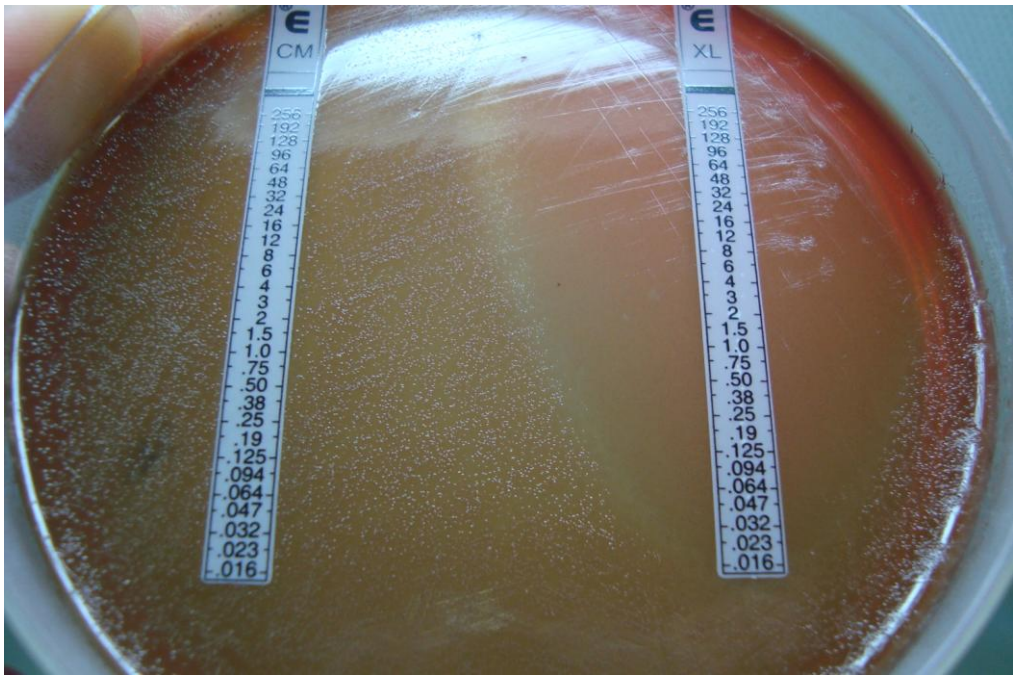
Neisseria meningitidis TTE14 CM MIC =8 µg/ml; XL MIC = 0.064 µg/ml



Pseudomonas aeruginosa PA resistant to CM and XL

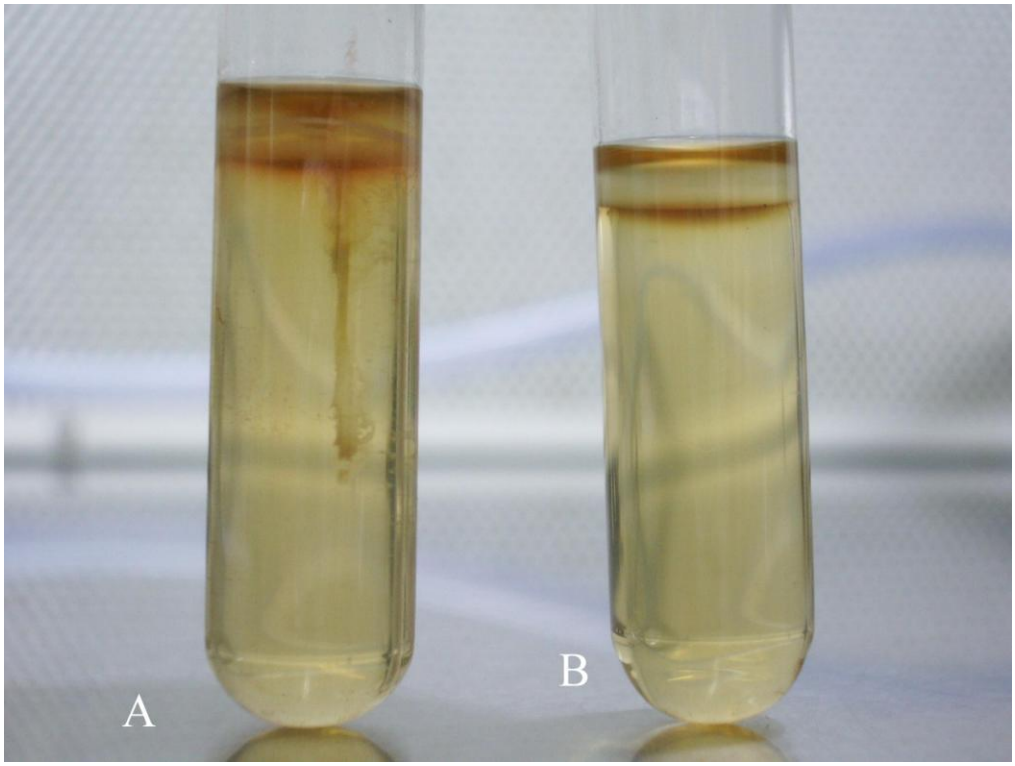


Streptococcus sanguinis TTE2 CM MIC = 16 μg/ml; XL MIC = 0.094 μg/ml

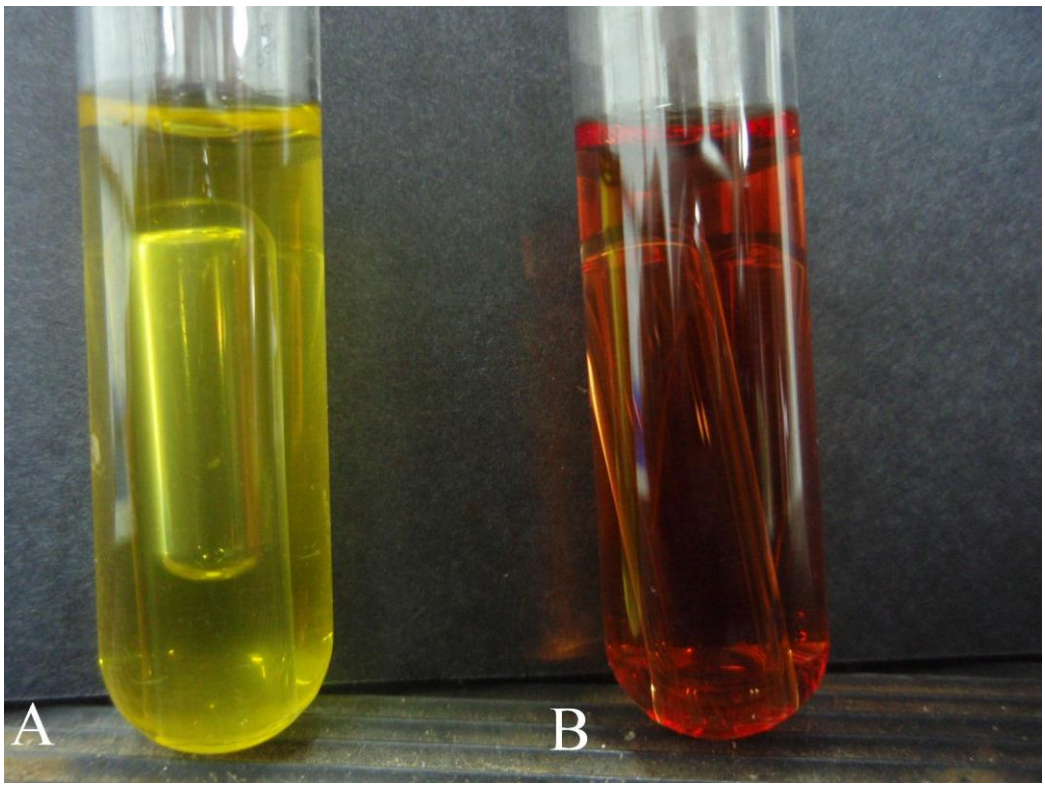


Streptococcus mitis F resistant to CM; XL MIC = 0.016 µg/ml

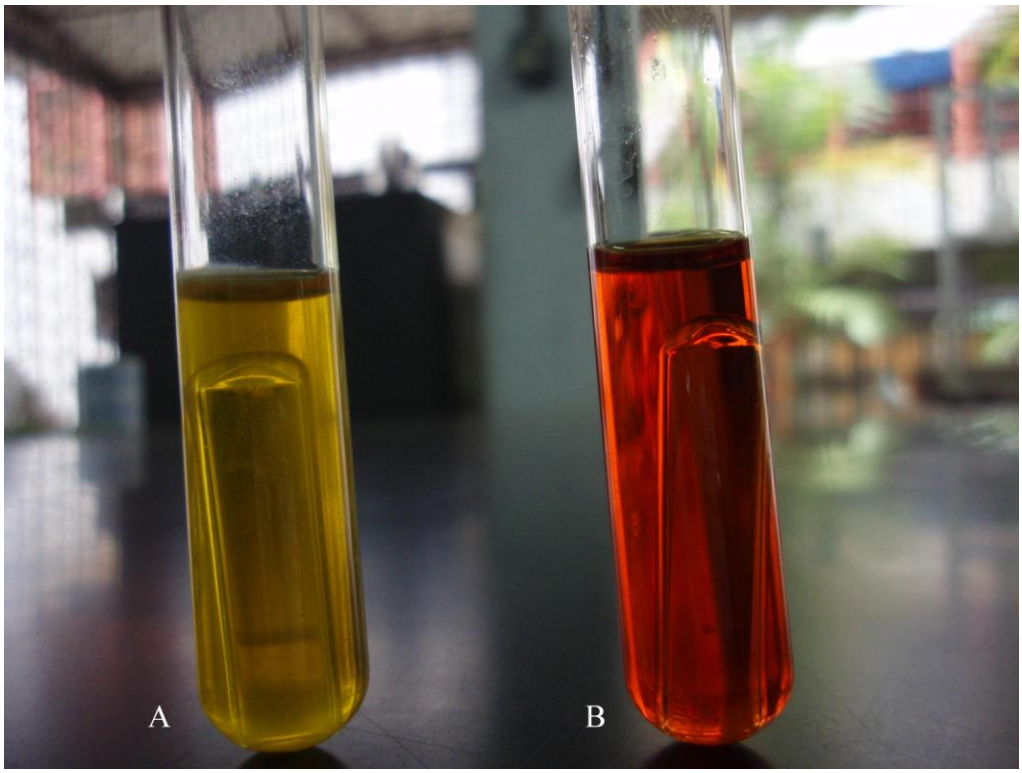
Appendix 10
Biochemical tests



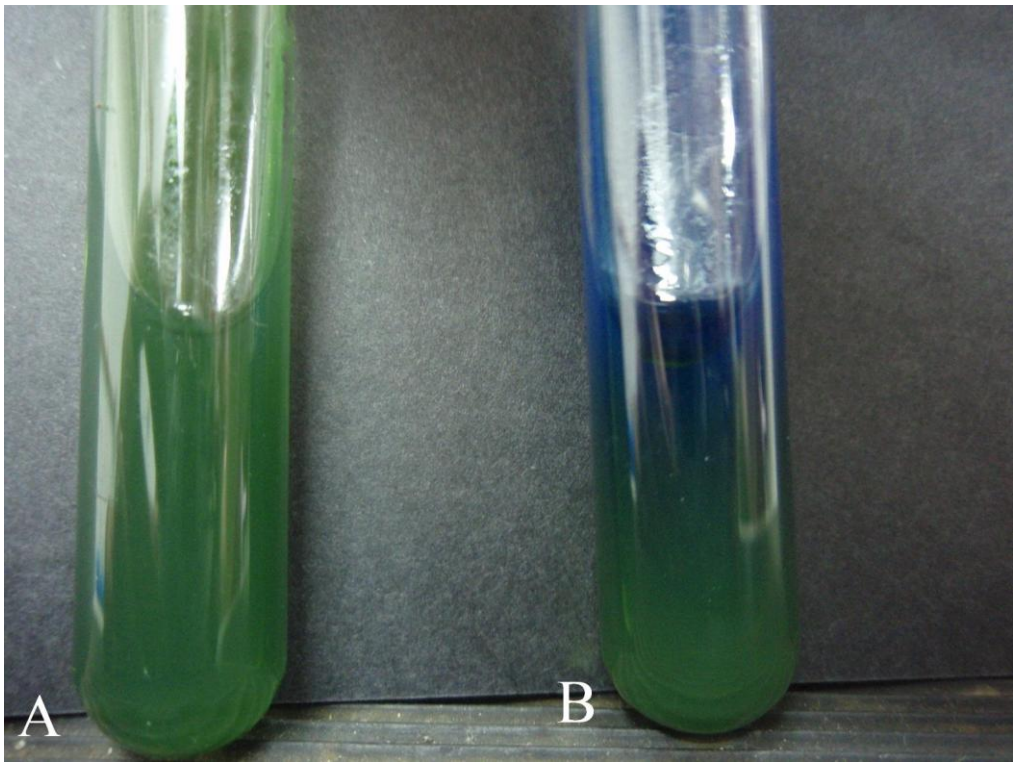
SIM test
A – inoculated (H_2S and indole test were negative) B – control



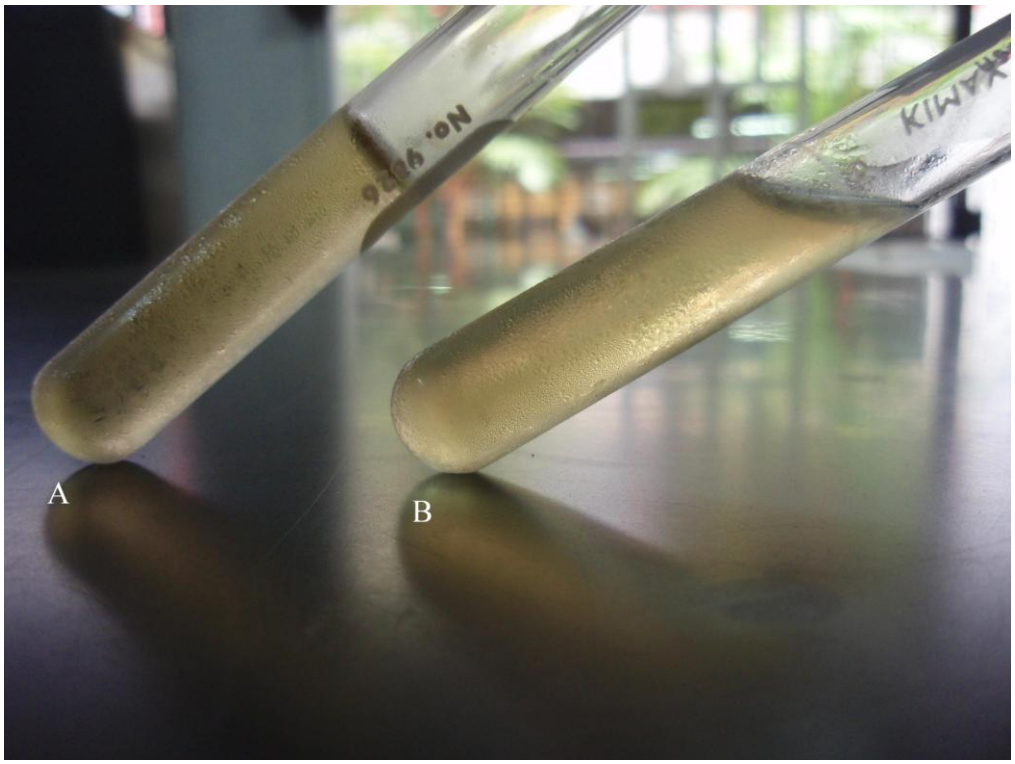
Phenol red fermentation test
A – positive with gas formation B – negative



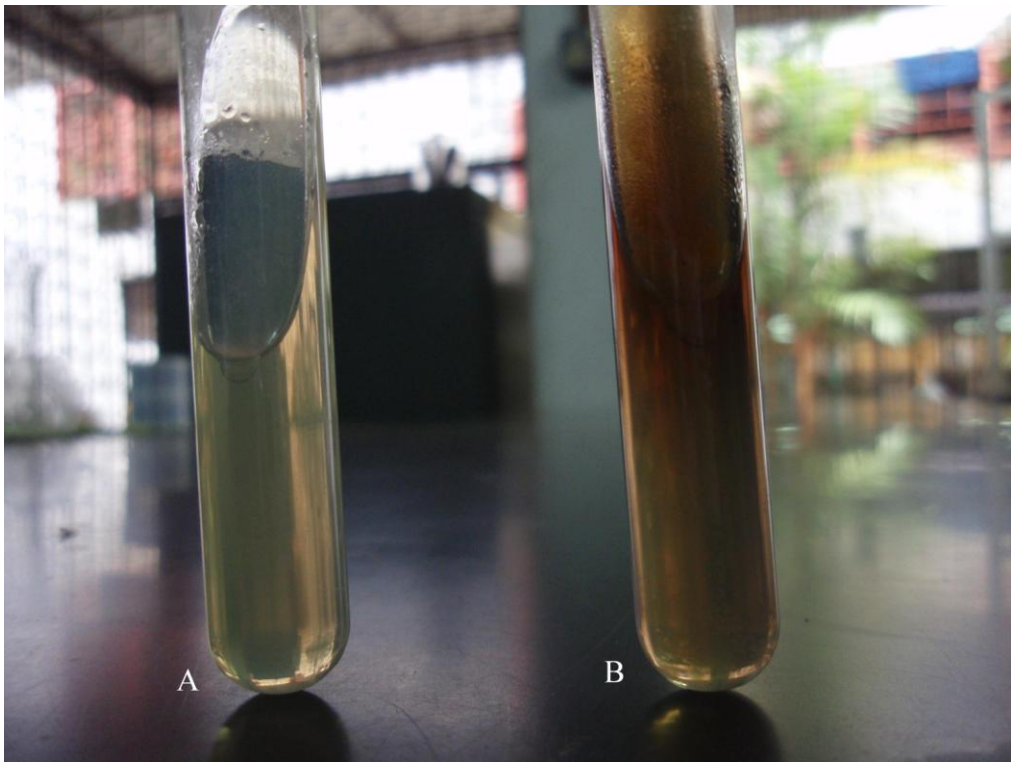
Phenol red fermentation test
A – positive without gas formation B – negative



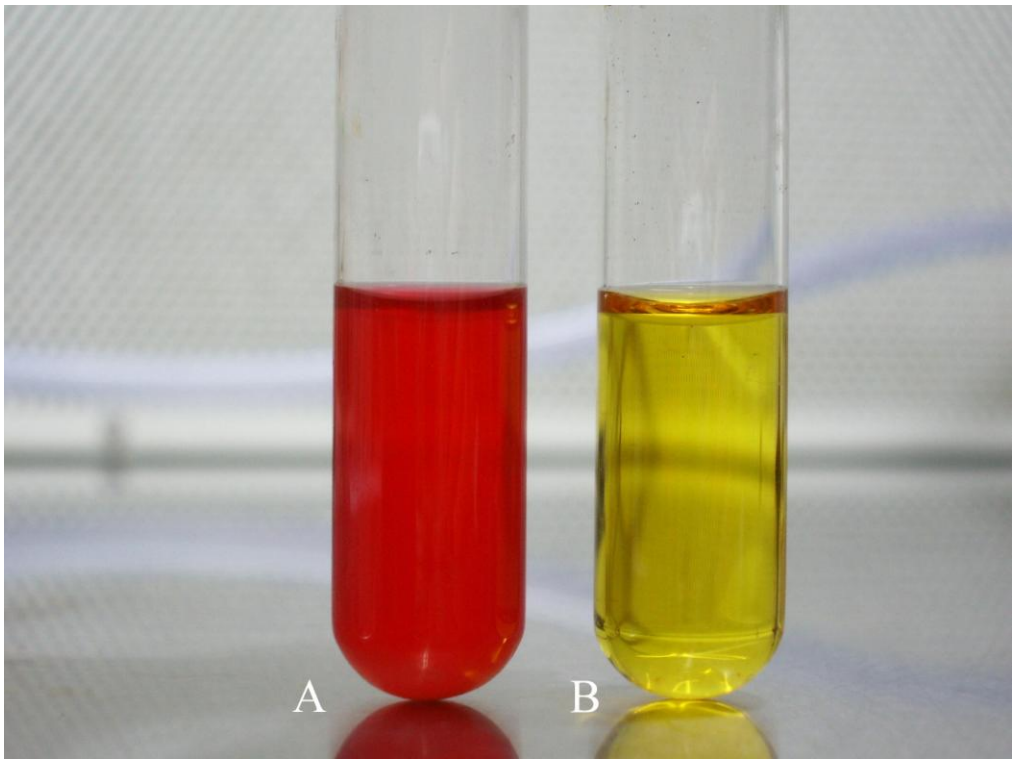
Simmon citrate test
A – negative B – positive



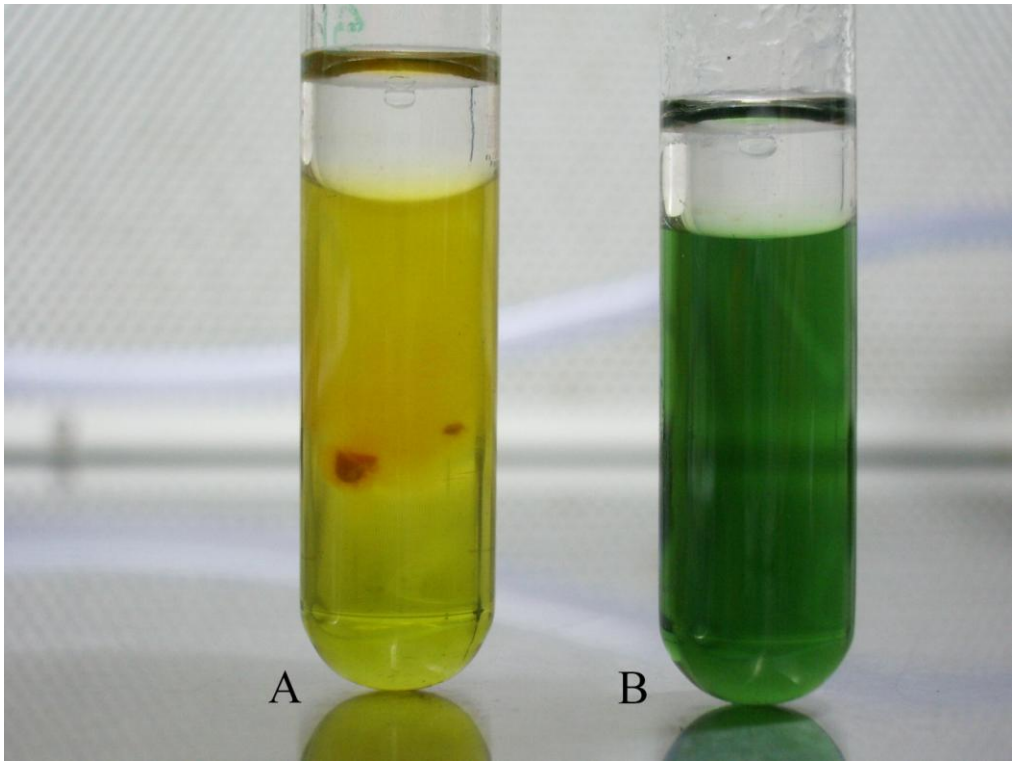
Gelatin hydrolysis
A – control B – positive



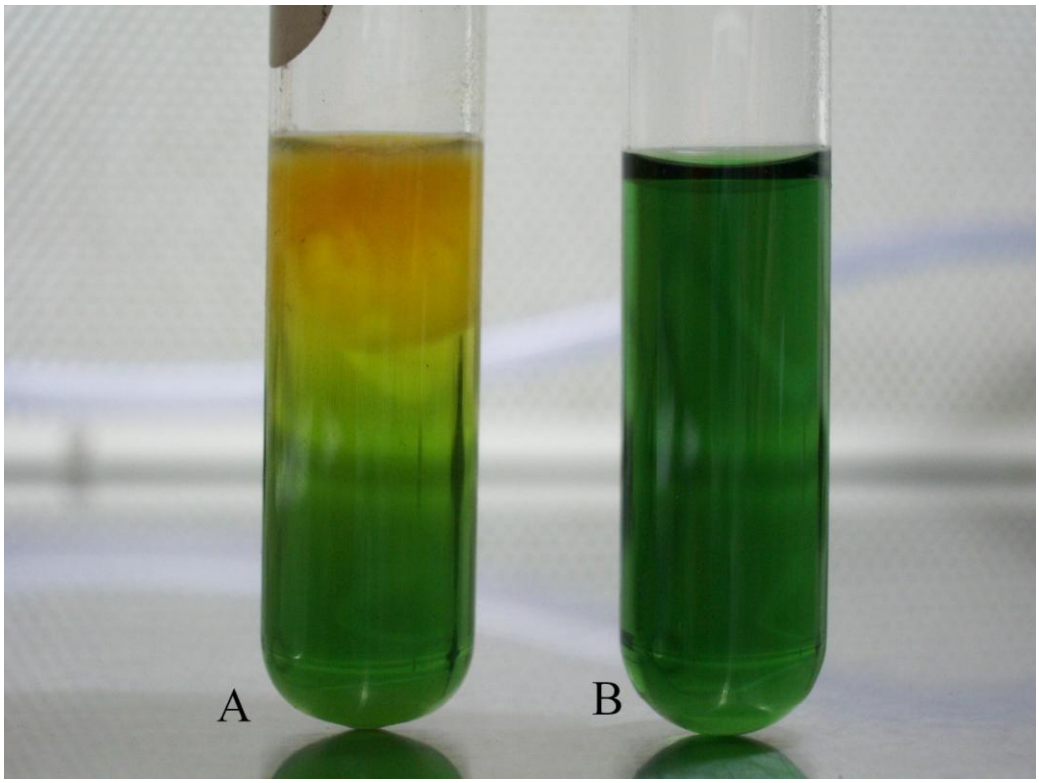
Esculin hydrolysis
A – control B – positive



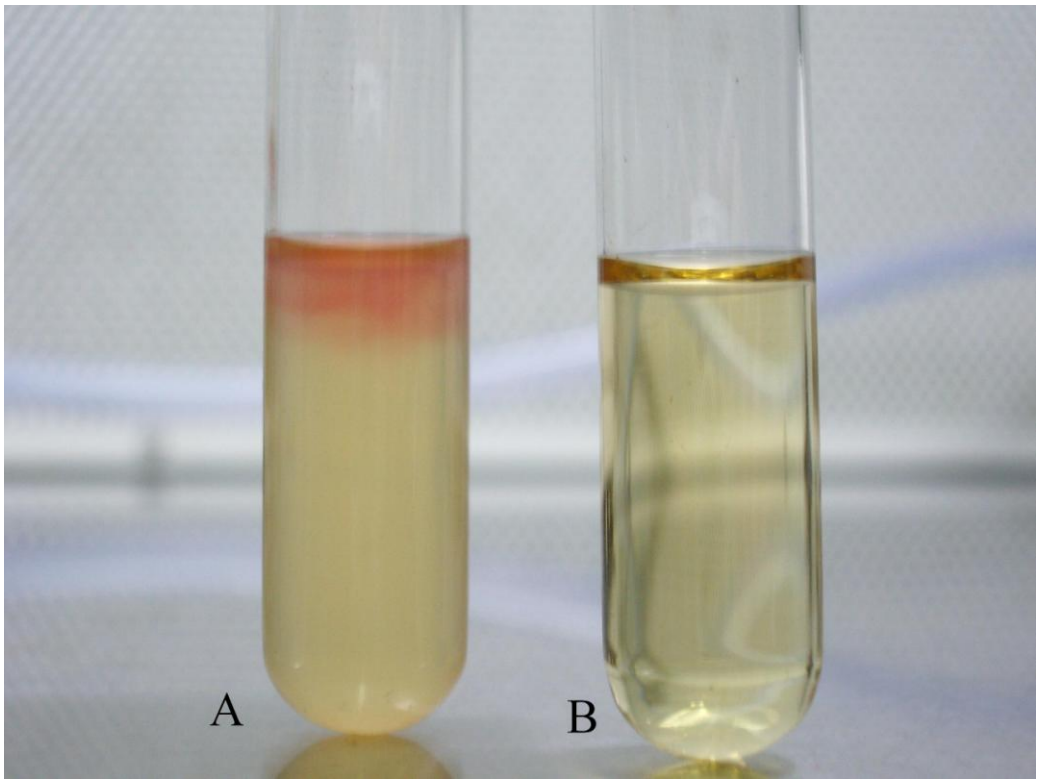
MR test
A – positive B - control



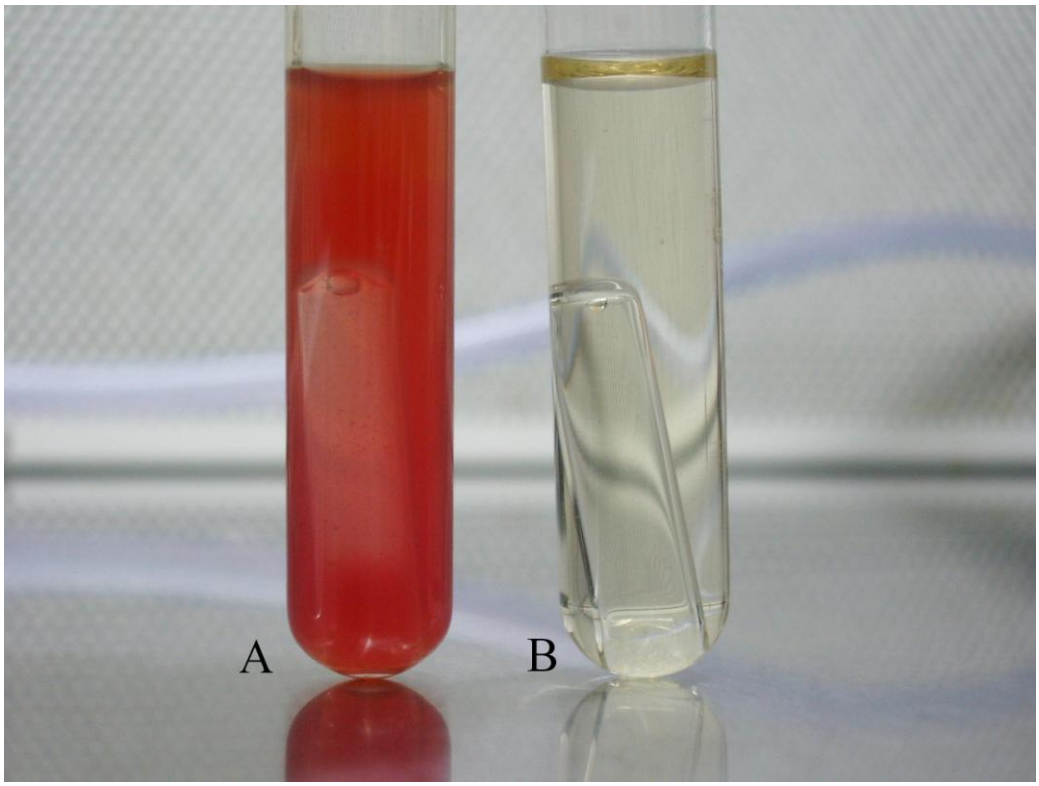
OF test
A – fermentation B - control



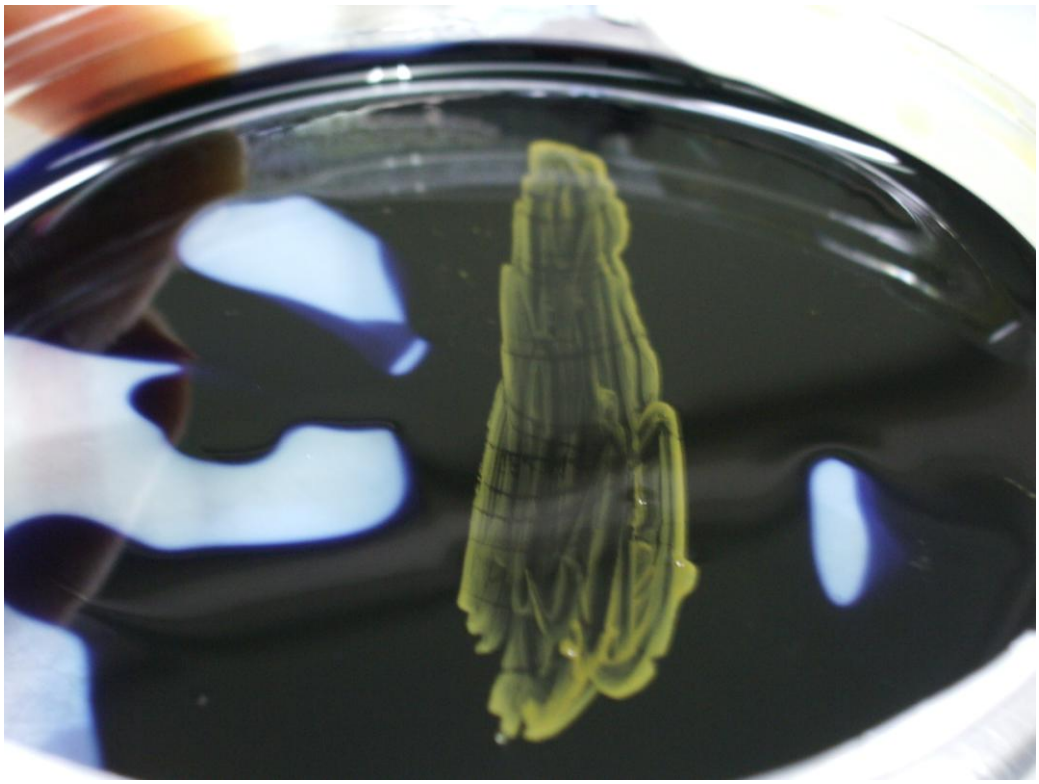
OF test
A – oxidative B - control

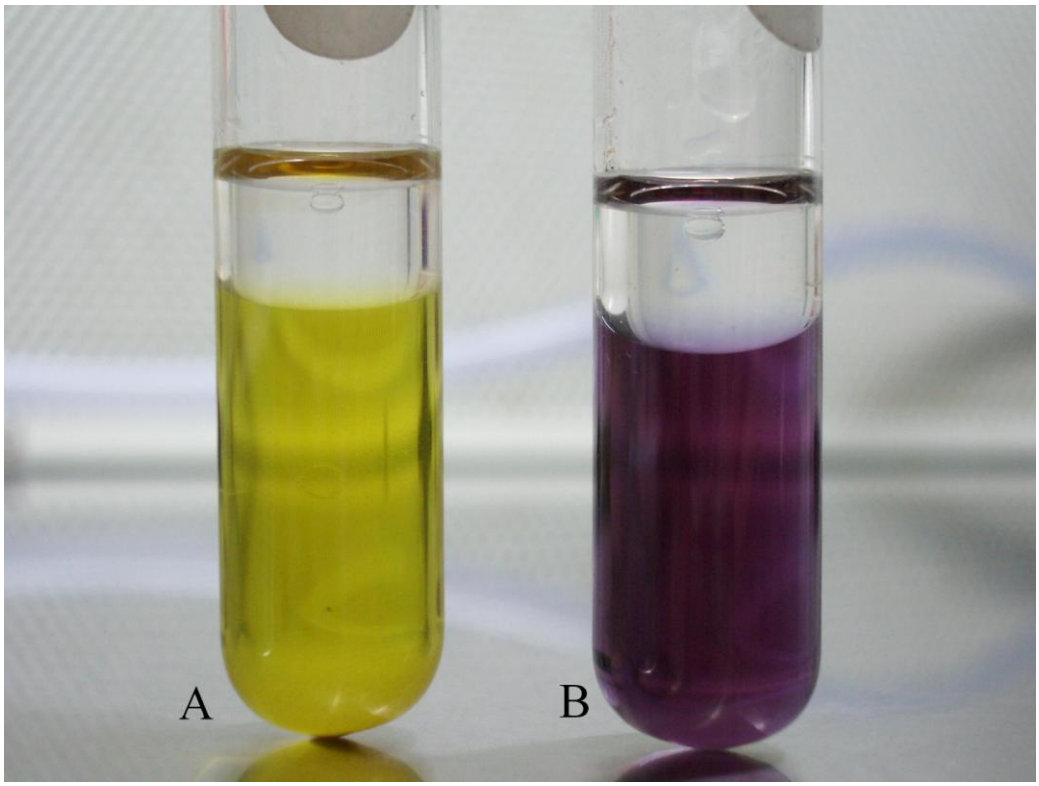


VP test
A – positive B – control

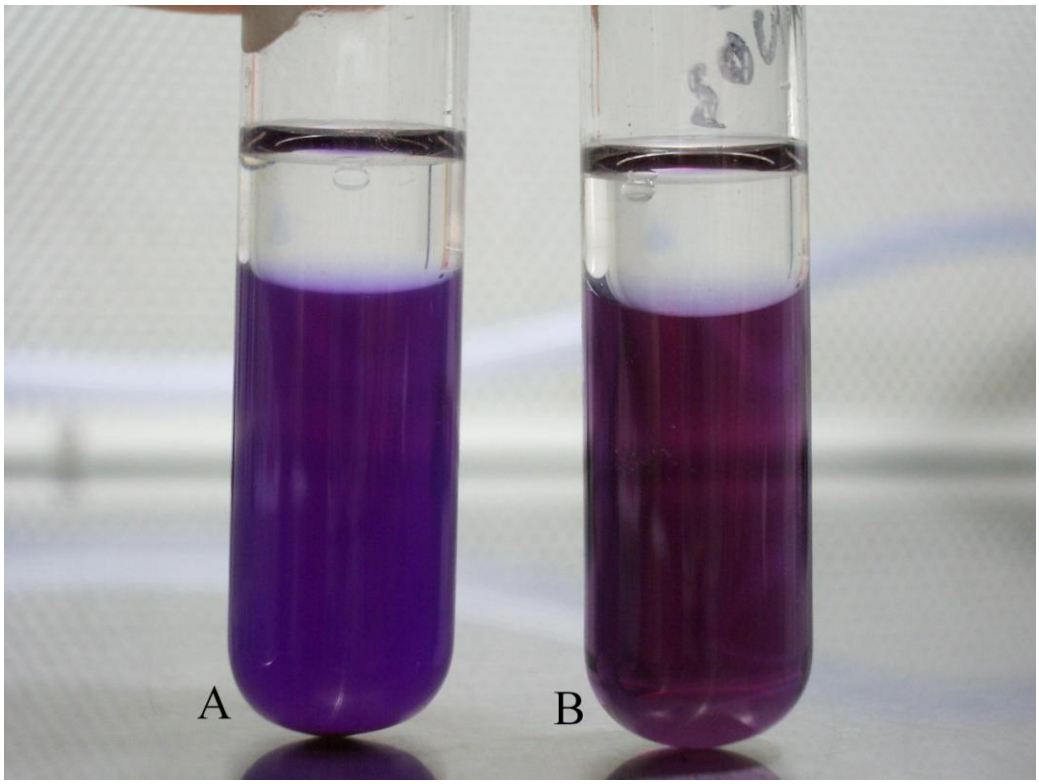


Nitrate reduction
A – positive B - control


Starch hydrolysis
Negative result

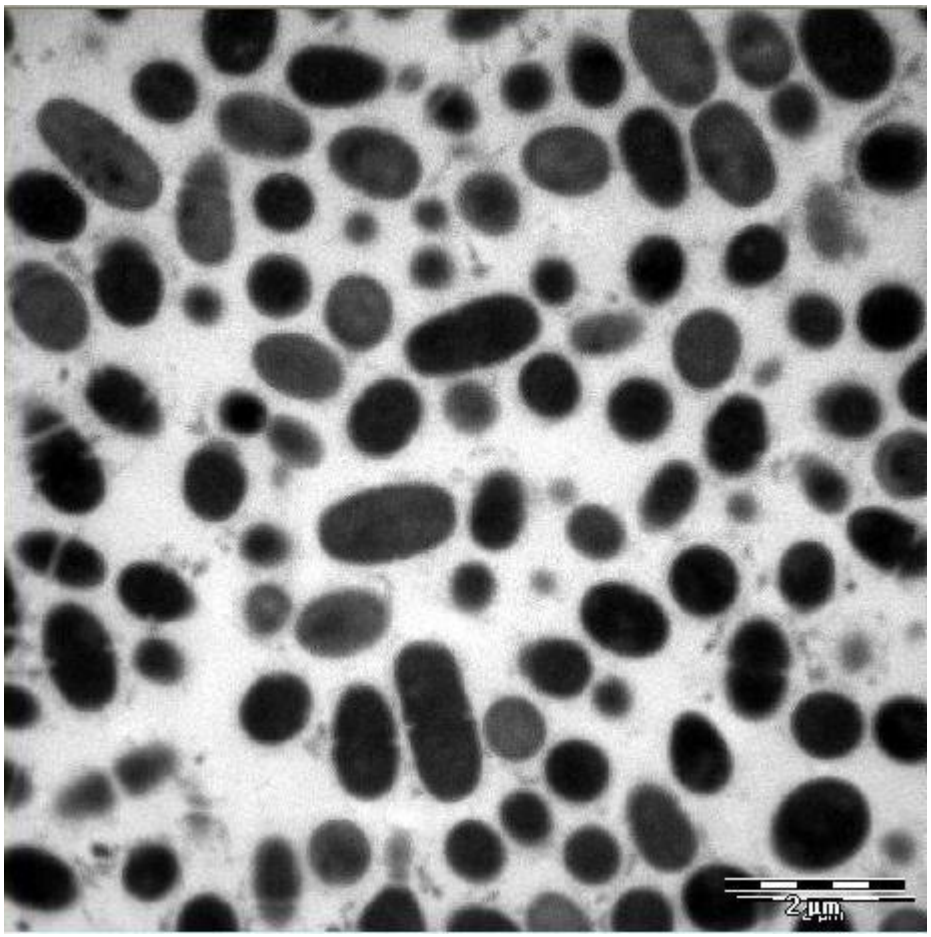


Amino acid decarboxylase test
A – negative B – control

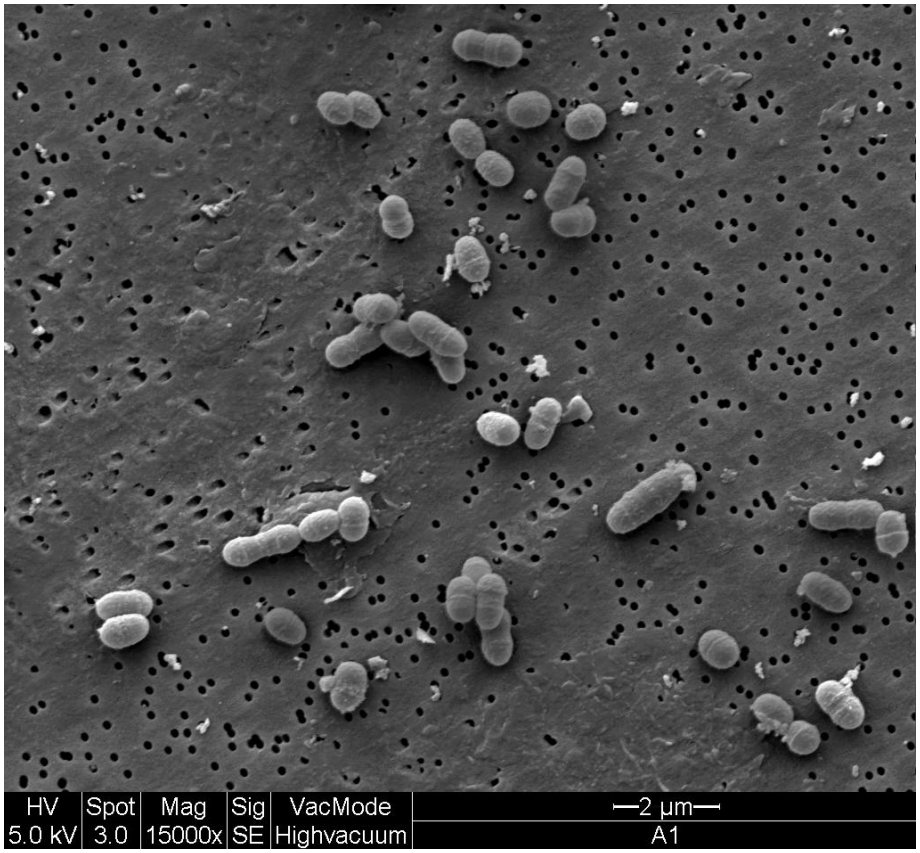


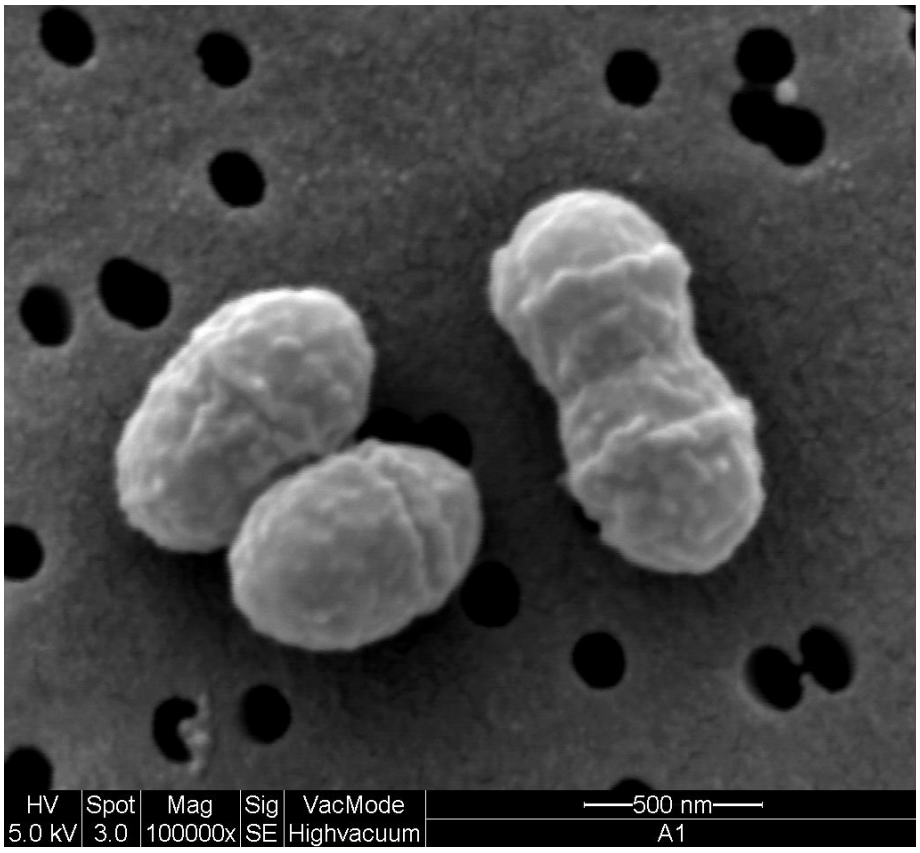
Amino acid decarboxylase test
A – light purple (positive) B - control

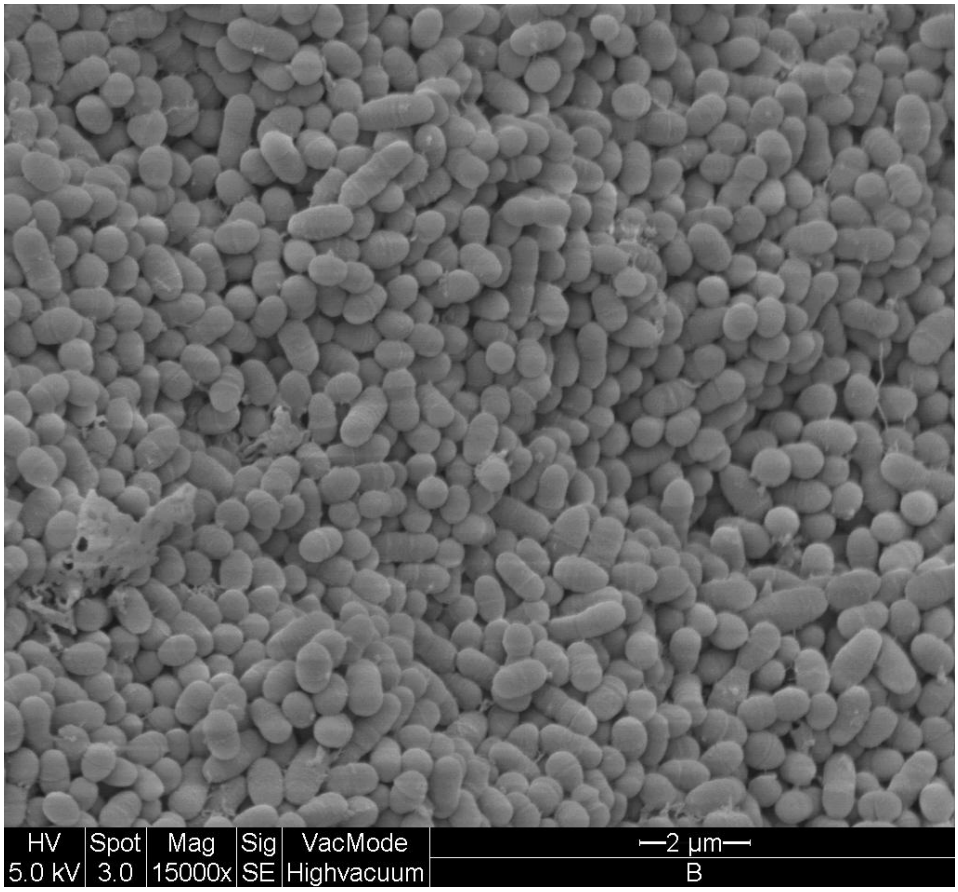
Appendix 11
TEM of *Streptococcus salivarius*



Appendix 12
SEM of *Streptococcus salivarius*

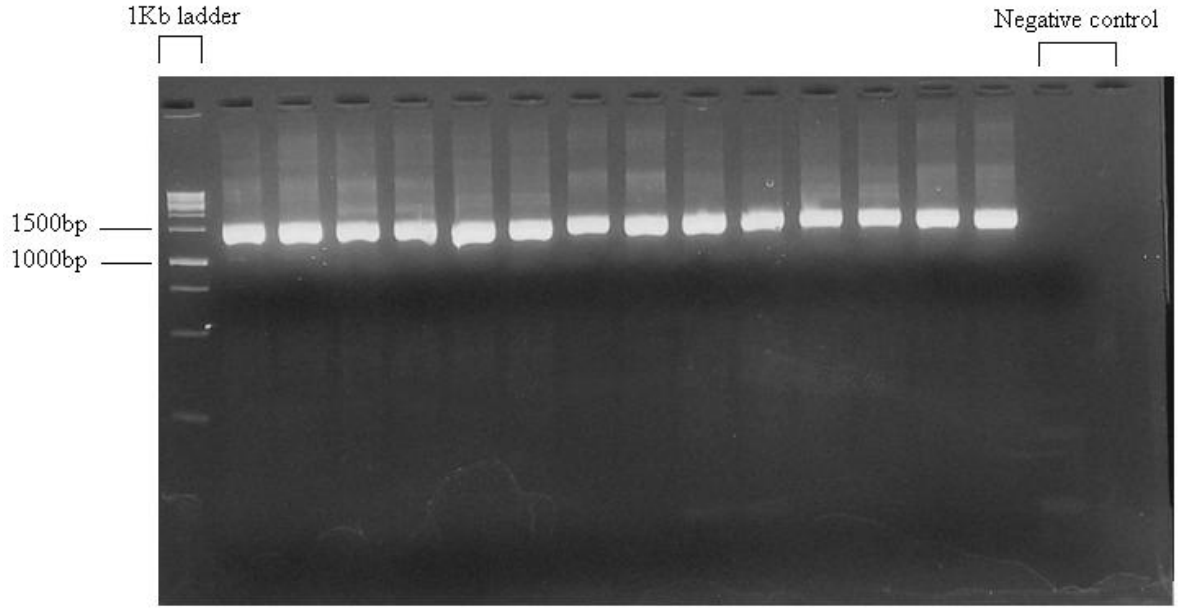




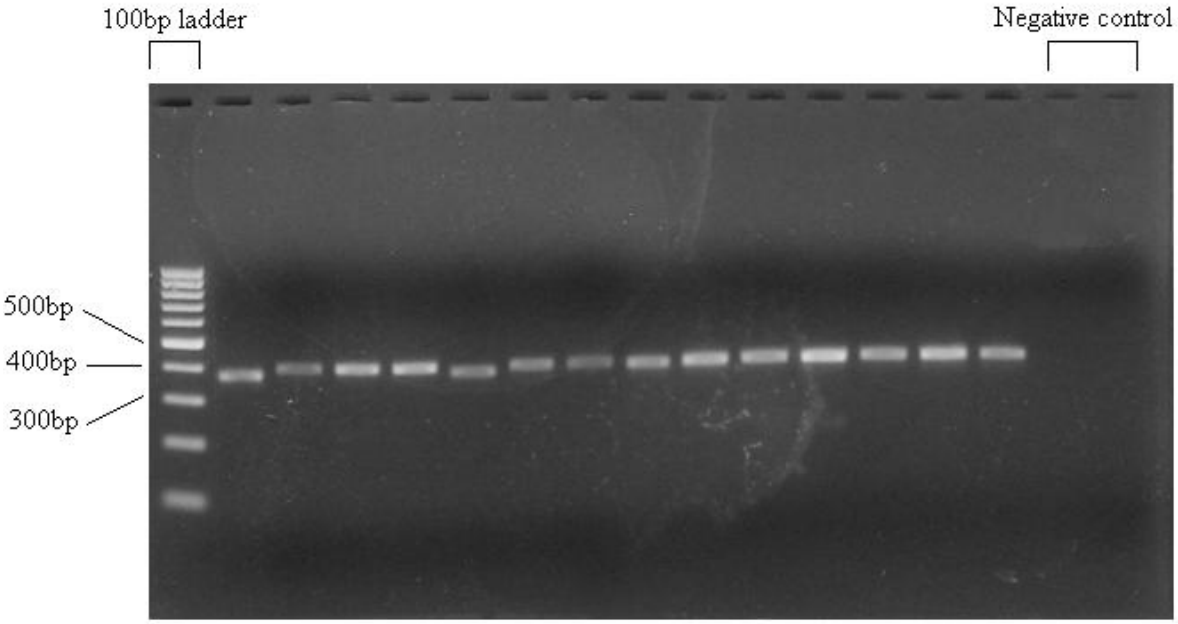

HV
5.0 kV
Spot
3.0
Mag
15000x
Sig
SE
VacMode
Highvacuum
2 µm
B

Appendix 13
Gel pictures

16S rDNA PCR products



***rnpB* gene PCR products**



Appendix 14

Publications and poster presentations