GENOTYPIC IDENTIFICATION OF ORAL BACTERIA USING 16S rRNA GENE IN CHILDREN WITH AND WITHOUT EARLY CHILDHOOD CARIES IN KELANTAN

by

SALAHDDIN ABDULQADER OTHMAN

AL-HUDHAIRY

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IDENTIFIKASI BAKTERIA ORAL SECARA GENOTIP MENGGUNAKAN GEN 16S rRNA DI KALANGAN AWAL KANAK-KANAK YANG MEMPUNYAI KARIES DAN TIADA KARIES DI KELANTAN

ABSTRAK

Mikroorganisma oral adalah salah satu faktor penyebab utama dalam kejadian karies gigi diperingkat awal kanak-kanak (ECC), tetapi mikroorganisma ini masih belum dikenal pasti sepenuhnya. Kajian ini bertujuan untuk mengenal pasti genotip bakteria oral dalam 12 kanak-kanak bebas karies (CF) dan 36 kanak-kanak yang mempunyai ECC, berumur 71 bulan dan ke bawah dengan menggunakan urutan gen ribosom 16S RNA. Dalam kanak-kanak ECC, plak dan dentin sampel telah diambil dari permukaan enamel yang utuh, bahagian luar permukaan lesi berkaviti dan dari lesi dentin. Bagi kanak-kanak CF, sampel plak dikumpulkan dari permukaan gigi sihat pada garis dasar dan selepas satu tahun susulan. DNA genomik telah diekstrak daripada kesemua sampel, tertakluk kepada 16S rRNA PCR amplifikasi dan produk akhir telah diklon ke pCR®2.1-TOPO® Vektor. Lima klon positif yang dipilih secara rawak dikumpul dari setiap permukaan telah dihantar untuk penjujukan. Pengenalpastian klon bakteria telah dilakukan dengan menggunakan carian BLAST terhadap pangkalan data GeneBank. Sebanyak 660 klon telah dikumpulkan daripada kanak-kanak yang mendaftar. Dari kumpulan ECC, 540 klon telah didapati dari tiga permukaan yang berlainan manakala, dalam kumpulan CF, 120 klon dikumpulkan dari permukaan gigi sihat pada garis dasar dan selepas satu tahun. Beberapa perbandingan telah dilakukan di antara bakteria oral yang telah dikenal pasti menggunakan ujian Pearson Chi-square atau ujian Fisher exact bagi pembolehubah kategori yang berbeza, manakala ujian McNemar digunakan untuk membuat

perbandingan di antara kategori individu. Sebanyak 39 genus bakteria oral telah dikenal pasti dari kumpulan ECC. Di peringkat genus, *Streptococcus* sp. adalah bakteria yang paling ketara di kalangan kumpulan ECC. *Fusobacterium* adalah lebih tinggi di permukaan enamel utuh manakala *Lactobacillus* adalah lebih tinggi di dalam dentin ($p < 0.05$). Di peringkat spesies, *Fusobacterium nucleatum subsp. polymorphum* dikesan di permukaan utuh (33.3%) manakala *Streptococcus mutans* dikesan pada bahagian luar permukaan lesi berkaviti dan dari lesi dentin (33.3% dan 52.7% masing-masing). Sebanyak 18 genus bakteria oral telah dikenal pasti dari kumpulan CF pada garis dasar dan selepas 1 tahun susulan, tetapi tidak terdapat perbezaan yang signifikan di antara kumpulan. Di peringkat spesies, *Fusobacterium nucleatum subsp. polymorphum* didapati paling tinggi dalam kumpulan CF. Selepas susulan, *Corynebacterium matruchotii* adalah paling tinggi di kalangan mereka yang kekal bebas karies, manakala *Porphyromonas catoniae* adalah paling tinggi di kalangan mereka yang mendapat karies. Kesimpulannya, *Streptococcus* sp. amat berkaitan dengan perkembangan karies pada kanak-kanak ECC terutama untuk *Streptococcus mutans*. *Lactobacillus* sp. adalah terhad pada lesi karies gigi yang dalam. *Fusobacterium* sp., *Leptotrichia* sp., dan *Corynebacterium* sp. mungkin mempunyai peranan dalam mengekalkan persekitaran yang sihat.

GENOTYPIC IDENTIFICATION OF ORAL BACTERIA USING 16S rRNA GENE IN CHILDREN WITH AND WITHOUT EARLY CHILDHOOD CARIES IN KELANTAN

ABSTRACT

Oral microorganisms are considered as one of the primary aetiological factors in Early Childhood Caries (ECC) but they have not been completely identified. The present study aimed to identify oral bacterial genotypes in 12 caries-free children (CF) and 36 children with ECC aged 71 month and below by using 16S ribosomal RNA gene sequence. In ECC children, plaque and dentine samples were collected from intact enamel surfaces, over cavitated lesion and from dentine lesion surface. For CF children, plaque samples were collected from sound tooth surfaces at baseline and after one year follow up. The genomic DNA was extracted from all samples, subjected to 16S rRNA PCR amplification and the end products were cloned into pCR®2.1-TOPO® Vector. Five randomly selected positive clones collected from each surface were sent for sequencing. Identification of the bacterial clones was performed using BLAST search against GeneBank database. A total of 660 clones were collected from enrolled children. From ECC group, 540 clones were obtained from three different surfaces while, in CF group, 120 clones were collected from sound tooth surfaces at baseline and after one year. Several comparisons were performed between those identified oral bacteria using Pearson Chi-square test or Fisher's exact test between different categorical variables, while McNemar test was used to compare among the individual categories. A total of 39 oral bacterial genera

were identified from the ECC group. At genus level, *Streptococcus* sp. was the most predominant bacteria among ECC group. *Fusobacterium* sp. is significantly higher in the intact enamel while *Lactobacillus* sp. is significantly higher in the dentine surface ($p < 0.05$). At species level, *Fusobacterium nucleatum subsp. polymorphum* was detected in the intact surface (33.3%) while *Streptococcus mutans* was detected over the carious lesions and dentine (33.3% and 52.7% respectively). A total 18 oral bacteria genera were identified from CF group at baseline and after 1 year follow up, but there were no significant differences between groups. At species level, *Fusobacterium nucleatum subsp. polymorphum* is found highest in the CF group. After follow up, *Corynebacterium matruchotii* is highest in those who remained caries free, while *Porphyromonas catoniae* is highest in those who developed caries. In conclusion, *Streptococcus* sp. is strongly associated with caries progression in children with ECC especially for *Streptococcus mutans*. *Lactobacillus* sp. is restricted to deep carious lesions. *Fusobacterium* sp., *Leptotrichia* sp., *and Corynebacterium* sp. may play a role in sustaining the healthy environment.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Dental caries is a significant public health problem even in the 21st century (Ramos-Gomez *et al.*, 2002). Early childhood caries (ECC) affects the pre-school children, can give rise to considerable amount of pain and suffering for the child and parent (Low *et al.*, 1999). In Malaysia, caries in 5-year old children has been found to be 76.2% (Malaysian Oral Health Division, 2005).

Since long time, the main implicated bacteria as a cause for dental caries has been identified as *Streptococcus mutans* (Berkowitz, 2003). Beighton (2005) in his review of microorganisms involved in high risk caries patients suggested that the essential role of mutans streptococci (MS) in the caries process is not proven, and is debatable (Beighton, 2005). He stated that other acidogenic and aciduric bacteria present in the mouth, like *Actinomyces* sp. and *Bifidobacterium* sp. can contribute to the caries progression. The main reason for the lack of such data so far, is because such taxonomy has not been cultured. Rapid PCR methods based on species-specific gene sequences can replace the use of selective media to identify MS and have the advantage of being more sensitive (Russell, 2008). Newer molecular techniques like PCR could considerably help in identification of newer bacteria associated with dental caries.

Becker *et al*. (2002) found ten novel phylotypes using 16S ribosomal DNA in a molecular analysis of all bacterial species associated with childhood caries. In addition, 23 previously known bacterial species were identified using reverse capture

checkerboard assay. Newer bacteria, like *Actinomyces gerenceriae* and other *Actinomyces* species were considered to play an important role in caries initiation (Becker *et al.*, 2002). A molecular analysis of microflora associated with carious lesions identified 31 novel, presumed uncultured taxa previously undescribed (Munson *et al.*, 2004). They also used PCR method and amplified the 16S rRNA genes to identify the oral bacteria.

Recently, Li *et al.* (2007) conducted a genetic profiling of the oral microbiota associated with severe early childhood caries obtained from pooled plaque samples from the bucco-gingival surfaces and the accessible proximal surfaces of the molars and canines (Li *et al.*, 2007). They found that the microbial diversity and complexity of the microbial biota in dental plaque are significantly less in severe ECC children when compared to caries-free children. Aas *et al.* (2005) studied the bacteria of dental caries in primary and permanent teeth in children and young adults and found specific bacterial species associated with health, caries initiation and caries production, with a subject to subject variation (Aas *et al.*, 2005). Hence, molecular methods based on PCR amplification of total DNA from plaque samples followed by sequencing of rRNA have led to an expanded list of caries-associated species (Russell, 2008). The findings support the ecological plaque hypothesis which suggests that caries is a result of a shift of the balance of the resident microflora driven by changes in local environmental conditions.

The key elements of the ecological plaque hypothesis is that the selection of "pathogenic" bacteria is directly coupled to the changes in the environment and that caries need not have a specific etiology, but any species with relevant traits can contribute to the disease process. Technical advances in continuous bacterial culture

and the biofilm concept have been crucial to the development of what has come to be known as the “ecological plaque hypothesis” in which repeated cycles of stress in the form of lowered pH due to consumption of fermentable carbohydrates lead to enrichment of acidogenic and aciduric species in plaque (Russell, 2009).

The clinical significance of such data gained could provide a better understanding of the dynamics of oral microflora that result in health or disease. Once more of the disease-associated microbes are identified, there are opportunities to develop diagnostic systems for early detection of changes in the oral ecology, detection of biomarkers for disease, and strategies for managing the disease process (Donly, 2009).

With this background, the main objective of this study is to identify the specific microflora from plaque and dentin, associated with and without early childhood caries in Malaysian children, using molecular methods.

1.2 Statement of problem

In Kelantan, the caries prevalence among preschool children is the highest in the Malaysian states (Malaysian Oral Health Division, 2007). The main causative factor in dental caries formation is the presence of cariogenic bacteria in the oral cavity. Microorganisms that have been implicated with the caries process can be identified by either conventional culture methods or molecular methods. Cultural methods are cumbersome and tedious. They also do not allow many bacteria to be identified. For example, different bacteria require different culture media and different transport media. Hence, there is a need for the identification of various bacteria in order to provide a better understanding of the dynamics of the disease in this high risk population using the molecular techniques.

1.3 Justification of the study

Malaysia is a multiracial country with varied dietary habits. Water fluoridation, being the most cost-effective measure in caries prevention, is present in most of the states except Kelantan and Terengganu where water fluoridation is only recently partly fluoridated (Shaharuddin *et al.*, 2009). Although caries is a multifactorial infectious disease, the high prevalence of early childhood caries reported in Kelantan calls for more studies to be directed towards better understanding of biological aetiological factor in this population.

Hence, this study aimed to identify the various species of bacterial microflora associated with early childhood caries as well as in children without early childhood caries, i.e. children who are caries-free. In addition, bacterial identification will also be studied in caries-free children after a follow up period of 1 year.

It is hoped that this study will improve our knowledge about the bacterial species that are associated with early childhood caries in high risk children such as those living in Kelantan. Future research can be directed toward various methods of controlling these bacteria either by minimizing their effect or inhibiting their presence.

1.4 Objectives of study

1.4.1 General objective

To identify the oral bacteria in caries-free children (CF) and children with early childhood caries (ECC)

1.4.2 Specific objectives

1. To identify the oral bacteria types from plaque of children without caries and from plaque and dentine of children with ECC with genetic profiling using 16S ribosomal RNA gene sequence (**CF_0**, **A**, **B** & **C**).
2. To compare types of oral bacteria from plaque samples on the tooth surfaces in caries-free children and over sound tooth structure in children with ECC with genetic profiling using 16S ribosomal RNA gene sequence (**CF_0** vs **A**).
3. To compare the types of oral bacteria from plaque samples on sound tooth structure of children with ECC and plaque samples over carious cavities in children with ECC with genetic profiling using 16S ribosomal RNA gene sequence (**A** vs **B**).
4. To compare types of oral bacteria from plaque samples over carious cavities in ECC children and dentine samples in children with ECC with genetic profiling using 16S ribosomal RNA gene sequence (**B** vs **C**).
5. To identify types of oral bacteria in caries-free children after one year (**CF_1**).

6. To compare types of oral bacteria from plaque samples in carious-free children after one year. (**CF_0** vs **CF_1**)

Figure 1.1 shows the 3 different sites of collected plaque and dentine samples from children who are caries-free (CF) and those with early childhood caries (ECC) for bacterial identification and comparisons between sites as well as between groups. In children with ECC, samples were collected from dental plaque on the intact enamel surfaces (A), dental plaque over the cavity lesion (B) and from dentine (C). For CF children, samples collected from sound tooth structure at the first observation (CF_0) and at the second observation (CF_1).

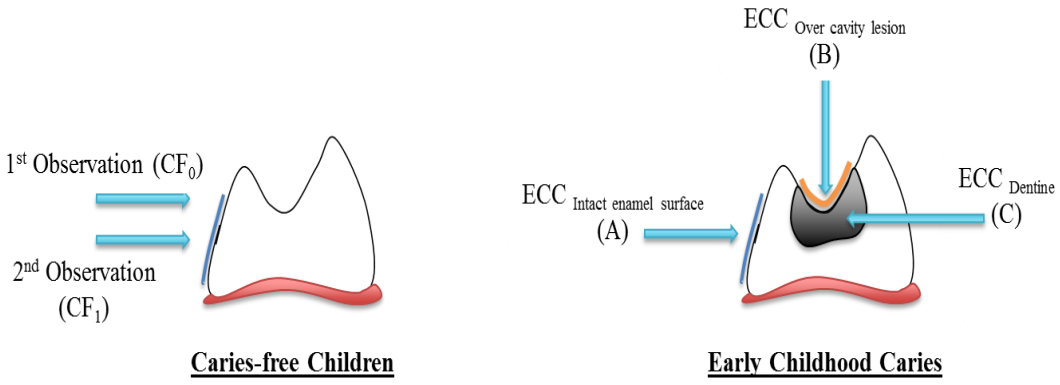

Figure 1.1 Sites of plaque and dentine collection

1.5 Hypothesis of study

1. The oral bacteria in children with and without ECC are different.
2. The bacteria in plaque over sound tooth surface and over carious lesion are different in children with ECC.
3. The bacteria associated with plaque and carious dentin is different in children with ECC.
4. The bacteria found in caries-free children will change after one year.

1.6 Conceptual model of study

As a type of dental caries, ECC is multifactorial disease that previously referred by Keyes and Jordan (1963) as an interaction between oral bacteria (dental plaque), carbohydrates (diet) and susceptible teeth (host) (van Houte, 1994).

This concept has been widely updated since that time due to continuous efforts in research on ECC. Figure 1.2 presents the conceptual framework of this study, which aimed to recognize the complex interaction between several predisposing factors to develop ECC at the end. These factors include biological (microbial, dietary and host) factors, the socio-demographic factors and behavioral factors.

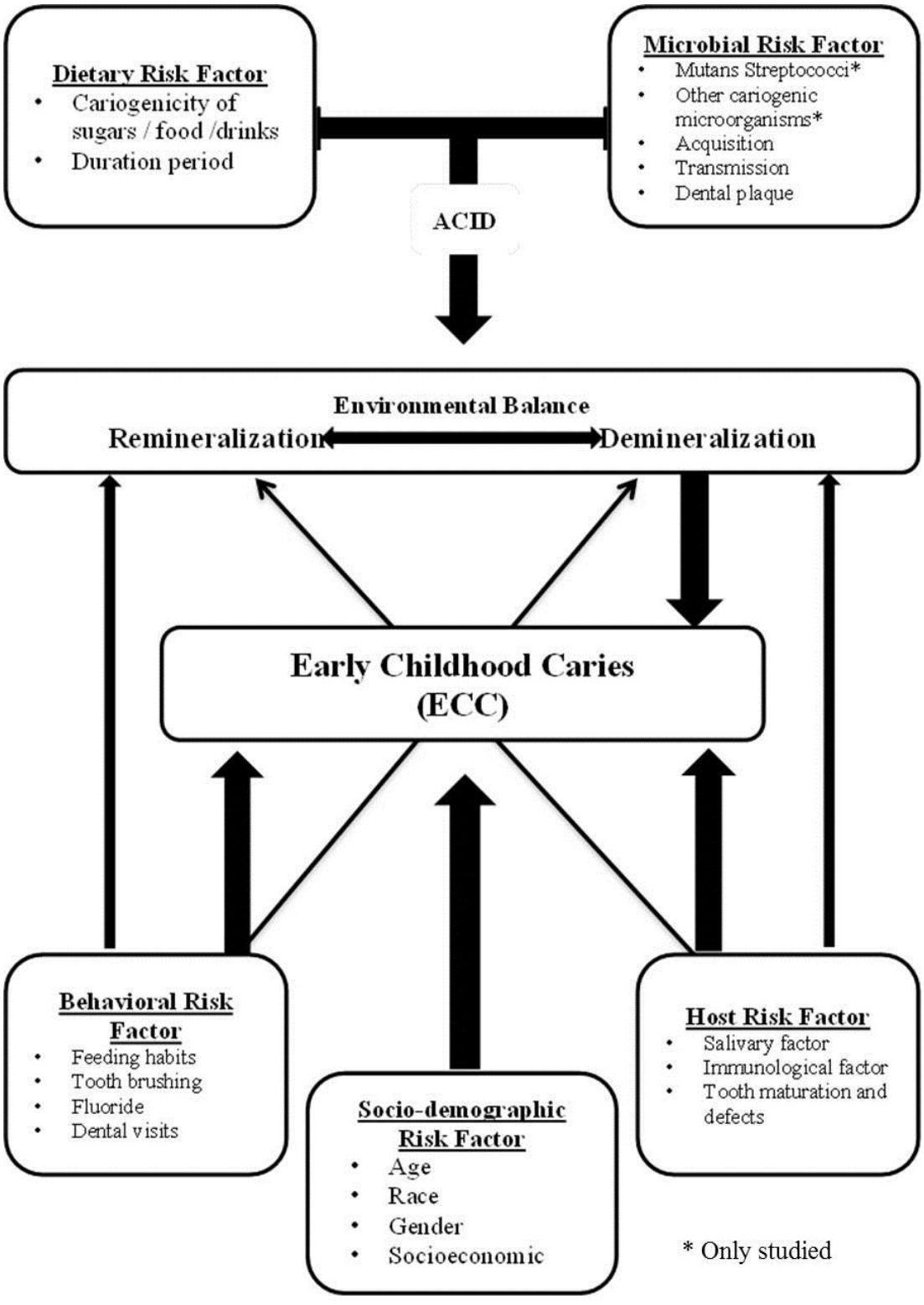

Figure 1.2 Conceptual framework of ECC.

CHAPTER 2

LITERATURE REVIEW

2.1 Early Childhood Caries (ECC)

2.1.1 ECC definition

Early childhood caries is a term that is recently used to refer to the presence of one or more decayed, missing or filled tooth surfaces in any primary tooth in pre-school children 71 months of age and younger (Drury *et al.*, 1999). In children younger than 3 years of age, any sign of smooth-surface caries is indicative of severe early childhood caries. From ages 3 through 5, 1 or more cavitated, missing (due to caries), or filled smooth surfaces in primary maxillary anterior teeth or a decayed, missing, or filled score of ≥ 4 (age3), ≥5 (age 4), or 6 (age 5) surfaces constitutes s-ECC. The term "Severe Early Childhood Caries (S-ECC)" refers to "atypical" or "progressive" or "acute" or "rampant" patterns of dental caries (American Academy of Pediatric Dentistry, 2014).

In the past, various names and terms have been used to refer the dental caries among young children such as nursing bottle mouth, baby bottle tooth decay, nursing caries, rampant caries, labial caries and maxillary anterior caries (Tinanoff, 1998; Ismail and Sohn, 1999). The new term “Early Childhood Caries”, reflects better the complex multifactorial aetiology of the disease (Edelstein *et al.*, 2009; American Academy of Pediatric Dentistry, 2014).

2.1.2 Prevalence of ECC

Early childhood caries (ECC) is a substantial public health problem in both developing and developed countries where its prevalence varies from one population to another. In United States of America, the prevalence of ECC in children aged 2-5 years old has been reported to be 27.5%, and 10% had S-ECC (Iida *et al.*, 2007). In the south-west Germany, 31.6% of children aged 3-5 years old had been reported with ECC while 9.5% reported with S-ECC (Bissar *et al.*, 2014).

In Thailand, ECC among infants aged 9-19 months has a high range which is between 20.8% - 82.8% (Vachirarojpisan *et al.*, 2004). As for Taiwan and Indonesia, the prevalence of ECC was found to be 56% and 81.2 % respectively (Tsai *et al.*, 2006; Sugito *et al.*, 2008).

In 2005, a Malaysian National Oral Health Survey in pre-school children aged 5 years revealed 76.2% had dental caries with a mean dmft (decayed, missing and filled teeth) of 5.5, while in 2007; 6 years old children had a mean dmft of 3.88. In the state of Kelantan, dmft was the highest at 9.22 (Malaysian Oral Health Division, 2005; Malaysian Oral Health Division, 2007) compared to other states in Malaysia.

2.1.3 The impact of ECC

Early childhood caries is a disease that can initiate early, then progresses rapidly in pre-school children who are at high risk, which will lead to an unpleasant impact on them and their parents if it was not well managed at the earliest.

Children with untreated ECC frequently suffers from intermittent or continuous pain due to the rapid caries progression, pulpitis and dento-alveolar abscesses resulting from a dental infection which, subsequently lead to limited or inability to chew nutritious foods as well as causing disturbance in their sleeping habits (Kayalvizhi *et al.*, 2014). Following dental pain, children with untreated ECC may have to go through aggressive dental treatments such as pulp therapy and tooth extractions. In primary dentition, any premature loss of deciduous teeth may lead to aesthetic, phonetic problems and/or malocclusion in the permanent dentition stage. Furthermore, these children are at a greater risk for morbidity and mortality associated with severe infection hospitalizations and treatments under general anesthesia. These consequences, as well as the general health of these children will be further jeopardized if they also suffer from medically compromised conditions.

The untreated ECC has also an effect on the pre-school children's body weight, growth and their life style quality. A study conducted by Acs *et al.* (1992) revealed that the weight of the pre-school children with ECC was significantly less than those who are caries-free. More specifically, only 1.7% of caries-free children weighed less than 80% of their ideal weight compared to 8.7% of children with ECC. A subsequent study confirmed that 13.7% of children with ECC weighed less than 80% of their ideal weight prior to dental treatment. Following dental rehabilitation, children with ECC showed an immediate increase in weight (Acs *et al.*, 1992; Acs *et al.*, 1999).

A report by Sheiham (2006) summarizes the effect of untreated ECC on children's growth, weight and their life style in three plausible mechanisms. First, food intakes were reduced due to pain and discomfort during eating as a result of

untreated ECC in association with infection. Second, pain due to severe ECC can disturb their sleeping habits which may affect glucocorticoids production and growth. For the third possible mechanism, anemia of chronic disease is considered as a result of erythropoiesis inhibition due to cytokines reactions in response to the severe untreated dental caries with pulpitis as well as chronic dental abscesses (Sheiham, 2006). Additionally, untreated ECC has a negative impact on the children's educational development where it restricts their school activities and decreases their school attendance due to emergency room visits to manage their pain and dental infections. It also affects their parents economic status due to high cost of dental treatment and their frequent taken time off from employment (Fung *et al.*, 2013).

2.1.4 ECC risk factors

Caries is a dynamic process which involves the demineralization and re-mineralization of tooth structure. Cariogenic bacteria in the biofilm (plaque) especially mutans streptococci and lactobacilli produced strong organic acid in the presence of fermentable carbohydrates. This acid creates an ideal environment for further growth of mutans streptococci. The acids diffuse rapidly and dissolve the enamel and dentine. This demineralization process usually takes months to years to progress to cavitation or dental caries (Featherstone, 2008). Protective effect of the saliva and fluoride lead to the re-mineralization and produced new crystals, fluoroapatite in the tooth structure which is stronger to acid attack (Featherstone, 2008).

The caries balance between demineralization and re-mineralization depends on the balance between pathological factors and protective factors. Pathological factors include acid producing bacteria, frequent eating and drinking of fermentable carbohydrates and subnormal salivary flow and function. Protective factors are such as salivary quantity and quality, fluoride and use of antibacterial agents such as xylitol and chlorhexidine (Featherstone, 2008).

Early childhood caries is a multifactorial disease that developed as a result of interaction between several factors similar to those of other dental caries types with unclear predisposing factors (Seow, 1998). Since ECC is an infectious disease, this thesis will focus the reviews on the risk factors from a microbiologic perspective.

2.1.4.1 Dietary risk factors

Children with ECC have frequent and prolonged consumption of sugars contained in fruit juices and many infant formula preparations. Such sugars are readily metabolized by *Streptococcus mutans* and lactobacilli to organic acids that demineralize enamel and dentin. The use of nursing bottles enhances the frequency of exposure. This type of feeding behavior, together with the decreased oral clearance and salivary flow rate during sleep, intensifies the risk of caries. Furthermore, caries-promoting feeding behaviors amplify the magnitude of dental reservoirs of *Streptococcus mutans* (Berkowitz, 2003).

When food is placed in the mouth and chewed, saliva production is increased and food is quickly swallowed. That is why it is believed that diet has little effect in the composition of the oral microbiota. One exception, however, is the fermentable carbohydrate. Some oral bacteria are talented in taking up sugars and fermenting

them to produce acid, thus causing demineralization. A persistent high intake of dietary carbohydrates can result in an altered microbiota, dominated by aciduric species (Takahashi and Nyvad, 2011). The salivary microbiome in caries-active subjects differs from that present in caries-free counterparts (Yang *et al.*, 2012; Wade, 2013).

2.1.4.2 Microbiological risk factors

As an infectious disease, several types of oral bacteria have been reported to have contributed in the development of ECC. These bacteria include mutans streptococci (MS), Lactobacillus species and *Bifidobacterium* species. *Streptococcus mutans* and *Streptococcus sobrinus* are species of MS group in which *Streptococcus mutans* plays a major role in the development of ECC (Chhour *et al.*, 2005; Berkowitz, 2006; Beighton *et al.*, 2008). Further review on the microbiological risk factors will be discussed in the next section.

2.1.4.2.1 Acquisition and transmission of *Streptococcus mutans*

As mutans streptococci (*Streptococcus mutans* and *Streptococcus sobrinus*) has been implicated as the most virulent cariogenic microorganisms, many studies have been directed to examine the critical period when this organism starts to colonize in an infant's mouth. This is because, an early colonization by *Streptococcus mutans*, are considered as a major risk factor for ECC and future dental caries.

Previous literatures reported that MS could not be detected among predentate infants because the mouth's feeble nature which disrupts the ability of MS to adhere

to its epithelial surfaces unless either primary incisor or primary molar teeth has erupted (Catalanotto *et al.*, 1975; Gibbons and Houte, 1975; Berkowitz *et al.*, 1980; Tedjosasongko and Kozai, 2002). Consequently, the concept that *Streptococcus mutans* required a non-shedding oral surface for persistent oral colonization became a basic principle of oral microbial ecology (Berkowitz, 2003).

However, more recent studies such as those conducted by Wan and colleagues in Australia have demonstrated that *Streptococcus mutans* was indeed can be detected in the predentate infants' mouth which was acquired in infants at the age of 3 months (Wan *et al.*, 2001). Another study conducted in Thailand, reported that *Streptococcus mutans* was detected in infants as early as 2 months age (Tankkunnasombut *et al.*, 2009). A study on 57 children, aged 6-8 months revealed that colonization of *Streptococcus mutans* was present in 55% plaque samples and 70% of tongue scraping samples. This revelation was acquired through the utilization of DNA probe technology (Tanner *et al.*, 2002). These recent studies on acquisition of *Streptococcus mutans* raise doubt that a non-shedding oral surface is required for colonization.

The early acquisition of mutans streptococci could occur either by vertical or horizontal transmission. In vertical transmission, the cariogenic microorganisms were transferred from parents to their infants. Mothers were considered as the major reservoir where the *Streptococcus mutans* strains found to be genetically identical with those in their children. The cariogenic microorganisms in horizontal transmission were transferred among siblings, classmates or in nursery. Several studies considered that the vertical transmission is the most common way for

transmission, whereas other studies found that the horizontal transmission is more commonly among children (Tedjosasongko and Kozai, 2002; Mitchell *et al.*, 2009).

2.2 Human oral microbiome

The human oral microbiome is a term that is used to refer to all of the microorganisms that have been found in the human oral cavity down to the distal esophagus (Dewhirst *et al.*, 2010). It was difficult to describe the exact composition of oral microbiome because the human oral cavity is frequently exposed to exogenous bacteria from food, water, air and humans via social contacts.

2.2.1 Healthy oral microflora

In describing oral microbiome structure, several studies have been conducted to expand the knowledge on the bacterial diversity of human oral cavity among healthy individuals.

In a healthy oral cavity, various oral bacteria have been found with wide diversity among the communities in the soft and hard tissues as well as in the saliva. The bacterial communities' compositions found in the soft tissues were more similar between each other than those that colonized on the hard tissues. Furthermore, the bacterial communities were individually different among those found sub- and supra-gingival sites of teeth (Mager *et al.*, 2003; Papaioannou *et al.*, 2009; Segata *et al.*, 2012).

The microbial profiles of soft tissue at genus level were characterized by abundance of *Streptococcus*, *Prevotella*, *Fusobacterium*, *Actinomyces*, *Veillonella* and *Leptotrichia*. In contrast, the obligate anaerobic genera such as *Fusobacterium* and *Prevotella* were predominantly colonized in the subgingival area of tooth surfaces, while the facultative anaerobic genera such as *Streptococcus*, *Leptotrichia*,

Actinomyces, Rothia, Capnocytophaga, Cornybacterium, Haemophilus and *Kingella* predominantly colonized the supra-gingival area of tooth surfaces (Aas *et al.*, 2005; Segata *et al.*, 2012).

At species level, the bacterial diversity varies between literatures due to their different approach in the detection methods. *Streptococcus mitis* was commonly found in most of soft tissue sites as well as *Streptococcus oralis, Gemella morbillorum, Gemella hemolysans, Veillonella parvula* and *Prevotella melninogenica*. For tooth surfaces, *Streptococcus gordonii, Streptococcus sanguis, Veillonella parvula, Actinomyces naesludii* and *Neisseria mucosa* were more commonly found in supra-gingival tooth surfaces, while *Leptotrichia buccalis, Streptococcus intermdius* and *Actinomyces gerencseriae* were more frequently found in the sub-gingival tooth surfaces (Mager *et al.*, 2003; Papaioannou *et al.*, 2009).

2.2.2 Common cariogenic microorganisms

2.2.2.1 Brief history

The full lists of cariogenic bacteria are still not yet revealed as research in the area are still in progress. Much research has suggested that mutans streptococci (*Streptococcus mutans* and *Streptococcus sobrinus*) have been closely associated with the development of dental caries (van Houte, 1994; Tanzer *et al.*, 2001; Okada *et al.*, 2002). This is because, first MS are frequently isolated from cavitated carious lesions; second, MS induce caries formation in animals when fed with a sucrose-rich diet; third, MS are highly acidogenic and aciduric and fourth, MS are able to produce water-insoluble glucan, which promotes bacterial adhesion to the tooth surface and to other bacteria (Takahashi and Nyvad, 2008) Additionally, its role in initiating the

dental caries on the smooth surfaces of the teeth of adult and children was confirmed in a systematic review by Tanzer and his colleagues (Tanzer *et al.*, 2001).

Lactobacilli are also implicated as important contributory bacteria in caries process, but their role in induction of lesions is not supported (Tanzer *et al.*, 2001). Failure to correlate Lactobacilli count with caries experience indicates that Lactobacilli may initiate caries only with the concurrent existence with *Streptococcus mutans*, possibly by increasing the acid production in plaque (Harris *et al.*, 2004). Other bacteria species that might be associated with dental caries development were reported such as, *Bifidobacterium* sp. and *Actinomyces* sp. (Corby *et al.*, 2005; Aas *et al.*, 2008).

2.2.2.2 Pathogenesis of dental caries

2.2.2.2.1 Dental plaque

As a chronic infectious disease, the first step in developing dental caries, (in this case ECC), is achieved from dental plaque formation. First of all, acquired pellicles were formed as a result of adsorption of salivary proteins as well as glycoproteins to a cleaned enamel tooth surface. This acquired pellicles allowed adhesion of pioneer oral microorganisms to it (cell to surfaces interaction) founding initial colonizers. Then, the initial colonizers can co-aggregate (cell to cell interaction) other oral bacterial such as *Streptococcus mutans* to finally develop the dental plaque (Kolenbrander *et al.*, 2006). Marsh and Martin (2009) summarize the plaque formation as shown in Figure 2.1 (Marsh and Martin, 2009).

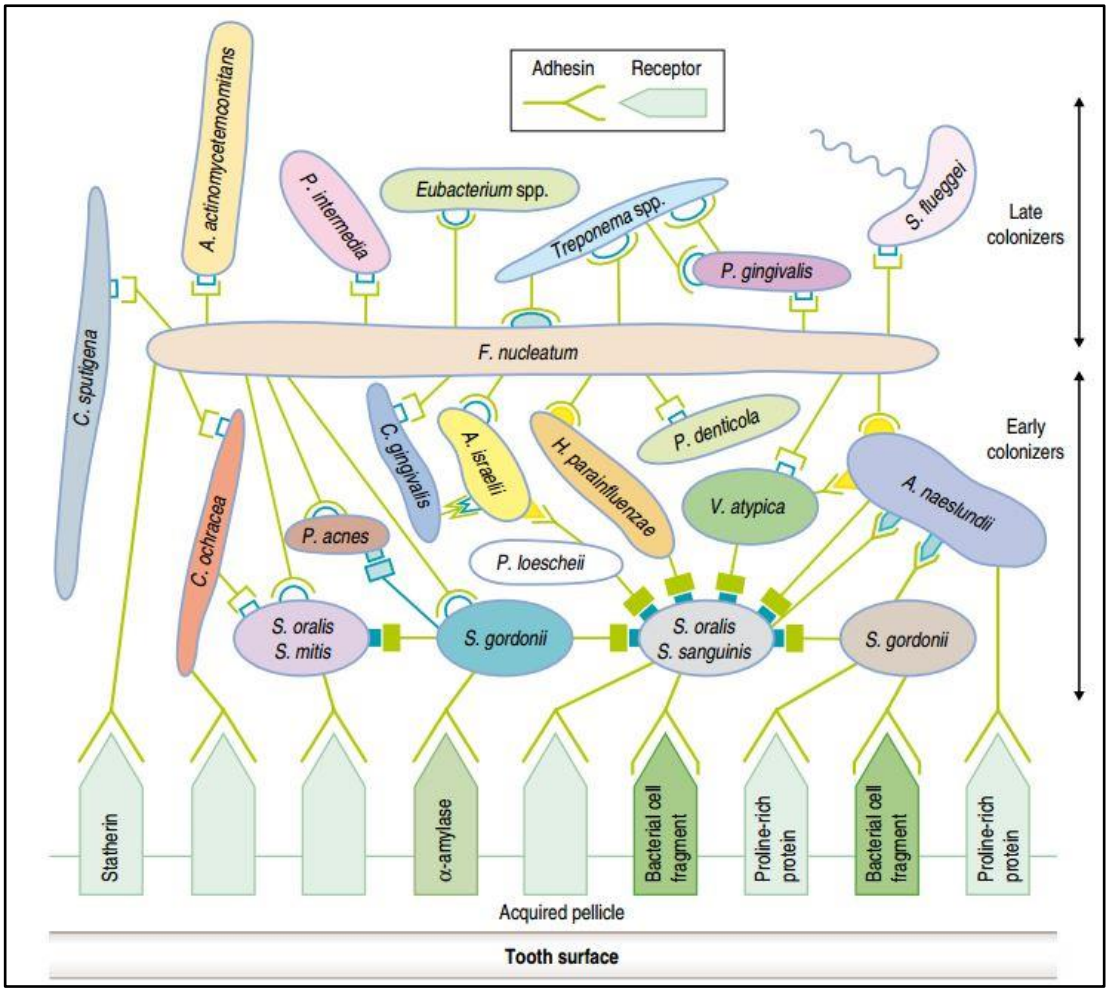

Figure 2.1 The patterns of coaggregation in dental plaque. Adapted from Marsh and Martin (2009).

Some of the adherent bacteria (mutans streptococci and non-mutans bacteria) produce extracellular polysaccharides (glucans and fructans) which contribute to plaque matrix as well as nutrient source for other plaque bacteria (Banas and Vickerman, 2003).

In matured plaque, various diversity of oral microflora was implied which influence caries development. For several years, the role of dental plaque in the etiology of dental caries was proposed by two main different hypotheses. The specific plaque hypothesis (Loesche, 1976) which suggested that, a single or a very limited number of oral microorganisms species are enrolled in caries development. While the non-specific hypothesis suggested that, the dental caries was developed as a result of interaction of all plaque microorganisms with the host (Theilade, 1986). An alternative hypothesis "ecological plaque hypothesis" proposed by Marsh (1994) explains that dental caries is the outcome of an imbalance in the resident plaque microflora due to changing in environmental conditions (Figure 2.2) (Marsh, 1994; Marsh, 2010).

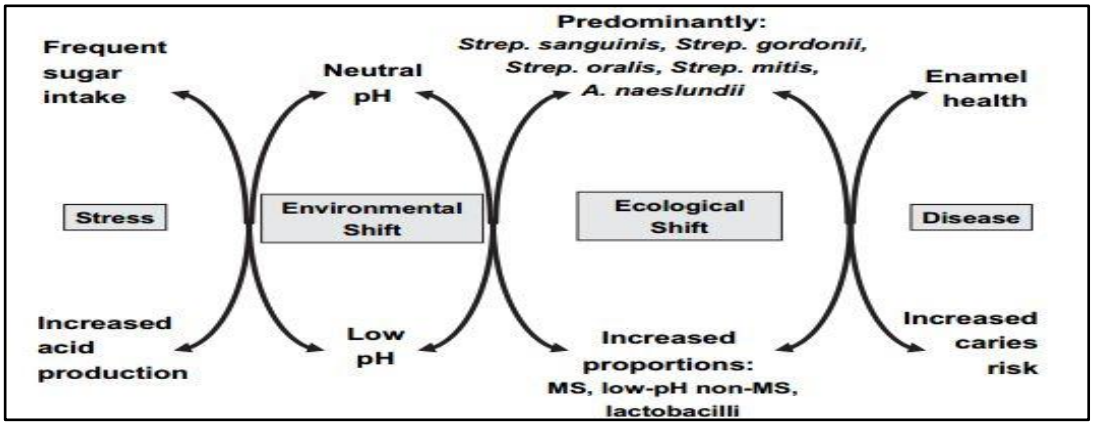

Figure 2.2 The ecological plaque hypothesis. Dental caries developed after frequent sugar intake followed by repeated lowering dental plaque pH and growth of aciduric and acidogenic bacterial species. Adapted from Marsh (2010).

2.2.2.2.2 The caries process

This process followed the dental plaque maturation, when the metabolic activities of the colonized bacterial reflected on surrounding environment; continuous and rapid fluctuation in plaque pH. Since the dental plaque cover intact enamel or carious tooth surfaces, the carious lesion might be developed or progressed based on mineral losses and gains processes. This caries process are considered as a ubiquitous and natural phenomenon as a consequence of decreasing salivary clearance mechanisms as well as richness of carbohydrates availability resulting in tooth surface demineralization or arresting the lesion progression due to tooth re-mineralization (Manji *et al.*, 1991; Kidd and Fejerskov, 2004; Varma *et al.*, 2008). With regards to this micro-dynamic concept, a new explanation has been suggested to consider the role of pH in oral biofilm in the caries development (Takahashi and Nyvad, 2008; Takahashi and Nyvad, 2011).

An extension for ecological plaque hypothesis was proposed to clarify the relativeness between the composition of dental plaque and the caries process (Takahashi and Nyvad, 2008). In this hypothesis, the caries process consists of 3 reversible stages (Figure 2.3).

The first stage is termed as the dynamic stability stage. The supra-gingival dental plaque is a dynamic microbial ecosystem in which non-mutans bacteria (non-mutans streptococci and *Actinomyces*) play a key role in maintaining the dynamic stability. By having the ability to produce acids from carbohydrates, these non-mutans bacteria can demineralize the enamel tooth surfaces and decrease the biofilm pH temporarily. This fall was raised to normal level by the homeostatic mechanism

in dental plaque because the acidification was mild and infrequent as well as the mineral balance was shifted toward tooth re-mineralization (Takahashi and Nyvad, 2008).

The second stage is termed acidogenic stage. In this stage, either the carbohydrates are frequently supplied or the salivary flow rate is strongly decreased leading to increasing of non-mutans bacteria acidic secretion. With dropping in pH, the plaque adaptively improved the acidogenicity and acidurance of the non-mutans bacteria. Furthermore, other aciduric strains, including 'low-pH' non-mutans streptococci and *Actinomyces* may increase their selectivity and with time, shift the mineral balance toward demineralization to start developing or progressing caries. However, the lesion in this stage could be arrested with restoring the mineral balance by decreasing the environmental acidification.

The third stage, the aciduric stage, it is a prolonged acidogenic stage in which more aciduric bacteria such as mutans streptococci, lactobacilli as well as Aciduric strains of non-mutans streptococci, *Actinomyces* and *Bifidobacterium* become dominant. Moreover, this stage is also reversible (Takahashi and Nyvad, 2008; Takahashi and Nyvad, 2011).