Bacterial Diversity in the Saliva and Plaque of Caries-free and Caries-active Filipino Adults

Candids Patrice A. Reyes¹ and Leslie Michelle M. Dalmacio¹

¹Department of Biochemistry and Molecular Biology, College of Medicine, University of the Philippines, Manila
²Department of Biology, College of Arts and Sciences, University of the Philippines, Manila

The oral cavity is inhabited by hundreds of bacterial species that play vital roles in maintaining oral health or in shifting to a diseased state such as dental caries. These microorganisms have been studied in the past using culture-dependent methods. However, due to the limitations of microbial identification through culture techniques, culture-independent techniques are currently being utilized to better understand the bacterial etiology of dental caries. In this study, molecular-based techniques were utilized to determine the bacterial profile of the saliva and supragingival dental plaque of caries-free and caries-active healthy Filipino adults. Bacterial DNA was amplified using primers for Eubacterial 16S rRNA gene (16S rDNA), diversity was assessed through denaturing gradient gel electrophoresis (DGGE), and the microorganisms were identified through sequencing of the 16S rDNA. Based on the DGGE profile and 16S rDNA sequences, the caries-free group exhibited a more diverse microflora compared with its caries-active counterpart. This suggests that part of the microflora may be inhibited or absent in a caries-active oral cavity. This is the first study that provides a baseline profile of the oral microbial diversity in caries-free and caries-active Filipino adults using culture-independent techniques. This profile may assist researchers and dental practitioners to gain better understanding of the microbial etiology of dental caries. It may also be used in future caries risk assessment and anti-caries vaccine development.

Key Words: bacterial diversity, caries-free, caries-active, dental caries, PCR-DGGE, 16S rRNA gene

INTRODUCTION

Dental caries is a disease that involves the destruction of the calcified tissues of the teeth. It is caused by the interplay of factors involving various oral bacteria in the presence of fermentable carbohydrates surrounding the tooth over a period of time (Lamont & Jenkinson 2010; Lundeen & Roberson 1995). It is considered by the World Health Organization (2003) as one of the most important global oral health burdens that affects people of various age groups all over the world. According to the National Monitoring and Epidemiological Dental Survey in the Philippines (1998), about 92.4% of Filipinos of various age groups have dental caries and the Philippines is the second worst among 21 WHO Western Pacific countries in terms of the Decayed, Missing and Filled Teeth (DMFT) Index. A national oral health survey among six-year-old public school children in the Philippines revealed that 97% of them suffer from dental caries (Monse et al. 2006).

Since oral bacteria are considered as one of the etiologic factors involved in caries development, various microbial studies have been conducted to better understand this oral problem. In the past, microbial studies were heavily
dependent on cultivation techniques. However, there are bacterial populations that cultivation methods fail to capture due to the selective media that are used (Madigan et al. 2004). As a result, bacterial species identified through culture-dependent methods are not truly representative of the oral microbial ecosystem, which poses limitations in our understanding of the oral microbiota in healthy and diseased states.

Molecular or culture-independent techniques are now utilized for studying bacterial communities (Muyzer et al. 1993). Polymerase chain reaction-based denaturing gradient gel electrophoresis (PCR-DGGE), which utilizes the 16S rDNA of microorganisms, is a good and highly sensitive molecular technique to identify oral microorganisms. DGGE reveals the biocomplexity of the various oral microbes of PCR-amplified gene products of similar lengths but with different sequences or GC-content. The resulting band profile, with each band representing a specific microorganism in the microflora, may be sequenced to identify the microorganisms in the sample (Kuramitsu et al. 2007).

Molecular techniques have been used in the identification of microorganisms in patients with various types of periodontal diseases and healthy oral state (Hutter et al. 2003; Paster et al. 2001), in studies on the microbial diversity of patients with squamous cell carcinoma (Hooper et al. 2006), and among patients with endodontic infections (Rolph et al. 2001). These culture-independent techniques have also been used in the study of plaque and carious teeth of 82-98-year-old patients with root caries (Preza et al. 2008), in the study of carious dentin among adult patients (Kim-Ly et al. 2005), in the study of carious tooth structure among 24-79-year-old adults (Munson et al. 2004), and in the study of saliva of caries-free (CF) and caries-active (CA) 18-22-year-old women (Li et al. 2005). However, no study of this kind has been conducted among Filipino adults with and without dental caries despite of its high prevalence in this population.

In order to better understand this oral disease which affects a large number of Filipinos, this study tested the hypothesis that there is a difference in bacterial diversity in the saliva and supragingival dental plaque of CF and CA Filipino adults. Saliva and supragingival dental plaque samples were taken from twelve healthy Filipino adults, six of whom were diagnosed with clinical signs of dental caries. Saliva was used as one of the sites for sample collection in this study since it is a fluid wherein all the other intraoral sites are immersed and could thus contain dislodged oral microorganisms from various intraoral sites (Marcotte & Lavoie 1998). Supragingival dental plaque was also included as a site for sample collection since it is made up of a community of microorganisms that may be found around the tooth surface which is located above the gingival margin. These microorganisms in dental plaque are embedded in a polymer matrix of host and bacterial origin. It is said to contain a diverse microbial community that may remain stable over time in a healthy state and it can harbor bacterial species that may predominate in diseased states (Marsh 2006). All PCR amplicons were run in DGGE and the DGGE profile was utilized to assess the biocomplexity of each group. Distinct DGGE bands were excised and sequenced to identify the oral bacteria in each group. The findings demonstrate a more diverse bacterial community among the caries-free individuals and support the hypothesis that there is difference in bacterial diversity between a healthy and a diseased state.

MATERIALS AND METHODS

Subject groups

Researches on the molecular analysis of oral microbial diversity utilized different subject population sizes. A previous study on the “core microbiome” of oral microbial communities used 3 healthy individuals (Zaura et al. 2009) while the study on the microbial diversity in advanced dental caries used 10 carious lesions (Chhour et al. 2005). In this study, 12 subjects were selected among healthy adult Filipino patients from the Clinical Department of the University of the Philippines College of Dentistry. The subjects were divided into two groups of 6 CF and 6 CA individuals. The caries status of all the subjects was measured using the DMFT index (decayed, missing and filled teeth) according to the criteria defined by the World Health Organization. The complete clinical examination included intra-oral examination and recording of medical and dental histories. The study protocol was approved by the Technical Review Board (TRB) and Ethical Review Board (ERB) of the Research Implementation and Development Office (RIDO) of the University of the Philippines Manila College of Medicine (Registration Code: GCS BS (BIO) 2009-35). The protocol was explained to each subject and a written informed consent was obtained during their initial visit.

Inclusion criteria

Subjects with and without dental caries were 22-34 years old. Caries-active (CA) was defined as an individual with at least one active carious lesion which was based on clinical examination. Caries-free (CF) was defined as an individual with a DMFT score of zero.

Exclusion criteria

Subjects with a history of antibiotic treatment within the past 3 mo prior to the initial clinical examination and sample collection were excluded. In addition, patients
using mouthwashes and lozenges and those who have undergone scaling and polishing within the past 30 days were also excluded. Female patients were also excluded on the basis of pregnancy or lactation.

Clinical sample collection
Saliva samples (5 mL) were collected in sterile centrifuge tubes and supragingival plaque samples were collected by isolating the teeth with sterile cotton rolls and scraping the supragingival plaque with sterile universal scalers (Hu-Friedy, USA). The supragingival plaque samples were placed in 1 mL 1X phosphate buffered saline (PBS) in 1.5 mL microcentrifuge tubes as previously described (Yoo et al. 2007). Another study has demonstrated that PBS may be utilized to extract cells from swabs and it does not affect subsequent DNA profiling (Martin et al. 2006). All samples were collected in duplicates at the Oral Medicine Section of the University of the Philippines College of Dentistry. These samples were stored and analyzed at the Genomics laboratory of the Department of Biochemistry and Molecular Biology, University of the Philippines College of Medicine.

Microbial DNA isolation
Microbial genomic DNA was isolated from the samples using the QIAamp DNA isolation kit (QIAGEN, Germany) following the manufacturer’s instructions and the cetyltrimethyl ammonium bromide (CTAB) DNA Isolation procedure (Doyle and Doyle 1987).

Briefly, in the CTAB DNA isolation (Doyle and Doyle 1987), all samples were initially centrifuged to collect the cell pellets which were resuspended in Tris-EDTA (TE) buffer. Sodium dodecyl sulfate (SDS) and protease K were added to the sample which was then incubated at 37°C for 1 h. Sodium chloride (NaCl) and 10% CTAB in 0.7 M NaCl were added to the mixture which was incubated at 65°C for 20 min. An equal volume of chloroform: isoamyl alcohol (24:1) was added and the mixture was centrifuged to separate the phases. RNase was added to the aqueous phase and this mixture was allowed to stand. An equal volume of isopropanol was added and mixed until the stringy white DNA pellet precipitated out of the solution and condensed into a tight mass. This was centrifuged and the supernatant was once again discarded. The washed and air-dried DNA pellet was dissolved in DNA-grade water and stored at -20°C until used for PCR. The concentration and purity of the extracted genomic DNA were evaluated by agarose gel electrophoresis and by UV spectrophotometry (260nm/280nm).

Amplification of the 16S rRNA gene
From the genomic DNA, the 16S rRNA gene was amplified using the eubacterial 8f and universal 1492r as forward and reverse primers, respectively (Vickerman et al. 2007). The PCR reaction mixture for saliva contained 0.5 mM each of 8f and 1492r primers (Eurogentec), Vivantis 1X buffer A, 2 mM magnesium chloride (MgCl₂), 0.8 mM dNTPs and 1 unit (U) Taq polymerase, and ≥50ng/µL DNA template. For plaque samples, the PCR reaction mixture contained 0.3mM of 8f and 1492r primers (Eurogentec), Vivantis 1X buffer S, 0.8 mM dNTPs and 1 unit/20 µL Taq polymerase, and ≥50ng/µL DNA template.

PCR was carried out in a standard thermocycler (VWR Doppio thermal cycler, USA) with the following set of conditions: initial denaturation of 94°C for 5 min, 35 cycles of denaturation (94°C for 1 min), annealing (52°C for 1 min) and extension (72°C for 1.45 min) and a final extension at 72°C for 5 min. The resulting PCR product, which was about 1.5Kbp, was confirmed by 1% agarose gel electrophoresis.

The 1.5 Kbp PCR products (≥50ng/µL) were used as template for a nested PCR that amplified the internal region of the 16S rRNA gene. The 341f-GC and 926r primer pair (Watanabe et al., 2001) were used as forward and reverse primers, respectively. The nested PCR reaction mixture contained 0.5 mM of each primer (Invitrogen) and Vivantis 1X buffer S, 0.8 mM dNTPs and 1U of Taq polymerase.

The nested PCR amplifications were also carried out in a standard thermocycler (VWR Doppio thermal cycler, USA) with the following set of conditions: initial denaturation of 94°C for 5 min, 35 cycles of denaturation (94°C for 1 min), annealing (53°C for 1 min) and extension (72°C for 1 min) and a final extension at 72°C for 30 min. The ~600bp PCR product was confirmed by agarose gel electrophoresis.

Denaturing gradient gel electrophoresis
DGGE was performed using the DCode Mutation Detection System (Bio-Rad, Hercules, CA, USA). A 30% to 70% linear denaturing gradient (100% denaturant is equivalent to 7 mol/L urea and 40% deionized formamide) was formed in 8% or 10% (w/v) polyacrylamide gels. The nested PCR products were directly loaded in each lane and electrophoresis in 0.5X TAE buffer was performed at 60°C for 14 hours using 60 V.

After electrophoresis, the gels were stained in ethidium bromide and were destained using sterile distilled water. DGGE profile images were digitally captured and recorded using a UV Transilluminator (Bio-Rad, Hercules, CA, USA). Distinct bands of PCR products in the DGGE gels were excised and placed in 50 µL sterile DNA grade water. The excised PCR product was eluted by crushing the gel, vortexing for 10 s, incubation at 37°C for 30 min, and centrifugation at 10,000 rpm for 1 min.
16S rDNA sequencing and identification

The solubilized excised DGGE band was reamplified using the same PCR mixture and condition for nested amplification of the internal region of the 16S rRNA gene. The PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN, Germany), following the manufacturer’s instructions. The concentration and purity of the purified PCR products were evaluated by 1% agarose gel electrophoresis and by spectrophotometry. About 25 µL of the purified PCR products, with a concentration of at least 40ng/µL, were sent to Macrogen, Korea for sequencing.

The bacteria from the saliva and supragingival dental plaque samples were identified by phylogenetic analysis using Molecular Evolutionary Genetic Alignment (MEGA) and sequence alignment of the 16S rRNA gene sequences with the available sequences present in the public databases using BLAST.

DGGE profile analysis

The DGGE gels were analyzed using Quantity One software (Bio-Rad, Hercules, CA, USA) to assess the bacterial diversity profile of the CF and CA samples. This software was utilized in detecting the various bands, matching them on assigned lanes and analyzing the similarity of the generated microbial fingerprints. In this study, it was assumed that common bands among the samples will migrate the same distance on the DGGE gel. Each lane was scored based on the presence or absence of each band found in its DGGE profile compared to the profile of all the other samples. The banding pattern of the microbial communities in the CF and CA groups was determined using the Dice similarity coefficient ($D_{SC} = [2j/(a+b)] \times 100$). The equation stands for $a =$ number of DGGE bands in lane 1, $b =$ number of DGGE bands in lane 2, and $j =$ number of common DGGE bands, and $D_{SC} = 100\%$ demonstrates complete similarity (Suchodolski et al. 2004). The authors designated that a similarity coefficient value of less than 50% was considered diverse while a similarity coefficient value of greater than 50% was considered similar (Nakatsu et al. 2000).

RESULTS

Clinical data

The clinical characteristics of the twelve healthy Filipino adults who participated in this study were recorded. The age range of the study subjects is 22-28 yr old. Both genders were equally represented. All the caries-free (CF) patients have a decayed, missing and filled teeth (DMFT) score of 0 while the caries-active (CA) patients have a DMFT score range of 2-8. The criteria of WHO for CF and CA individuals were followed.

Bacterial diversity profiles

The DGGE profiles of the 16S rDNA amplicons from the samples of CF and CA subjects revealed varied microbial diversity patterns for each group. Several bands from each sample were seen in the DGGE gel despite having just a single band in 1% agarose gel. Each DGGE band theoretically represents a bacterial population present in the sample (Kuramitsu et al. 2007). The diversity of the oral microbial population was qualitatively determined through the number of bands present in each sample, which is regarded as directly proportional to the diversity of the microbial community present in the sample (Nakatsu et al. 2000). Quantity One Software (Biorad) was utilized to detect the bands and to generate the similarity coefficient.

In the DGGE profile analysis of saliva in CF and CA adults (Figure 1B), about 4-7 bands were seen in each lane of the CF group while about 1-3 bands were found in each lane of the CA group. This finding is further supported by the 32.59% average similarity coefficient of the CF and CA saliva samples, which denotes diversity between these two groups.

The DGGE profile analysis of dental plaque in both groups (Figure 2) depicts a diverse microbial pattern as well. About 13 – 23 bands were seen in each lane of the CF group while about 4 – 10 bands were found in each lane of the CA group. This finding is further supported by the 20.65% average similarity coefficient of the CF and CA plaque samples which denotes diversity between these 2 groups.

All the identified bacteria from the saliva and supragingival dental plaque of CF and CA groups are presented in Table 1.

Several uncultured bacteria were found in this study. These “uncultured bacteria” are defined as bacteria that were identified through the amplification of their 16S rRNA genes obtained from isolated microbial genomic DNA, which avoids the bias derived from culture-dependent techniques of microbial study (Handelsman 2004).

Figures 3 and 4 presents the phylogenetic tree that was constructed from the 16S rDNA sequences obtained from the saliva and supragingival plaque of the CF and CA groups.

DISCUSSION

More DGGE bands were found in the Filipino CF group compared with their CA counterpart. A similar observation was seen in the DGGE-generated diversity profile analysis of the saliva of CF and CA women ages 18 to 22 yr old.
Figure 1A. (left) DGGE gel of CF and CA saliva samples and Figure 1B (right) DGGE profile analysis using Quantity One Software (Biorad). Lane A – CFM\textsubscript{1}, lane B – CFM\textsubscript{2}, lane C – CFM\textsubscript{3}, lane D – CAM\textsubscript{1}, lane E – CAM\textsubscript{2}, lane F – CAM\textsubscript{3}, lane G – CFF\textsubscript{1}, lane H – CFF\textsubscript{2}, lane I – CFF\textsubscript{3}, lane J – CAF\textsubscript{1}, lane K – CAF\textsubscript{2}, lane L – CAF\textsubscript{3}.

Figure 2A. (left) DGGE gel of CF and CA plaque samples and Figure 2B DGGE profile analysis using Quantity One Software (Biorad). Lane A – CFM\textsubscript{1}, lane B – CFM\textsubscript{2}, lane C – CFM\textsubscript{3}, lane D – CAM\textsubscript{1}, lane E – CAM\textsubscript{2}, lane F – CAM\textsubscript{3}, lane G – CFF\textsubscript{1}, lane H – CFF\textsubscript{2}, lane I – CFF\textsubscript{3}, lane J – CAF\textsubscript{1}, lane K – CAF\textsubscript{2}, lane L – CAF\textsubscript{3}.

(Li et al. 2005). They found a significantly increased diversity of oral microorganisms in CF individuals. This result suggests that a part of the oral microflora of CF individuals may be absent, inhibited or replaced in the CA state. Even with the use of conventional culture-dependent techniques in oral microbial studies, the total cultivable levels of bacteria in the CF group was found to be significantly greater (Li et al. 2005).
Table 1. Matched bacterial identities of the saliva and supragingival dental plaque of caries-free and caries-active Filipino adults.

<table>
<thead>
<tr>
<th>Type of Sample</th>
<th>Bacteria in CF and CA subjects</th>
<th>Bacteria in CF group only</th>
<th>Bacteria in CA group only</th>
</tr>
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<td>Saliva</td>
<td>Uncultured Weisella sp. clone 45d_B9 (GU929376.1)</td>
<td>Streptococcus cristatus strain ATCC 51100 (AY188347.1)</td>
<td>Streptococcus sp. oral taxon 071 strain F0408 (HM596298.1)</td>
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<td>Prevotella melanogenica strain JCM 6321 (AB547694.1)</td>
<td>Streptococcus infantis (AB008315.1)</td>
<td>Neisseria subflava strainToTA(FJ823143.1)</td>
</tr>
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<td></td>
<td>Prevotella melanogenica strain ATCC 25845 (CP002123.1)</td>
<td>Uncultured Streptococcus sp. clone GI-10-I07(GQ130081.1)</td>
<td>Neisseria subflava NJ9703 (AF479578.1)</td>
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<td></td>
<td>Uncultivated Prevotellaceae bacterium clone Goos136-81 (FJ221150.1)</td>
<td>Uncultured bacterium clone nby389f01e1 (HM821289.1)</td>
<td>Lactobacillus plantarum isolate L3-1 (AB550298.1)</td>
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<td></td>
<td>Uncultured Porphyromonas sp. clone PBI_5 (AM942601.1)</td>
<td>Uncultured bacterium clone nby306d03c1 (HM816034.1)</td>
<td>Uncultured bacterium isolate DGGEgel 2 band 25 (FJ999852.1)</td>
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<td>Uncultured bacterium clone E41-267 (DQ636840.1)</td>
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<td>Uncultured bacterium clone ncd940h05c1 (HM306984.1)</td>
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<td>Uncultured bacterium clone nby560d09c1 (HM836553.1)</td>
<td>Uncultured bacterium clone ncd1104a12c1 (HM333506)</td>
<td>Uncultured bacterium clone Dec08_05D (GU117456.1)</td>
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<td>Uncultured bacterium clone myd4_aaa04b07 (EU505416.1)</td>
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<td>Uncultured proteobacterium isolate DGGE gel band3 (DQ105621.1)</td>
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<td>Uncultured bacterium clone E67Ca-9 (DQ638344.1)</td>
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<td>Uncultured bacterium clone AJD1IVNA0 (GU019551.1)</td>
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<td>Uncultured bacterium clone B4_590 (EU765797.1)</td>
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<tr>
<td></td>
<td></td>
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<td>* in parenthesis are the database accession numbers</td>
</tr>
<tr>
<td>Supra-gingival</td>
<td>Uncultured Streptococcus sp clone 6B3111 (FJ976265.1)</td>
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<td>Streptococcus sp. oral cloneAA007(AY005046.1)</td>
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<td>dental plaque</td>
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<td>Uncultured Streptococcus sp. clone C181_E02_13K5 (GU075365.1)</td>
<td>Leptotrichia sp. oral taxon 225 strain FEA2 (GQ422731.1)</td>
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<td>Uncultured Streptococcus sp. clone 2 (DQ346439.2)</td>
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<td>Capnocytophaga sp.oral cl. AH015 (AY005074.1)</td>
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<td>Leptotrichia buccalis clone WWP_S3_C18 (GU415713.1)</td>
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<td>Uncultured Capnocytophaga sp. clone 10B374 (FJ976319.1)</td>
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<td>Uncultured Streptococcus sp. clone C181_E02_13K5 (GU075365.1)</td>
<td>Uncultured Capnocytophaga sp. clone G5-008-G03 (FJ192591.1)</td>
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<td></td>
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<td>Uncultured Streptococcus sp. clone C181_E02_13K5 (GU075365.1)</td>
<td>Neisseria subflava strainToTA (FJ823143.1)</td>
</tr>
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<td>Uncultured Actinobacteria bacterium clone QEDV2 (CU919137.1)</td>
<td>Uncultured Streptococcus sp. clone C181_E02_13K5 (GU075365.1)</td>
<td>Uncultured Neisseria sp. cl. 502G08 (AM420196.1)</td>
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<td>Uncultured bacterium clone ncd496f06c1 (HM328576.1)</td>
<td>Uncultured Streptococcus sp. clone C181_E02_13K5 (GU075365.1)</td>
<td>Uncultured Neisseria sp. isolate DGGE gel band S9J18 (EF531953.1)</td>
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<td>Uncultured Streptococcus sp. clone C181_E02_13K5 (GU075365.1)</td>
<td>Uncultured bacterium clone nby389f01e1 (HM821289.1)</td>
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<td>Enterococcus faecium strain LHIACA_37.4 (FJ656791.1)</td>
<td>Unidentified bacterium clone p27k110k (FJ3479048.1)</td>
<td>Uncultured bacterium clone ncd1103b07c1 (HM337602.1)</td>
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<td>Uncultured bacterium clone ncd1015b02c1 (HM344148.1)</td>
<td>* in parenthesis are the database accession numbers</td>
</tr>
</tbody>
</table>
Several members of the Streptococcaceae family were identified in the saliva and plaque of both the CF and CA adult Filipino subjects. A number of species that belong to this family are considered initial colonizers of the oral cavity (Lamont & Jenkinson 2010). The results of this study confirmed previous reports that some *Streptococcus* species are associated with healthy states (Aas et al. 2008; Corby et al. 2005), while others are associated with diseased states (Simon 2007; Lee et al. 1986). The most commonly associated and strongly implicated member of this family with dental caries is *Streptococcus mutans* (Tortora et al. 2007). However, it is interesting to note that this bacterium was not recovered from the CA Filipino subjects. A similar finding was observed in a previous study implying that dental caries can occur in the apparent absence of this bacterium (Marsh 2006). There are also non-*mutans* *Streptococcus* that have been found solely in the CA samples and which have been associated with childhood caries (Aas et al. 2008; Corby et al. 2005). In this study, *Streptococcus* sp. oral taxon 071 strain F0408
and *Streptococcus* sp. oral clone AA007 has been identified only in the CA adults. This may therefore have a significant contribution in caries progression among adults. In line with this observation, the possible role of the other members of the Streptococcaceae family in maintaining a healthy or diseased state must be studied in the future.

Members of the family Fusobacteriaceae were also recovered in the dental plaque of both groups. They are most often seen in great number in supra- and subgingival plaque of healthy and diseased oral cavities. They also facilitate the accumulation and adhesion of other oral bacteria in the biofilm (Lamont and Jenkinson 2010).

Other bacteria present in both CF and CA groups are presented in the second column of Table 1. Their presence in both groups may signify that they form part of the resident microflora. They may also participate in the tipping of the microbial ecology to a healthy or diseased state. Two families of the phylum Bacteroidetes, a group of bacteria that includes several anaerobes that coaggregate with other bacteria in the oral cavity (Chhour et al. 2005), was seen in the saliva of both the CF and CA Filipinos. The Porphyromonadaceae family includes species that initially colonize the oral cavity by interacting with the bacteria in supragingival plaque such as the oralis group of oral streptococci (Daep et al. 2008). Members of this family have been linked to various oral diseases like periodontal diseases, dental caries and persistent apical infections in root canal treated teeth (Haraldsson 2005; Nadkarni et al. 2004). Bacteria that belong to this family are rarely seen in healthy mouths (Haraldsson 2005). The Prevotellaceae family, on the other hand, is found in supragingival and subgingival plaque, saliva, buccal mucosa and tonsil area of the oral cavity (Haraldsson

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**Figure 4.** Phylogenetic tree of bacteria in plaque samples.
2005). They are frequently seen in healthy individuals and are not commonly associated with diseases. They are among the first anaerobic bacteria that colonize the mouth of infants and they are also known to inhabit the oral cavity by coaggregating with other bacterial species (Haraldsson 2005). At times, it has also been isolated in periodontal abscesses (Newman et al. 2002). It is considered as a periodontal pathogen since its presence increases the severity of periodontal diseases (Allison & Hillman 1997) but it may also be found in healthy gingival crevices where they occur in lower proportions (Marcotte & Lavoie 1998). Because of its presence in the healthy and diseased state, it may be considered an opportunistic bacterium that resides in the oral cavity. In addition, a previous study (Chhour et al. 2005) isolated members of this family in advanced carious lesions.

A member of the family Lactobacillaceae, specifically uncultured Weissella species clone 45d_B9 was isolated from the saliva of both the CF and CA groups. This family represents a group of lactic acid-producing bacteria which are aciduric as well. They have been studied for their possible probiotic role but they have also been associated with the development of dental caries. Aside from their opportunistic role in the caries progression, some Weissella species have been observed to coaggregate with Fusobacterium nucleatum which is a bridge-organism that promotes coaggregation of other bacteria. Thus, Weissella species have been implicated in the prevention of maturation of dental plaque (Kang et al. 2005).

Uncultured Actinobacteria bacterium clone QEDV2 has been found in the CF and CA plaque samples in this study. This microorganism has also been identified in healthy human oral cavities (Aas et al. 2008).

Another bacterium found in the CF and CA plaque samples in this study is Enterococcus faecium strain LHICA_37_4. Enterococci were among the first bacteria that were shown to induce dental caries in gnotobiotic animals. They have been known to be acidogenic and aciduric and they are not commonly seen in the human oral cavity (Tanzer et al. 2001). However, for the bacterium Enterococcus faecium strain LHICA_37_4, this is the first known report for its isolation in the oral cavity of CA humans.

An interesting observation is the isolation of members of the family Neisseriaceae only in the CA group of both the saliva and plaque. This family inhabits the oral cavity and mucous membranes of healthy and diseased states (Tortora et al. 2007). Being part of the normal microflora, it has been recovered on the surface of the tongue among healthy Malaysian subjects (Philip et al. 2008). This observation may be attributed to the nature of saliva as a physiologic fluid that coats various intraoral sites and as a carrier of several microorganisms that have been shed off from the different intraoral sites (Marcotte & Lavoie 1998). Members of this family are part of the earliest colonizers of clean surfaces of the teeth and they consume oxygen as plaque initially forms. In addition, they facilitate subsequent attachment of facultative and obligate anaerobes as late colonizers of dental plaque (Samaranayake 2006).

Another important observation is the occurrence of Lactobacillus plantarum only in the saliva of the CA Filipino subjects. Lactobacilli rapidly metabolize dietary sugars found in certain foods such as rice, which is the staple food for most Filipinos. The acids produced by lactobacilli create an environment conducive for their growth and survival and at the same time cause decalcification of teeth (Marsh 2006). Previous studies have demonstrated the association of increased proportions of acidogenic and aciduric bacteria like Lactobacillus species in dental caries (Munson et al. 2004; Caufield et al. 2007).

Several uncultured and yet-to-be identified bacteria were found in all the subjects of both the CF and CA groups. A number of these uncultured bacteria were found to cluster with the Streptococcaceae, Porphyromonadaceae, Prevotellaceae, Neisseriaceae, Flavobacteriaceae, Dietziaceae and Lactobacillaceae families (Figures 3 and 4). Some of these are the first known report of their presence in the human oral cavity, while others were previously obtained from the upper respiratory tract, esophagus, skin, or from environmental samples (Grice et al. 2009, 2010).

CONCLUSIONS AND RECOMMENDATIONS

The data obtained from this study supports the efficiency of molecular techniques such as PCR-DGGE and the 16S rRNA gene in analyzing and identifying various oral bacteria in a complex microbial community such as the one found in the saliva and supragingival dental plaque. The observed bacterial profile of the Filipinos compared with other populations may be brought about by the difference in their diet or oral hygiene practices. However, the possibility of this correlation has not been covered in this study and has to be studied in the future. Other factors influencing the shifts in the oral microbial diversity from a healthy to a diseased state may also be the focus of future studies.

It is also highly recommended that further studies on the abundance of the identified bacteria be pursued to provide more information on the bacterial etiology of dental caries. In the future, similar studies of this kind may also be conducted among the children and the elderly to complete the overall picture of the Filipino oral bacterial microflora.
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