Comparison of Oral Microbiome in Siblings Using Next Generation Sequencing

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Comparison of Oral Microbiome in Siblings Using Next Generation Sequencing

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마지막으로 저의 모든 것이자 살아가는 의미인 가족들에게 감사의 말씀을 전합니다. 아버지, 어머니, 큰언니, 큰형부, 예원, 예윤, 작은언니, 작은형부, 소희, 소정과 시아버지, 시어머니, 형님, 아주버님, 다은이, 평소에 바쁘다고 소홀하고, 표현하지 못해서 죄송합니다. 정말 사랑하고 감사합니다. 생각만해도 마음이 따뜻해지는 나의 작은 영웅 김재원군과 큰 영웅 김정인씨에게 이 논문을 바칩니다. 사랑합니다.

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Comparison of Oral Microbiome in Siblings Using Next Generation Sequencing

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The purpose of this study was to identify the oral microbiome in siblings with or without dental caries using next generation sequencing to verify the ecological changes in health and caries state and to identify the horizontal transmission. To investigate the composition of microbiome, 14 young siblings including 7 in caries group and 7 in caries-free group (average of 4.3 years) were enrolled in 7

families. Supragingival plaques were collected from cervico-buccal area of posterior teeth. All samples were analyzed by pyrosequencing based on 16S rRNA gene V1-V4 hypervariable regions. At the phylum level of caries-group, *Firmicutes* increased and *Proteobacteria, Bacteroidetes, Fusobacteria,* and *Actinobacteria* decreased. At the genus level of caries-group, *Streptococcus* increased and *Haemophilus, Capnocytophage,* and *Leptotrichia* decreased. At species level of caries group, *Veillonella dispar, Veillonella tobetsuensis, Veillonella rodentium, Actinomyces viscosus,* and *Streptococcus sanguinis* increased and *Streptococcus mitis, Capnocytophage sputiger, Capnocytophage leadbeltt* and AF366267 decreased. Similarity between the siblings was evident in UniFrac distance ($p<0.05$). In conclusion, these results showed the ecological change of oral microbiome in caries, the same as the previous researches. In addition, it appeared that the horizontal transmission contributed to the colonization of oral microbiome in siblings. We suggested that the strategy of caries prevention and management have to be considered in more holistic and personalized approach.

Keywords: Oral microbiome, Next generation sequencing, Pyrosequencing, Dental caries, Sibling, Ecological plaque theory, Horizontal transmission

Comparison of Oral Microbiome in Siblings with or without Dental Caries Using Next Generation Sequencing

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I. INTRODUCTION

Since Miller (Miller, 1890) announced the Chemoparasitic theory in 1890, there were two conflicting opinions about the role of bacteria in dental caries. Non-specific plaque theory was that the dental caries were caused by indigenous microorganism in plaque and treated by its elimination (Theilade, 1986). In contrast, specific plaque theory was that the dental caries caused by a single

specific pathogen, and treated by elimination of pathogen or leaving non-pathogenic plaque (Loesche, 1976). Prevention was focused on inhibiting the transmission or development of vaccination (Emilson and Krasse, 1985). In the early and middle 1900s, *lactobacillus* was indicated to be a certain bacteria (Green et al., 1957; Howe, 1917). It was appeared only in advanced caries lesion and the amount was generally less (Glass, 1952). In 1960, Fitzgeral and Keys experimented the successful generation of dental caries by transmission of *mutans streptococci* in the hamster (Fitzgerald and Keyes, 1960). Since, through a number of studies, the virulence of *Streptococcus mutans* (*S. mutans*), such as acidogenegic and aciduric potential, tooth-adhesion property, intra- and extra-cellular polysaccharide production – have been discovered (Holbrook, 1993; Krasse, 1988; Masuda et al., 1979; Tanzer, 1989; Thenisch et al., 2006). However, the amount of *S. mutans* and the dental caries showed correlation in population not in the individual level (Lang et al., 1987; Sullivan et al., 1989). In addition, it was found in experiments that other bacteria were involved in occurrence of early dental caries (de Soet et al., 2000; van Houte et al., 1996; Van Houte et al., 1991; van Ruyven et al., 2000)

Ecological plaque theory reconciles these two theory (Marsh, 1994). Commensal or residual oral microorganisms maintain the health status by adapting to the environmental changes. But if the pathological changes were persisted, microorganisms cause pathological conditions. In the development of dental caries, dental plaque in the healthy oral environment was dominant with non-

mutans streptococci (MS) and *actinomyces*, balancing reminralization and demineralization (dynamic stability state) (Takahashi and Nyvad, 2008). However, along with the pathological changes, such as highfrequent diets, reduction of salivary flow, acid environment (acidogenic state), dominant bacteria were changing to acidogenic and aciduric bacteria, such as *S. mutans* and *lactobacillus* (aciduric state) (Takahashi and Nyvad, 2011). As a result, the accumulated acid in dental plaque enhances demineralization and visual lesion, named dental caries was exposed in oral cavity.

The recent understanding of the human microbiome by the development of molecular biology was the background of ecological theory. A microbiome was "the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space" (Group et al., 2009). Human microbiome plays an important role in metabolic processes, homeostasis, nutrition, protection against deleterious infections, and even heredity (Do et al., 2013; Human Microbiome Project, 2012; Wade, 2013). In addition, it was associated with the human disease, such as allergy, rhinitis, atopy, obesty, immune disease, enteritis and heart disease (Aagaard et al., 2013).

Dental plaque was considered as a tooth-related biofilm, composed of a tooth-related microbiome embedded within a self-produced matrix of extracellular polymeric substance (Seneviratne et al., 2011; Tanzer, 1989; Wade, 2013). Oral microbiome was naturally transmitted from mothers, siblings, and environment and selected to reside by the oral environment (Berkowitz and Jordan, 1975;

Caufield et al., 1988; Kulkarni et al., 1989). Acquisition in oral cavity starts with birth and soon the *streptococcus* species including *S. salivarius, S. mitis* and *S. oralis* increase as 'pioneer group' (Hegde and Munshi, 1998; Nyvad and Fejerskov, 1987). Nutrition, micro-environment such as pH and redox potential and adhesions on tooth surface made by the pioneer group make other microorganisms survive in dental plaque and increase the diversity of microbiome (microbial succession) (ten Cate and Zaura, 2012). Finally, a stable climax community resides but, it could be changed by its environmental changes (Kononen et al., 1999; Li et al., 2003; Marsh and Devine, 2011).

The molecular biology was essential to reveal the microbiome, because previous cultural method can identify only 50% of oral microorganisms (Nyvad et al., 2013; Wade, 2011). The molecular biology of microbiome was based on 16S rRNA which was 1000pb in length, easy to read and to distinguish, common in all microorganism (Tanner et al., 2011). Many methods using 16S rRNA, such as denaturing gradient gel electrophoresis (Ling et al., 2010), polymerase chain reaction (PCR) (Marchant et al., 2001; Zhang et al., 2012), 16s rRNA gene microarray (Tanner et al., 2011; Tanner et al., 2012), DNA-DNA checkerboard hybridization (Becker et al., 2002; Lima et al., 2011), fluorescent *in situ* hybridization (Dige et al., 2014), and DNA sequencing(Gross et al., 2010; Munson et al., 2004) have been conducted in oral microbiology.

However, only DNA sequencing can identify *'de novo'* species (Nyvad et al., 2013). DNA sequencing was the process of determining the precise order of

nucleotides within a DNA molecule and has been used in applied fields such as diagnostic, biotechnology, virology and biological systematics (Pettersson et al., 2009). The 1st generation DNA sequencing, known as Sanger sequencing, was exact but time-consuming and expensive (Zaura, 2012). The 2nd generation DNA sequencing, so called Next generation sequencing (NGS), was possible to read more and economically faster (Xu and Gunsolley, 2014). There were several kinds of NGS, but pyrosequencing was the most appropriate method in microbiology due to the longest read length. Therefore, pyrosequencing has been used in oral microbiology and researches about dental caries, such as the healthy state (Keijser et al., 2008; Zaura, 2012; Zaura et al., 2009), white spot lesions (S. H. Kim et al., 2012), various caries states (Jiang et al., 2014; Tanner et al., 2012), and impact of pH (Kianoush et al., 2014). In addition, the heritability of oral microbiome was researched in twins (Corby et al., 2007; Stahringer et al., 2012). According to these researches, holistic change of microbiome in dental caries including *S. mutans* has been revealed. However, there was no research in sibling with or without dental caries.

The purpose of this research was to identify the oral microbiome in siblings with or without dental caries using NGS. We expect to verify the ecological changes in health and caries state and to the horizontal transmission.

II. Materials and Methods

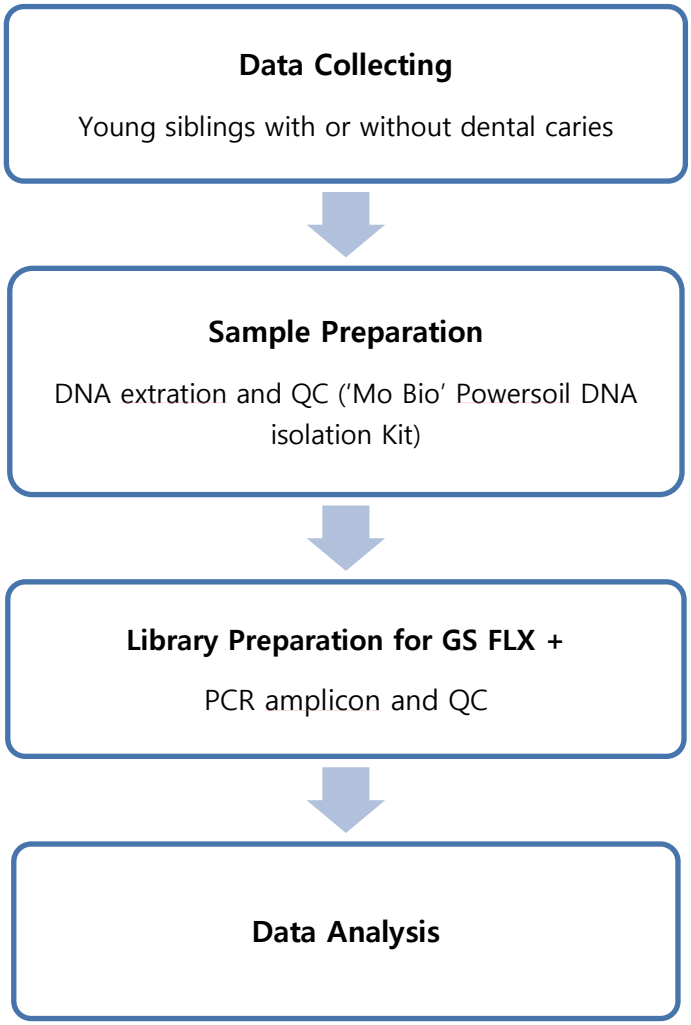
1. Ethics statement

Approval from the Institutional Review Board of the Dental Hospital, Yonsei University (#2-2014-0028) was obtained for this research, and written consents were obtained from the parents of all participants.

2. Data collecting

We presented the schematic flow chart of experimental design in Fig. 1. Participants were recruited from the department of pediatric dentistry, Dental Hospital, Yonsei University. Inclusion criteria were i) children under 12 years with primary or mixed dentition and ii) siblings with 5 more caries experienced teeth and without caries. Exclusion criteria were i) children visiting dental clinic for orthodontic treatment or dental trauma and ii) having antibiotics within last a month.

All caries surfaces were scored by DMFT system in participants with caries. For collecting dental plaque of participants, a sterile cotton swab was rubbed across the cervico-buccal area of the plaque 2-3 times under pressure in each child. Each plaque sample was obtained by pooling from multiple teeth. Samples were placed in a sterile 1.5ml microcentrifuge tube and frozen for storage.



- QC = quality control

Figure 1. Flow chart of experimental design

3. Sample preparation

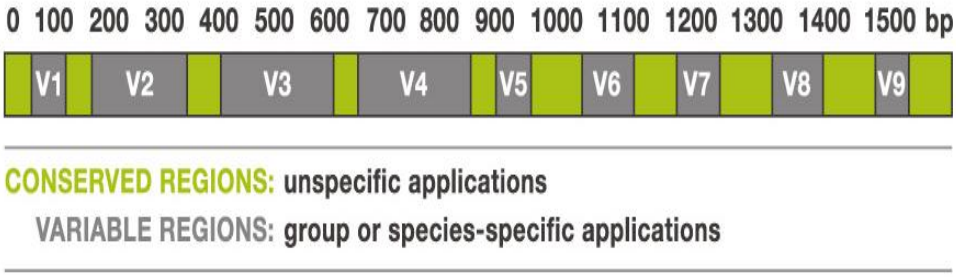
DNA was extracted using PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, samples were put in the PowerBead Tubes and homogenized with solution C1 for 10 minutes. Homogenized supernatants were transferred to 2 ml collection tubes and centrifuged with solution C2-C5 four times. DNA was eluted with deionized water and stored at -20℃. DNA concentration and purity were quantified using a NanoDrop Spectrophotometer (ND-1000, Thermo Scientific, Waltham, MA, USA) by measuring absorbance at 260 and 280 nm.

4. PCR amplification and pyrosequencing

PCR amplification was performed using primers targeting from V1 to V4 regions of the 16S rRNA gene with extracted DNA (Fig. 2 (A)). For bacterial amplification, barcoded primers of 27F (5'-CCTATCCCCTGTGTGCCTTGGCAGTC-TCAG-AC-AGAGTTTGATCMTGGCTCAG-3'; underlining sequence indicates the target region primer) and 800R (5'-CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-X-AC-GAGTTTGATCMTGGCTCAG-3'; 'X' indicates the unique barcode for each subject) was designed (Fig. 2 (B)). The amplifications were carried out under the following conditions: initial denaturation at 95 ℃ for 5 min, followed by 30 cycles of denaturation at 95 ℃ for 30 sec, primer annealing at 55 ℃ for 30 sec, and extension at 72 ℃ for 30 sec, with a final elongation at 72 ℃ for 5 min. The PCR product was confirmed by using 2% agarose gel electrophoresis and visualized under a Gel Doc system (BioRad, Hercules, CA, USA). The amplified products were purified with the QIAquick PCR purification kit (Qiagen, Valencia, CA, USA). Equal concentrations of purified products were pooled together and removed short fragments (non-target products) with Ampure beads kit (Agencourt Bioscience, MA, USA). The quality and product size were assessed on a Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA) using a DNA 7500 chip. Mixed amplicons were conducted emlusion PCR, and

then deposited on Picotiter plates. The sequencing was carried out at Chunlab, Inc. (Seoul, Korea), with GS FLX Plus system (Roche, Branford, CT, USA) according to the manufacturer's instructions.

(A)



CONSERVED REGIONS: unspecific applications

VARIABLE REGIONS: group or species-specific applications

(B)

Forward primer (Primer A, Lib-L):
5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG-{MID}-{template-specific sequence}-3'

Reverse primer (Primer B, Lib-L):
5'-CCTATCCCCTGTGTGCCTTGGCAGTCTCAG-{template-specific sequence}-3'

(1) blue text: identifies the GS FLX Titanium Primer A and Primer B sequences, here in modified for use with GS FLX Titanium emPCR Kits (Lib-L).

(2) red text: key sequence

(3) Orange box: MID optional Multiplex Identifier (MID) tag

(4) Green box: template specific region

- PCR ; polymerase chain reaction

Figure 2. Variable region of 16S rRNA gene and PCR amplicon; (A) GS FLX plus uses the V1 – V4 and 27F-800R as primer; (B) Production of amplicon library

5. Pyrosequencing data analysis

The basic analysis was conducted according to the previous descriptions in other studies (Fig. 3) (Chun et al., 2010; Hur et al., 2011; B. S. Kim et al., 2012). Obtained reads from the different samples were sorted by unique barcodes of each PCR product. The sequences of the barcode, linker, and primers were removed from the original sequencing reads. Any reads containing two or more ambiguous nucleotides, low quality score (average score < 25), or reads shorter than 300bp, were discarded. Potential chimera sequences were detected by the bellerophone method, which was comparing the BLASTN search results between forward half and reverse half sequences (Huber et al., 2004). After removing chimera sequences, the taxonomic classification of each read was assigned against the EzTaxon-e database (http://eztaxon-e.ezbiocloud.net) (O. S. Kim et al., 2012), which contains 16S rRNA gene sequence of type strains that have valid published names and representative species level phylotypes of either cultured or uncultured entries in the GenBank database with complete hierarchical taxonomic classification from the phylum to the species. The richness and diversity of samples were determined by Chao1 estimation and Shannon diversity index at the 3% distance. Random subsampling was conducted to equalize read size of samples for comparing different read sizes among samples. The overall phylogenetic distance between communities was estimated using the Fast UniFrac

(Hamady et al., 2010) and visualized using principal coordinate analysis (PCoA). To compare OTUs between samples, shared OTUs were obtained with the XOR analysis of CLcommunity program (Chunlab Inc., Seoul, Korea).

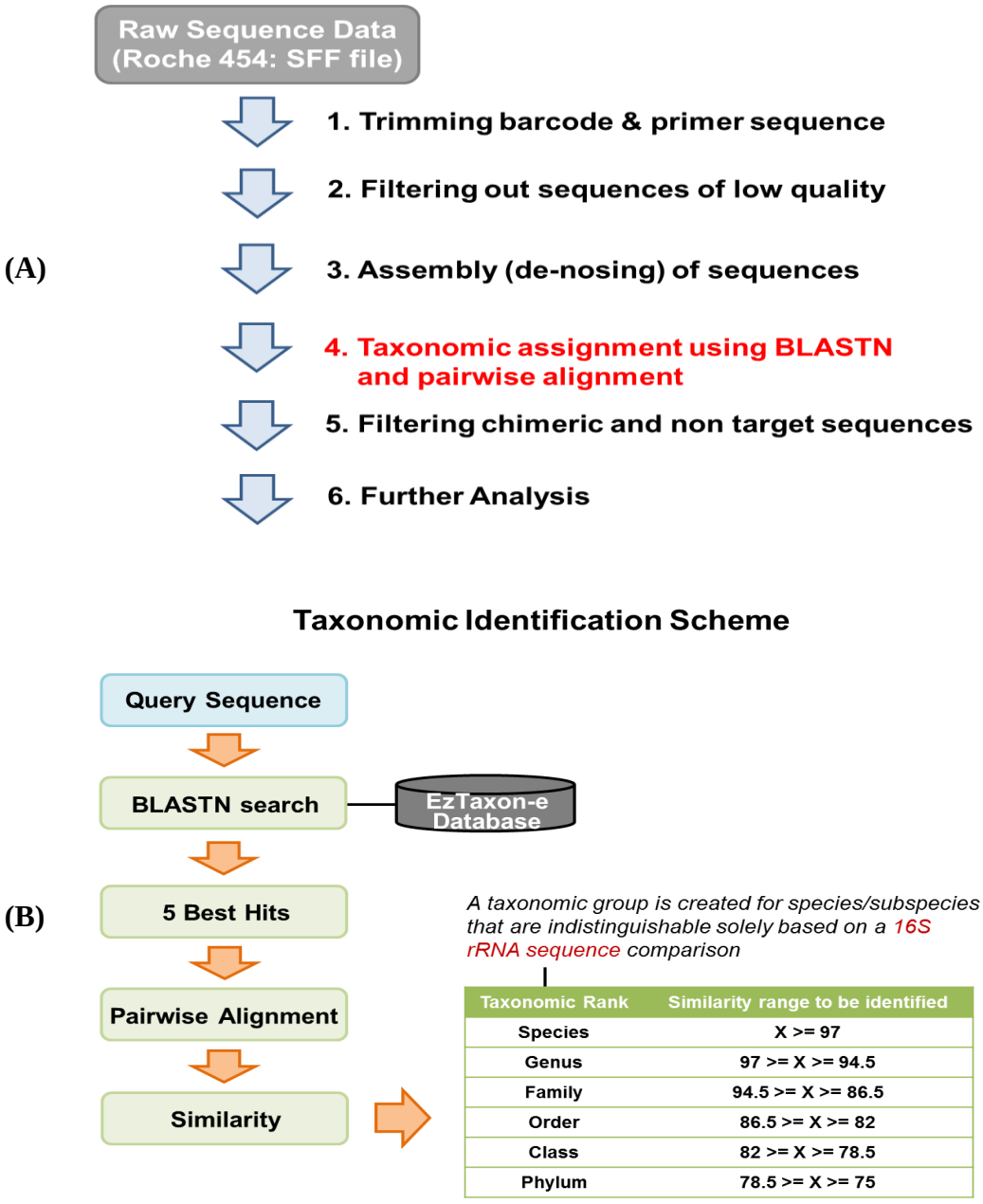


Figure 3. Data analysis procedure; (A) Raw sequence data produced by 454 pyrosequencing was processed in 6 steps. (B) Detailed explanation of the taxonomic identification ((A)-4).

III. RESULTS

1. Demographics and clinical information

7 children with caries and 1 children without caries in 7 families were participated in this research (Table 1). Mean age was 4.3 years, ranging from 1 to 10. The gender ratio was almost 1:1 (8 males and 6 females). In caries group, average DMFT index was 7.3, ranging from 5 to 15. Especially, # 51 showed the highest DMFT index, 15.

Table 1. Demographic and clinical information of the participants

	Caries group				Caries-free group		
Family group	ID	Age (Year)	Gender	DMFT	ID	Age (Year)	Gender
1	11	1	M	6	12	4	F
2	21	4	M	5	22	2	M
3	31	5	M	6	32	3	M
4	41	4	F	5	43	6	F
5	51	3	M	15	52	2	M
6	61	6	F	6	62	3	F
7	71	10	F	8	72	7	M

- Gender ; M=male, F=Female
- DMFT = decayed, missing, and filling teeth

2. Taxonomic identification and operational taxonomical unit (OTU) diversity

PCR amplification and subsequent pyrosequencing of the 16S rRNA gene hypervariable region V1-V4 of 15 samples resulted in 624,630 raw reads (Table 2). 391,325 were used for analysis after filtering. Operation taxonomical unit (OTU) diversity was an operational definition of a species or a group of species often used only when DNA sequence data were available. OTUs were defined at the 3% divergence (97% similarity) threshold using the average neighbor clustering algorithm. OTUs for all children were average 523. OTUs for the children without caries (average 562) were more than OTUs for the children with caries (average 479), but found no significant difference by independent t-test (α=0.05).

The richness and diversity of the samples were determined by Shanon diversity index at the 3% distance using taxonomy-dependent clustering - taxonomy-based clustering (TDC-TBC) method (Fig. 4). The alpha-diversity (Shannon) index of the caries group was lower than caries-free group. It demonstrated that the caries group displayed lower bacterial diversity. There was significant difference between the caries and caries-free group by Wilcoxon signed-rank test (p=0.022).

Table 2. Taxonomic identification

Group	ID	Number of raw reads	Number of final reads	Number of OTU
Caries	11	43,755	24,475	466
	21	63,625	46,354	185
	31	43,101	22,552	460
	41	35,815	19,669	649
	51	41,936	25,175	397
	61	43,994	23,667	554
	71	42,811	25,073	645
	Average	**45,214**	**26,709**	**482**
Caries-free	12	46,346	26,448	597
	22	51,476	36,767	203
	32	41,503	22,366	526
	42	38,726	21,400	755
	52	29,112	16,591	389
	62	40,570	20,858	750
	72	54,760	30,959	872
	Average	**43,213**	**25,056**	**585**

- OTU = operational taxonomic units

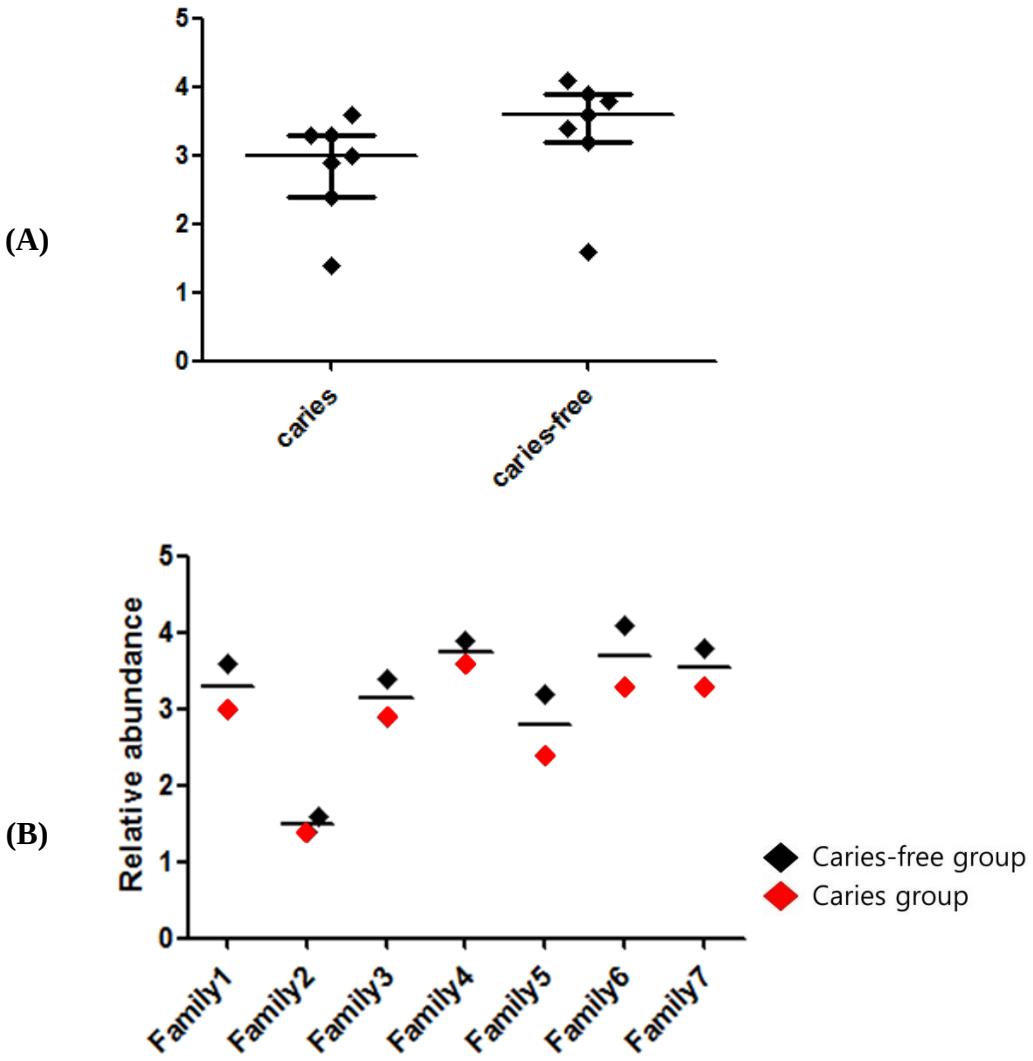


Figure 4. Comparison of the richness and diversity of samples at 3% distance using TDC-TBC (taxonomy-dependent clustering - taxonomy-based clustering) method; In alpha-diversty (Shannon) index, the caries group showed lower bacterial diversity, but there was significant difference by Wilcoxon signed-rank test (p=0.022).

3. Comparison of the composition of the microbiome between caries and caries-free group

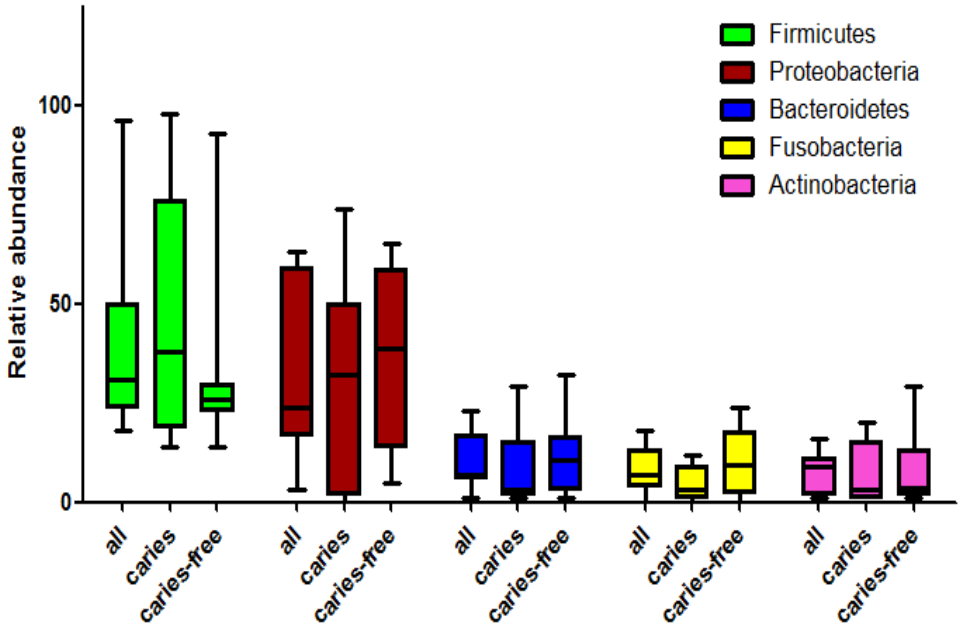
(1) Comparison of all samples at phylum, genus, and species level

At phylum level, five major bacterial phyla, *Firmicutes, Proteobacteria, Bacteroidetes, Fusobacteria* and *Actinobacteria,* were identified (over 5% of all microbiome) (Fig. 5 (A)). In caries group, *Firmicutes* was increasing and *Proteobacteria, Bacteroidetes, Fusobacteria,* and *Actinobacteria* were decreasing.

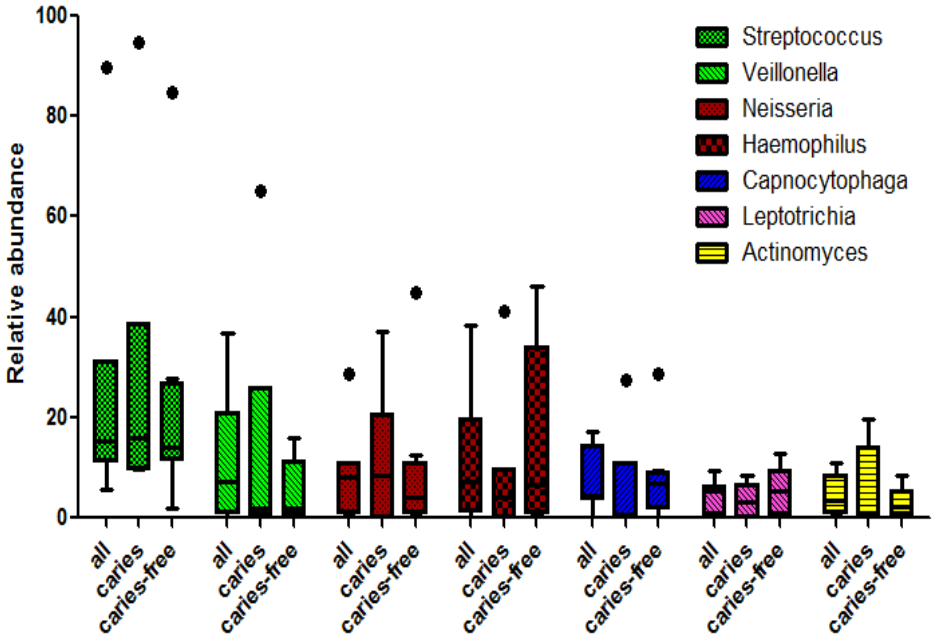
At genus level, 7 major bacterial genera, *Streptococcus, Veillonella, Neisseria, Haemophilus, Capnocytophaga, Leptotrichia* and *Actinomyces,* were identified (over 5% of all microbiome) (Fig. 5 (B)). In caries group, *Streptococcus* and *Neisseria* were increasing and *Haemophilus, Capnocytophage,* and *Leptotrichia* were decreasing.

At species level, 14 major bacterial species, *Streptococcus dentisani, S. mitis, S. sanguinis, Veillonella dispar, V. tobetsuensis, Haemophilus parainfluenzae, H. paraphrohaemolytic, Neiseria flava, N. subflava, Lautropia mirabills, Capnocytophaga sputigena, C. granulosa, Leptotrichia hongkongensis,* and *JQ464944,* were identified (over 5% of all microbiome) (Fig. 5 (C)). In caries group, *Veillonella dispar, Veillonella tobetsuensis, Veillonella rodentium, Actinomyces viscosus,* and *Streptococcus sanguinis* were increasing and *Streptococcus mitis, Capnocytophage sputiger, Capnocytophage leadbeltt* and AF366267 were decreasing.

(A)


Relative abundance
100
50
0
Firmicutes
Proteobacteria
Bacteroidetes
Fusobacteria
Actinobacteria
all
caries
caries-free

(B)


Relative abundance
100
80
60
40
20
0
Streptococcus
Veillonella
Neisseria
Haemophilus
Capnocytophaga
Leptotrichia
Actinomyces
all
caries
caries-free

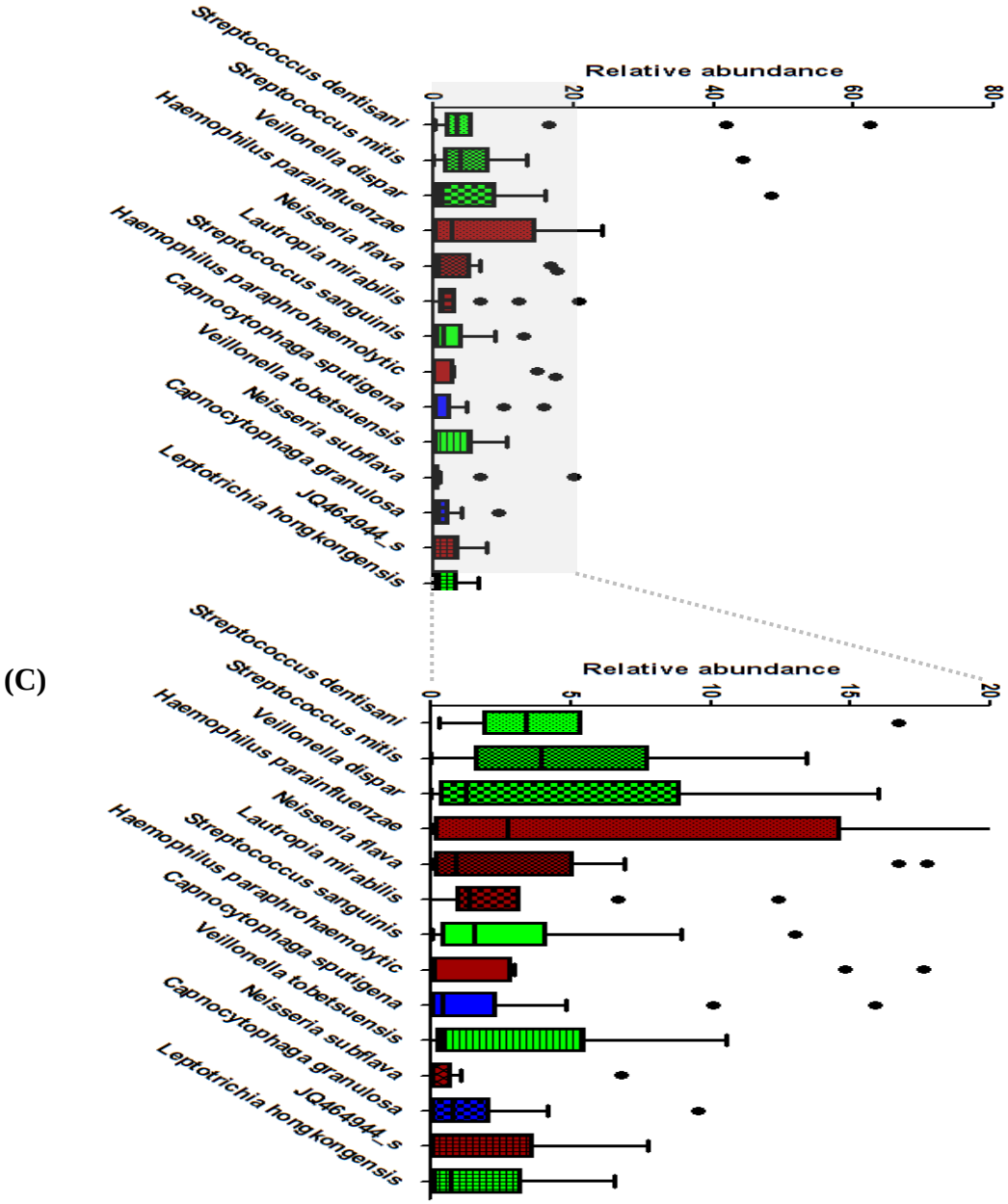


Figure 5. The relative abundance of oral microbiome by Box-and-whiskers plot; (A) Relative abundance of bacterial phyla were identified (over 5% of all microbiome). (B) At genus level, 7 major bacterial genus were identified (over 5% of all microbiome). (C) At species level, 14 major bacterial species were identified (over 5% of all microbiome).

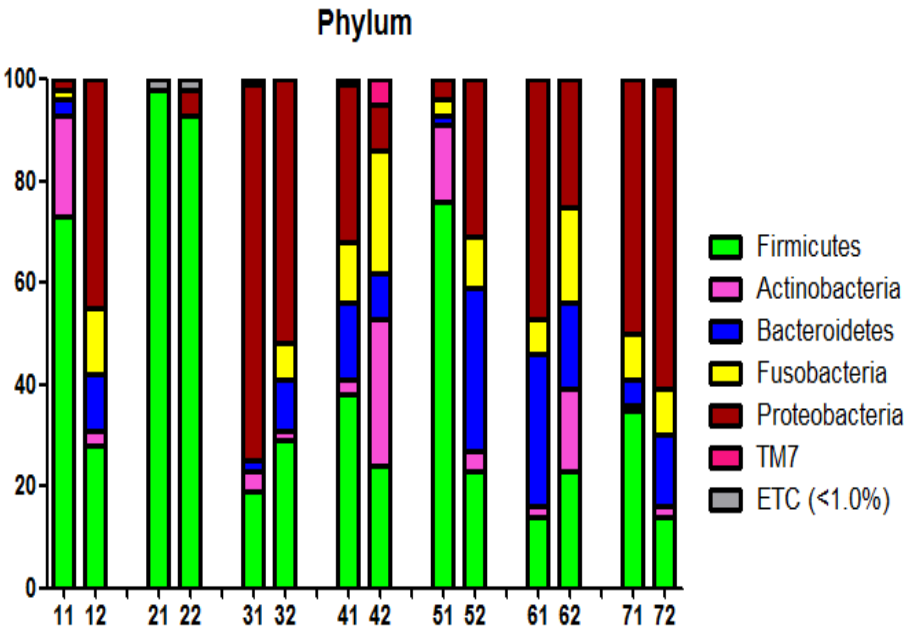
(2) Comparison in individuals at phylum, genus, and species level

At phylum level, individuals showed various composition of microbiom. But, *Firmicutes* increased in the caries group of 5 families (samples # 11,21 41,51,71) (Fig. 6 (A)).

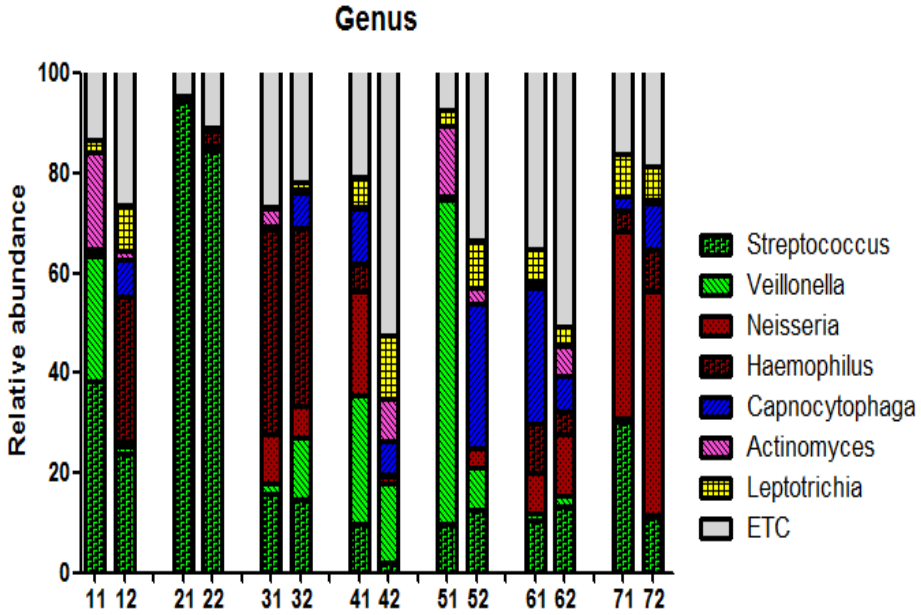
At genus level, *Streptococcus* increased in the caries group of 5 families (samples # 11,21,31,41,71) (Fig. 6 (B)). Also, *Veillonella* increased in the caries group of 3 families (samples # 11,41,51), especially # 51 showing the most DMFT index among all samples.

We compared samples # 51 and 52, becuase # 51 have the most DMFT index and it might reveal the notable ecological changes in dental caries in child (Fig. 6 (C)). At species level, 14 major bacterial species, *Streptococcus dentisani, S. mitis, S. sanguinis, Veillonella dispar, V. tobetsuensis,* In the sample # 51, *Veillonella dispar, Veillonella tobetsuensis, Veillonella rodentium, Actinomyces viscosus*, and *Streptococcus sanguinis* increased and *Streptococcus mitis, Capnocytophage sputiger, Capnocytophage leadbeltt* and AF366267 decrease.

(A)


Phylum
100
80
60
40
20
0
11 12 21 22 31 32 41 42 51 52 61 62 71 72
Firmicutes
Actinobacteria
Bacteroidetes
Fusobacteria
Proteobacteria
TM7
ETC (<1.0%)

(B)


Genus
Relative abundance
100
80
60
40
20
0
11 12 21 22 31 32 41 42 51 52 61 62 71 72
Streptococcus
Veillonella
Neisseria
Haemophilus
Capnocytophaga
Actinomyces
Leptotrichia
ETC

(C)

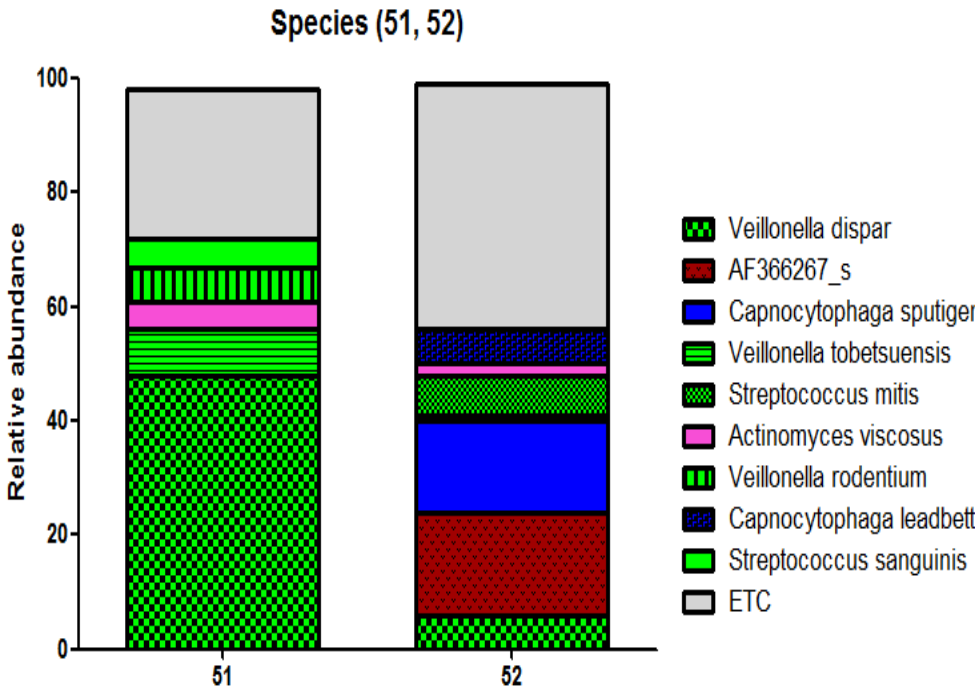


Figure 6. The relative abundance of oral microbiome; (A) Relative abundance of bacterial phyla were identified (over 5% of all microbiome). (B) At genus level, 7 major bacterial genera were identified (over 5% of all microbiome). (C) At species level, 9 major bacterial species were compared in sample # 51 which showed the most DMFT index and # 52 (over 5% of all microbiome).

4. Correlation of the siblings

Comparison of the sharing of microbiome of siblings permited an assessment of the horizontal transmission. The metric used for comparison was the weighted UniFrac distance (Lozupone et al., 2006; Lozupone and Knight, 2005), a widely used qualitative (presence/absence) community comparison measure based on phylogenetic information. UniFrac values range from 0 (identical communities) to 1 (maximum difference). We compared siblings to unrelated individuals and there was a statistical difference by a two-tailed t-test with unequal variance ($p<0.05$) (Fig. 7).

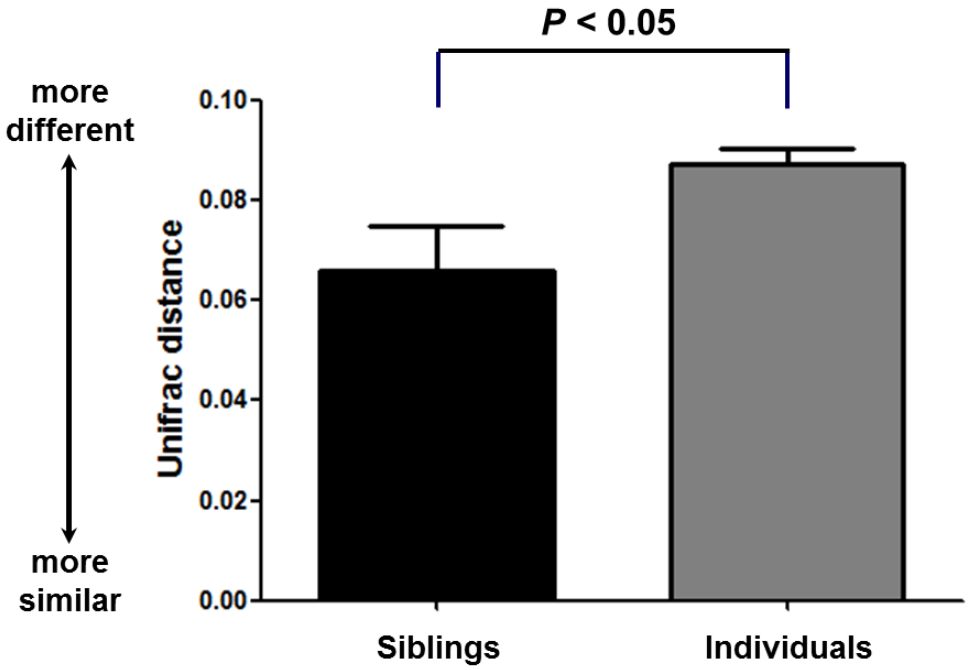


Figure 7. Mean pairwise weighted UniFrac distance for subsets of samples; Mean pairwise weighted UniFrac distance reflecting siblings and individuals. Means were shown ± SEM, and p-values were calculated by a two-tailed t-test with unequal variance.

IV. DISCUSSION

For this study of the oral microbiome, cross-sectional samples from 15 young siblings with or without dental caries were collected. Microbiome analysis was conducted using 16S rRNA gene cloning and pyrosequencing, and bacteria were identified using an oral 16S rRNA database. This open-ended approach allowed a holistic approach at the microbiome associated with caries and health in children and the horizontal transmission.

Participants in this study were children under 12 years with primary or mixed dentition. This period was the most vulnerable time to dental caries in life and the early established microbiome might influence the entire oral health (Kohler and Andreen, 2010; Kohler et al., 2003). In addition, the microbiome was changing with ages, but it was quiet stable without external stimulation before the pubescent (Crielaard et al., 2011). Dental plaques were collected in the cervico-buccal area of posterior teeth in all samples. In spite of the site-specific characteristics (Bowden et al., 1975; Van Houte and Green, 1974), we expected to identify the basic commensal or residual microbiome in the same location.

In this research, oral microbiome in carie and caries-free groups showed several similarities with the previous researches. At first, the number of OUT was lower in caries group. Several previous researches showed the diversity decreased with increasing caries within subject (Arif et al., 2008; Gross et al., 2012; Gross et al., 2010; Jiang et al., 2011). The reason was supposed that acid-senditive species

were eliminated in acidic environment produced by acidogenic specied and only a few aciduric species survived. This loss of diversity might damage the resilience of bacterial community in oral disease.

The change of microbiome in caries and caries-free group was similar with the previous researches, particularly in phylum level (Table 3). Oral microbiomes in health and caries status in previous researches were listed in Table 4. At the phylum level of caries-group, *Firmicutes* increased and *Proteobacteria, Bacteroidetes, Fusobacteria,* and *Actinobacteria* decreased. At the previous researches, *Proteobacteria, Actinobacteria* and *Bacteroidetes* were all health associated (Gross et al., 2010; Stahringer et al., 2012). The reason was the acidogenic and aciduric species, such as *S. mutan, S. sobrinus, non-MS,* and *Lactobacillus*, were included in Firmicutes.

At the genus level of caries-group, *Streptococcus* and *Neisseria* increased and *Haemophilus, Capnocytophage,* and *Leptotrichia* decreased. At species level of caries group, *Veillonella dispar, Veillonella tobetsuensis, Veillonella rodentium, Actinomyces viscosus*, and *Streptococcus sanguinis* increased and *Streptococcus mitis, Capnocytophage sputiger, Capnocytophage leadbeltt* and AF366267 decreased.

Table 3. Oral microbiome in healthy and caries status in previous researches (Corby et al., 2007; Crielaard et al., 2011; Gross et al., 2012; Jiang et al., 2014; Tanner et al., 2011; Tanner et al., 2012)

Healthy	Phylum	5 Major : *Bacteroidetes, Fusobacteria, Actinobacteria, Firmicutes, Proteobacteria*
	Genus	*Capnocytophage, Fusobacterium, Porphyromonas, Leptotrichia, Selenomonas, Abiotrophia, Comamonas, Tannerella, Eikennella, Paludibacter, Treponeme, Actinobaculum, Stenotrophomonas, Aestuariimicrobium, Peptococcus*
	Species	*Streptococcus parasanquinis, S. mitis/oralis, S. sanguinis, S. cristatus, S. salivarius, streptococcus sp.* *Gemella. morbillorum, G. haemolysans, Actinomyces defective, Porphyromonas catoniae, Neisseria flavescens*
Caries	Phylum	Increasing: *Firmicutes* Decreasing: *Bacteroidetes, Actinobacteria, Proteobacteria*
	Genus	*Streptococcus, Veillonella, Cryptobacterium, Lactobacillus, Megasphaera, Olsenella, Scardovia, Shuttleworthia, Cryptobacterium*
	Species	*Streptococcus mutans, , S. salivarius, S. sobrinus, S. parasanquinis, Scardovia wiggsiae, S. exigua, Veillonella granulicatella, Lactobacillus sp., Prevotella sp., Atopobium sp., Olsenella sp., Actinomyces sp.*

Especially, *Veillonella* was abundant in sample # 51 showing the highest DMFT index. The genus of *Veillonella* has regularly been found in the oral cavity. These genera have been distinguished from each other based on phenotype, but they cannot be reliably differentiated based on 16S rRNA sequence. *Veillonella* rely soley on lactate and other organic acids as an evergy source (Rogosa, 1964). *Veillonella* was found to be significantly associated with caries in children in previous molecular studies as well (Aas et al., 2008; Gross et al., 2012; Tanner et al., 2011). *Veillonella* was highly correlated with the total of all known acid producing species. This was not surprising given its reliance on lactate as its nutrient source, and has potential clinical utility since *Veillonella* levels may serve as a sensitive biologic indicator and early warning of acid production. Gross et al. suggested that *Veillonella*, but not S. mutans or other acid-producing species, was the microbial risk factors, predicting future caries (Gross et al., 2012).

In contrast, the correlation of dental caries and well-known cariogenic species, such as *S. mutans, S. sobrinus,* and *Lactobicillus* was insignificant. S. mutans was higly acidogenic and aciduric, and considerable clinical and laboratory data implicates this species as the primary pathogen in human dental caries. S. sobrinus was closely related to S. mutans, and these species were often referred to collectively as the mutans streptococci. S. sobrinus was associated with caries in the current study, and appeared to be the primary pathogen in some subjects. Lactobacilli were rarely detected, and when present were at very low levels in this cohort of young children with the earliest stages of caries. Lactobacilli have

shown a robust association with more advanced caries in many studies and in older children. The reason was supposed that the location of sample collecting was cervico-buccal surface, not the caries site. If we collected at the caries progressing site, the more acidogenic and aciduric microbiome would be revealed than this result due to the site specific colonization.

We identified horizontal transmission in siblings in this research. In previous researches, intrafamilial transmission of *S. mutans* including horizontal transmission (Domejean et al., 2010; Hoshino et al., 2012) and vertical transmission (Caufield and Walker, 1989; Mannaa et al., 2012) was approved. However, the result of this study and the recent researches (Stahringer et al., 2012) by NGS approved the transmission of the whole oral microbiome. Even, Corby et al.(Corby et al., 2007) used the term 'Heritability' and suggested that the relative abundance of oral microbial species was in part determined by the host genome. Heritability was the proportion of observed differences on a trait among individuals of a population that were due to genetic differences and factors including genetics, environment and random chance can all contribute to the variation between individuals in their observable characteristics (Raj and van Oudenaarden, 2008). In the same vein, the human microbiome was considered as human second genome (Grice and Segre, 2012). This heritability could be used in personalized dental medicine.

Personalized dental medicine was personalized medicine in dentistry, which is the medical model that proposes the customization of healthcare using molecular

analysis – with medical decisions, practices, and/or products being tailored to individual patient. In this model, diagnostic testing is often employed for selecting appropriate and optimal therapies based on the context of a patient`s genetic content (Eng et al., 2012). In fact, the genetic approach to the dental caries is difficult because it is multifactorial disease. But, Werneck et al. (Werneck et al., 2011) represent the genetic risk factors controlling caries susceptibility. In addition, Li et al (Li et al., 2007) revealed the genetic profiling of the oral microbiome and suggested that the diversity and complexity of the microbiome were significantly less in caries children. Host factors such as tooth and salivary factors and dietary factors were influenced by genes in some ways. (Brook, 2009; Kim et al., 2005; Poulter et al., 2013; Vitorino et al., 2005; Wendell et al., 2010; Wright et al., 2011). These genes could be the sources of personalized dental medicine for dental caries.

We also think about the prevention and management of dental caries in more holistic and ecologic aspect. The targets of specific plaque theory, which was believed in world-wide, were the blockage of transmission and the vaccination, but the transmission of microbiome including *S. mutans*, *s. sobrinus*, and *Lactobacillus* was natural from family or environment. We should try to reduce the number of transmitted pathologic microbiome by the improvement of hygiene in family and environment and to make the oral environment not suitable for the selective colonization of the pathologic microbiome. In addition, the acquisition timing was not limited in the ‘window of infectivity’, all the time from the birth

was critical until the stable and healthy climax community was built. The timing of the climax community and the influencing factor was needed to be researched further. Also, the oral hygiene care of pregnant women has to be emphasized far more for reducing the cariogenic microbiome in empirically and theoretically.

The one of the limitations of this research was a small number of participants. In addition, the duplication or triplication of sampling would make more accurate research. And, the collected amount of sample # 21 and 22 were so small that a couple of species were dominant. Larger scale and precise research is needed for overcoming this limitation.

V. CONCLUSIONS

In conclusion, these results showed the ecological change of oral microbiome in caries, such as the reducing of the diversity. In addition, it appeared that the horizontal transmission contributed to the colonization of oral microbiome in siblings. We suggested that the strategy of caries prevention and management have to be considered in more holistic and personalized approach.

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국문요약

차세대시퀀싱을 이용한

형제간의 구강마이크로바이옴의 비교연구

연세대학교 대학원 치의학과

이 효 설

지도교수 : 이제호

본 연구의 목적은 치아우식증의 유무가 다른 형제에서의 구강미생물균을 차세대시퀀싱으로 동정하여 비교하는 것으로 건강한 상태와 치아우식증에서의 생태학적 변화를 조사하고 수평적 전이를 확인하는 것이다. 구강미생물의 조성을 파악하기 위해, 7 가족에서 14명의 어린이(평균 4.6 세)가 참여하였고, 7명은 치아우식증이 있고 7명은 치아우식증이 없었다. 구치부 협측치경부에서 치은연상치태를 채취하였다. 시료를 16S rRNA 유전자의 V1-V4 부위에 대해 파이로시퀀싱(pyrosequencing)으로 분석하였다. 개개인은 자신의 형제와 더 비슷한 결과를 보였다. 문(門, Phylum) 영역에서는 *Firmicutes*이 증가하였고, *Proteobacteria*와 *Bacteroidetes, Fusobacteria, Actinobacteria*가 감소하였

다. 속(屬, Genus) 영역에서는 *Streptococcus*가 증가하고, *Haemophilus*와 *Capnocytophage, Leptotrichia*가 감소하였다. 종(種, Species) 영역에서는 *Veillonella dispar*와 *Veillonella tobetsuensis, Veillonella rodentium, Actinomyces viscosus*가 증가하고 *Streptococcus mitis*와 *Capnocytophage sputiger, Capnocytophage leadbeltt*, AF366267가 감소하였다. 형제간의 유사성은 UniFrac distance로 유의성있게 나타났다 ($p<0.05$). 결론적으로 본 연구의 결과는 이전의 연구와 유사하게 치아우식증 발생에 있어 구강미생물의 생태학적 변화를 보였다. 또한, 수평적 전이가 형제간의 구강미생물의 집락에 영향을 주는 것으로 나타났다. 치아우식증의 예방과 관리 전략을 수립할 때 보다 더 전체적인 접근이 필요하다.

핵심되는 말: 구강미생물, 차세대시퀀싱, 파이로시퀀싱, 치아우식증, 형제, 생태학적 치태가설, 수평적 전이